Development of process technologies for the valorisation of fish and shellfish processing waste streams: Integration of sub- and supercritical fluids with deep eutectic systems

Liliana Andreia Amaro Rodrigues

Dissertation presented to obtain the Ph.D. degree in Engineering and Technology Sciences, Chemical Engineering

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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Aos meus pais.

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CONTENTS

Development of process technologies for the valorisation of fish and shellfish processing waste streams: Integration of sub- and supercritical fluids with deep eutectic systems

Abstract

The world's strong dependency on fossil resources for energy generation and products manufacturing has arisen serious environmental problems and economic concerns. Therefore, the search for alternative solutions that enable an improvement of the quality of life of present and future generations, while sustaining the environment through the significant reduction of the exploitation and consumption of fossil fuels and the implementation of clean processing technologies, has become of the utmost importance. The path for a sustainable future lays, therefore, in the implementation of the biorefinery concept as a strategy for the establishment of a circular bioeconomy, aiming at producing a wide range of marketable products and energy, by using renewable biomass resources as feedstock.

The generation of waste during food processing is most of the times unavoidable. However, the valorisation of such residues can contribute to maximize financial return, and to simultaneously address the problem of the increasing amount of waste disposal, as well as feedstock depletion.

Fish and shellfish are a major source of surpluses when processed for human consumption, generating residues equivalent to 20 to 75 % of the harvested fish total weight. In particular, the canning process or the processing of crustaceans and molluscs are regarded as some of the biggest sources of seafood-processing wastes and by-products, generating more than 2 million tonnes of waste per year in the European Union alone. Nevertheless, after processing, fish and shellfish residues still harbour significant amounts of value-added products such as fatty acids, proteins and derivatives, pigments, biopolymers, and minerals, which have shown potential to be applied in the food and beverage, pharmaceutical, biomedical, nutraceutical, cosmetic, agrochemical, or biotechnology industries, among others.

Within this context, the main goal of this PhD thesis was to design and develop sustainable approaches to convert canned sardine and brown crab shell residues, into value-added products. The strategy applied combined the use of supercritical carbon dioxide (sc-CO_2), subcritical water (scW), and deep eutectic systems (DES), for the extraction of fatty acids,

protein derivatives and hydrolysates, carotenoids (with a special focus on astaxanthin, AXT), and chitin from these waste streams.

Depending on the process, an attempt was made to maximize the extraction of the target compounds, by studying the impact of different process parameters on their recovery:

(i) Sc-CO_2 extraction was applied for the recovery of fatty acids from canned sardine residues, by varying conditions of pressure (300, 425, and 550 bar), temperature (35, 55, and 75 °C), and CO_2 flow rate (5, 15, and 25 g/min);

(ii) Protein derivatives were extracted from canned sardine residues using betaine/polyol-based DES, and the operating conditions studied included temperature (25, 45, and 80 °C), extraction time (6 and 18 h), and solid-liquid ratio (1:8, 1:16, 1:40, and 1:80 g/g);

(iii) AXT was extracted from crab shells using terpene/fatty acid-based DES, by evaluating the impact of operating temperature (30, 45, and 60 °C) and extraction time (2, 6, and 24 h);

(iv) scW was applied for the extraction of protein hydrolysates from crab shells, and the impact of operating temperature (150, 200, and 250 °C), solid-liquid ratio (1:5, 1:10, and 1:15 g/mL), and heating rate (3 and 6 °C/min) was assessed;

(v) And finally, chitin was recovered from crab shells using choline chloride/organic acid-based DES, by testing variations in the operating conditions of temperature (50, 80, and 130 °C) and processing time (2.5, 3, and 4 h).

Whenever DES were used as solvents, their physicochemical and toxicological properties were also assessed.

To evaluate the potential application of sardine/crab extracts as functional ingredients, as well as their safety, different bioactivity assays were selected depending on the target molecule, including cytotoxicity, and antioxidant, antimicrobial, antiproliferative, and anti-inflammatory potential. Additionally, as a proof of concept, the most promising processes were experimentally scaled-up (up to a 70-fold increase in scale), integrated, and their environmental impact evaluated through life cycle assessment (LCA).

Results presented throughout the chapters of this thesis have shown that the extraction methodologies applied could successfully recover the different target molecules, performing similarly to, or in some cases better than, the conventional methods tested. The main

products obtained were: (i) fatty acid-rich extracts with antiproliferative, antioxidant, and anti-inflammatory potential; (ii) protein derivative-rich extracts that have shown to be promising antioxidants and antimicrobials; (iii) AXT-rich extracts with antiproliferative and antimicrobial effects; (iv) protein hydrolysate-rich extracts that have shown to be antioxidants; and (v) chitin that has shown to be highly pure and porous, with interesting features regarding thermal stability, degree of acetylation, and crystallinity. In general, all extracts resulting from the application of alternative extraction methods performed better in terms of bioactive potential than the extracts resulting from the processes using conventional solvents, particularly when DES were used as solvents and were maintained as part of the final product. In addition, results have also shown that the processes could be integrated, thus allowing the minimisation of feedstock consumption, while preserving most of the characteristics of the final products. Globally, the slightly different bioactive effects or physicochemical properties obtained for some of the samples resulting from process integration did not seem to have a strong impact on the products' final application, except for chitin, which revealed to have decreased its purity by 1.5-fold due to a contamination arising from the extraction of AXT. Finally, LCA evaluation has shown that it is possible for the integrated processes reported herein to be competitive, particularly if more reliable and accurate models, energy integration, water recycling and re-use, replacement/reduction of fossil resources by renewable energy, increased equipment efficiency and reduction of resources (including solvents/chemicals), recycling and re-use of some of the solvents/chemicals, among others, are considered at commercial scale.

Overall, this research suggests that the process technologies developed during this PhD thesis may enable the utilization of currently undervalued marine-processing waste streams in the development of new functional food, health, or cosmetic/personal care bioactive products, thus contributing to the future implementation of a seafood waste-based biorefinery and the establishment of a circular bioeconomy model.

Resumo

A forte dependência da matriz energética global, bem como dos processos produtivos, em relação aos recursos fósseis tem suscitado graves problemas ambientais e diversas preocupações económicas. Desta forma, a procura por soluções alternativas que permitam uma melhoria da qualidade de vida das gerações presentes e futuras, preservando simultaneamente o ambiente, assume uma importância primordial, nomeadamente através da redução significativa da exploração e consumo de combustíveis fósseis, bem como da implementação de tecnologias de processamento limpas. Contrariamente à utilização de recursos não renováveis, o percurso para um futuro sustentável reside, portanto, na implementação do conceito de biorefinaria como estratégia para a instituição de um modelo de bioeconomia circular, visando a produção de uma ampla gama de produtos e energia, utilizando como matéria-prima recursos renováveis, *i.e.*, biomassa.

Durante o processamento de alimentos, a geração de resíduos é muitas vezes inevitável. No entanto, a sua valorização pode contribuir tanto para a maximização do retorno financeiro, como para facilitar a resolução de problemas, tais como o crescente aumento da deposição de resíduos em aterro ou a sobre-exploração dos recursos naturais.

Quando processados para consumo humano, o peixe e o marisco são uma fonte importante de excedentes, gerando uma quantidade de resíduos equivalente a 20 a 75 % do peso total do pescado. Em particular, o processo de conserva de peixe ou o processamento de crustáceos e moluscos para consumo humano, são das maiores fontes de resíduos e subprodutos resultantes do processamento de pescado, gerando mais de 2 milhões de toneladas de resíduos por ano, apenas na União Europeia. No entanto, após o seu processamento, estes resíduos ainda conservam na sua composição quantidades significativas de produtos de alto valor acrescentado, tais como ácidos gordos, proteínas e seus derivados, pigmentos, biopolímeros e minerais, que têm vindo a demonstrar um enorme potencial para aplicações nas indústrias alimentar e de bebidas, farmacêuticas, biomédicas, nutracêuticas, cosméticas, agroquímicas, biotecnológicas, entre outras.

Tendo em conta este enquadramento, o trabalho desenvolvido nesta tese de doutoramento teve como principal objetivo a conceção e o desenvolvimento de abordagens sustentáveis que permitissem a conversão de resíduos de sardinha e sapateira em produtos de alto valor

acrescentado. A estratégia aplicada passou pela combinação de diversas tecnologias, incluindo a utilização de dióxido de carbono supercrítico (CO_2-sc), água subcrítica (Asc) e sistemas eutécticos (SE), para a extração de ácidos gordos, derivados e hidrolisados de proteínas, carotenoides (com especial foco na extração de astaxantina, AXT) e quitina a partir destes efluentes.

Dependendo do processo, por forma a maximizar a extração dos compostos alvo, estudaram-se diferentes parâmetros com reconhecido impacto na sua recuperação:

(i) A extração com CO_2-sc foi aplicada para a recuperação de ácidos gordos a partir de resíduos de sardinha, variando as condições de pressão (300, 425, e 550 bar), temperatura (35, 55, e 75 °C) e caudal de CO_2 (5, 15, e 25 g/min);

(ii) Os derivados de proteínas foram extraídos a partir de resíduos de sardinha utilizando SE à base de betaína/polióis, e as condições operacionais estudadas incluíram a temperatura (25, 45, e 80 °C), o tempo de extração (6 e 18 h) e o rácio sólido-líquido (1:8, 1:16, 1:40, e 1:80 g/g);

(iii) A AXT foi extraída a partir das cascas de sapateira utilizando SE à base de terpenos/ácidos gordos, através da avaliação do impacto da temperatura (30, 45, e 60 °C) e do tempo de extração (2, 6, e 24 h);

(iv) A Asc foi aplicada para a extração de hidrolisados de proteínas a partir das cascas de sapateira, avaliando-se o impacto da temperatura (150, 200, e 250 °C), do rácio sólido-líquido (1:5, 1:10, e 1:15 g/mL) e da velocidade de aquecimento do reator (3 e 6 °C/min);

(v) E finalmente, a quitina foi recuperada a partir das cascas de sapateira utilizando SE à base de cloreto de colina/ácidos orgânicos, testando variações nas condições de temperatura (50, 80 e 130 °C) e tempo de processamento (2,5, 3, e 4 h).

Sempre que os SE foram utilizados como solventes, as suas propriedades físico-químicas e toxicológicas foram também estimadas.

De forma a avaliar a potencial aplicação dos extratos de sardinha/sapateira como ingredientes funcionais, bem como a sua segurança, dependendo da molécula alvo selecionaram-se diferentes ensaios de bioatividade, incluindo citotoxicidade e potencial antioxidante, antimicrobiano, antiproliferativo e anti-inflamatório. Adicionalmente, de forma a validar o conceito, efetuou-se um aumento de escala para os processos mais

promissores (até 70 vezes a escala inicial), bem como a sua integração e avaliação de impacto ambiental, através da análise do ciclo de vida (ACV).

Os resultados apresentados ao longo dos capítulos desta tese demostraram que as metodologias de extração aplicadas permitiram recuperar com sucesso as diferentes moléculas alvo, tendo um desempenho semelhante, ou em alguns casos superior, ao dos métodos convencionais testados. Os principais produtos obtidos foram: (i) extratos ricos em ácidos gordos com potencial antiproliferativo, antioxidante e anti-inflamatório; (ii) extratos ricos em derivados de proteínas que demonstraram ser promissores antioxidantes e antimicrobianos; (iii) extratos ricos em AXT com efeito antiproliferativo e antimicrobiano; (iv) extratos ricos em hidrolisados de proteínas que demostraram ter potencial antioxidante; e (v) quitina de elevada pureza que apresentou uma superfície porosa e características interessantes no que diz respeito à estabilidade térmica, grau de acetilação e cristalinidade. Em geral, todos os extratos resultantes das extrações com solventes alternativos demonstraram ter um melhor desempenho em termos de potencial bioativo do que as amostras resultantes das extrações com solventes convencionais, particularmente quando os SE foram utilizados como solvente e foram mantidos como parte integrante do produto final. Além disso, os resultados apresentados demonstraram também a possibilidade de integração dos diversos processos, permitindo assim a minimização do consumo de matéria-prima e preservando simultaneamente, na sua grande maioria, as características dos produtos finais. Globalmente, as ligeiras diferenças detetadas em termos de efeitos bioativos ou características físico-químicas em algumas das amostras resultantes da integração dos processos, não aparentaram ter um forte impacto na aplicação final dos produtos, exceto no caso da quitina, cuja pureza diminuiu cerca de 1.5 vezes devido a uma contaminação resultante da extração da AXT. Finalmente, a avaliação de ACV demostrou que os processos integrados descritos neste trabalho podem ser competitivos, particularmente se se considerar a aplicação à escala comercial de modelos mais confiáveis e precisos, de uma forte integração energética, da reciclagem de água e da sua reutilização, da substituição/redução dos recursos fósseis utilizados por energias renováveis, do aumento da eficiência dos equipamentos e da redução dos recursos (incluindo solventes/químicos), da reciclagem e reutilização de alguns dos solventes/químicos, entre outros.

Globalmente, este trabalho de investigação sugere que as tecnologias de processamento desenvolvidas ao longo desta tese de doutoramento podem vir a possibilitar a utilização de efluentes resultantes do processamento de pescado, atualmente subvalorizados, no desenvolvimento de novos produtos bioativos, tais como alimentos funcionais, ou produtos de saúde e bem-estar, contribuindo assim para a futura implementação de uma biorefinaria baseada em resíduos de origem marinha e para a instituição de um modelo de bioeconomia circular.

List of publications

Papers published in international peer-reviewed scientific journals (6)

Rodrigues, L. A., Redovniković, I. R., Duarte, A. R. C., Matias, A. A., Paiva, A. (2021). Low-phytotoxic deep eutectic systems as alternative extraction media for the recovery of chitin from brown crab shells. *ACS Omega*, *6*(43), 28729-28741, doi: 10.1021/acsomega.1c03402

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Gargalo, C. L., Rodrigues, L. A., Paiva, A., Carvalho, A. Life cycle assessment of a canned sardine residue upcycling process in Portugal. *In preparation*.

Gargalo, C. L., Rodrigues, L. A., Paiva, A., Carvalho, A. Life cycle assessment for early-stage design: upcycling of crab shell. *In preparation*.

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Pitch presentations (1)

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CHAPTER 1

Introduction

CHAPTER 1

Contents

1. The biorefinery concept

Much of the world's energy and products remain heavily dependent on fossil fuels. However, the feasibility of the continued use of these non-renewable resources has already started to decrease, due to an increasing demand for energy (4 to 9% increase between 2019 and 2030[1]), the environmental concerns that their use have arisen (including the production of toxic waste, land degradation, greenhouse gas emissions, and water and air pollution[2]), and the recurrent price increases related to their depletion[3,4]. According to the Production Gap Report, to avert a potential climate catastrophe and meet the goals defined by the Paris Agreement (that aims to limit global warming to below 2 °C, compared to pre-industrial levels), countries must decrease their fossil fuel production by 6% per year between 2020 and 2030[5,6]. Therefore, a pressing need for solutions that have not only the ability to mitigate climate changes, but also to promote a reduction of the production and consumption of fossil fuels, has emerged[3,7]. In recent years, governments and society at large have begun to recognise the opportunities that can be offered by a sustainable economy based on renewable resources. The replacement of crude oil with biomass (*i.e.*, biological materials from living or recently living organisms) has emerged as a promising alternative for the development of products and energy, and it has been the driving force for the development and implementation of biorefineries[3,8].

The biorefinery concept is defined as the "sustainable processing of biomass into a spectrum of marketable products and energy"[9]. It involves a great diversity of technologies directed towards the fractionation of biomass resources into their building blocks, which can then be converted into value-added products, chemicals, and energy[3]. This concept is in some way analogous to a crude oil refinery, which produces the most diverse products and fuels using petroleum as raw material. Although biomass and fossil fuels present a completely different composition, it is possible to produce a similar range of end products by using renewable resources that can be equally competitive in the market, leading to a progressive replacement of fossil fuel-based products[3,7]. As reviewed by Ubando *et al.*, the biorefinery concept has already been successfully applied for the production of a wide range of bio-based products and energy from different feedstocks, such as lignocellulosic and algae biomass, and different wastes, including non-edible feedstocks and biogenic residues (such as manure), and food and microbial-treated wastes[4].

The concept of circular bioeconomy was introduced in the 1990s as an alternative to the traditional linear economy approach (and current economic development model), which is based on the "take, make, dispose" framework[4]. Aiming at reducing the impact of climate change, land and ecosystem degradation, and mitigating population growth effects, this concept has become a major European Union (EU) priority in terms of policies[10], since it has the potential to minimize the environmental burden associated to industrial processes, through the redesign of the product's life cycle and the recycling or reusing of by-products in a closed loop within the industrial network, thus limiting or hindering the consumption of raw biological resources, while reducing waste generation. In this way, the transition of oil refineries to biorefineries can be foreseen as a potential strategy to reduce the use of fossil resources, while supporting the transition from a linear economy to a circular bioeconomy[4,11]. Nevertheless, in order to promote a sustainable production, within the biorefinery concept that reduces or eliminates the use or generation of hazardous substances, the integration of the 12 principles of green chemistry[12], along with the application of low environmental impact technologies, is of the utmost importance[3].

2. The food waste-based biorefinery

Every year, around one-third of the global food production is lost or wasted, corresponding to 1.3 billion tonnes of food, which costs the world's economy about US$ 750 billion. Furthermore, as the world's population increases, these figures are expected to increase as well. For this reason, this highly ineffective food system has had a serious impact on all three pillars of sustainability (economic, environmental, and social), which has led to the development of different strategies, guidelines, and policies, not only to reduce, but also to repurpose food waste[13,14]. In particular, the EU is committed to halving per capita food waste at retail and consumer levels by 2030[15], within the European Green Deal[16] and in line with the United Nations' Sustainable Development Goals[17].

So far, although there has been an effort to repurpose some of these wastes for example for animal feed, composting, anaerobic digestion, or fermentation, the financial return has been usually negligible[11,13].

Food losses and waste are generated at all stages of the value chain, from production and harvest, post-harvest, processing, and distribution (185 kilograms per capita per year in

Europe), to household consumption (95 kilograms per capita per year in Europe)[18]. When processed at the consumer's stage, the waste composition presents a high heterogeneity level. However, when generated at the primary production or at the processing stages, the wastes present a high homogeneity in terms of composition, thus representing a potential viable feedstock for the production of bio-based value-added products and chemicals, within the biorefinery concept[11,19]. In fact, the conversion of these food wastes into marketable products can be up to 3.5-, 7.5-, and 10-fold more profitable than their conversion into fuel, animal feed, or electricity, respectively[13].

Figure 1 illustrates through a chord diagram some of the possible valorisation pathways for each type of food waste, the value-added products resulting from their valorisation, and their possible field of application.

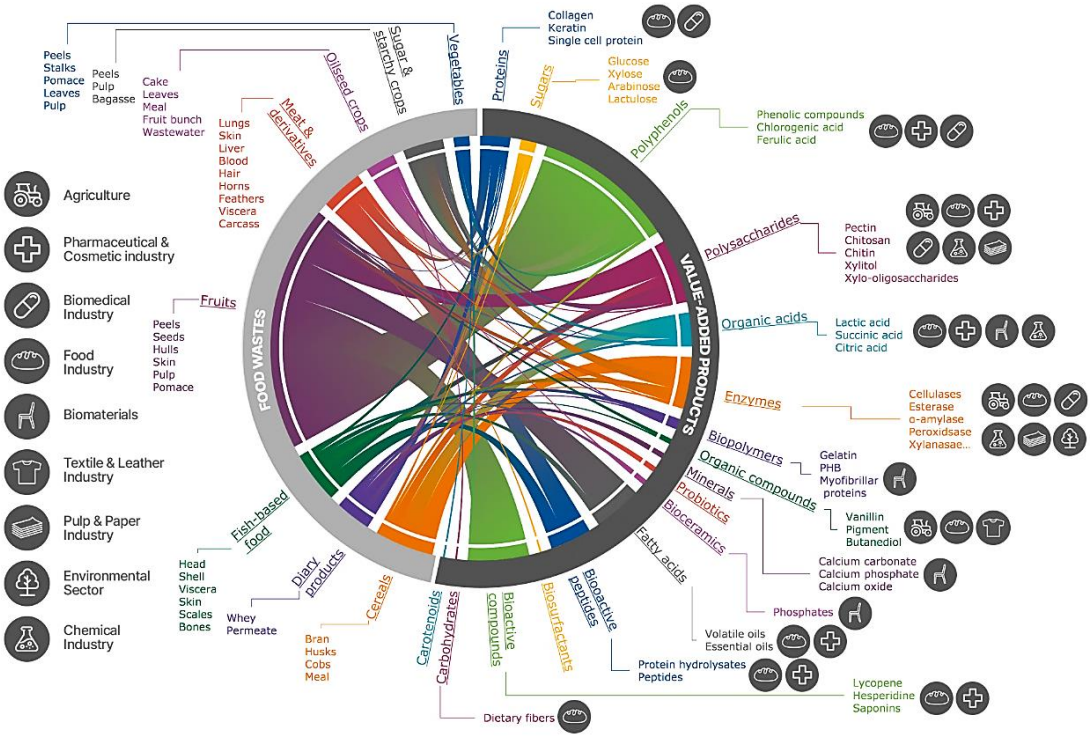

Figure 1. Valorisation pathways for different food wastes, resulting valued-added products, and respective area of application. (Adapted from Caldeira *et al.*)[19].

As can be observed in Figure 1, a significant amount of different research works has shown that the most diverse food residues can be used for the production of valuable ingredients, which can find applications in areas such as the food, pharmaceutical, cosmetic, or medical

industries, in the production of biomaterials, in agriculture, among others. In this way, although the use of food waste as feedstock in biorefineries is still at an early stage of development, it has been gaining interest in recent years, as new technologies for their valorisation are being explored[19]. Furthermore, food waste can represent an alternative to first-generation biomass feedstocks, which compete with food crops[4]. In this way, these recent developments are key to foster the implementation of new policies and to accelerate the implementation of food-waste based biorefineries.

3. Seafood by-products and wastes

Current fishing practices have been consistently recognized as unsustainable. Seafood discards have been a key contributing factor to this problem, not only due to their recognized economic and environmental impact, but also due to the loss of potential food resources[20]. On the other hand, the global fish processing market expansion, which is projected to reach a volume of more than 37 megatonnes and a value of around US$ 223 billion this year (driven by the high nutritional profile and added health benefits of processed fish or fish products, the growth of aquaculture industry, and the increase in health consciousness towards value-added fish products[21]), will continue creating increasing quantities of by-products, accounting for up to 75% of the harvested seafood total weight[22,23]. Fish filleting, salting, and smoking are responsible for generating the largest amount of solid wastes and by-products (50 to 75% of the fish), corresponding to more than 3.1 million tonnes of waste per year in the EU alone[24]. Fish canning, an important economic sector in North-Western Spain and in Northern Portugal[25], is regarded as the second largest source of solid fish-processing wastes and by-products (30 to 65% of the fish), generating more than 1.5 million tonnes per year of waste in the EU. Crustaceans and molluscs are also responsible for generating considerable amounts of residues (20 to 50% of the fish), corresponding to approximately 500 thousand tonnes per year in the EU[24].

Until recently, seafood waste or by-products were often discarded, used directly as feed, or in silage and fertilizers, resulting in significant economic losses for fish processing companies. However, in the last two decades there has been an endeavour to find sustainable applications that can be more relevant in terms of revenue, so that these resources can be used more efficiently[22]. In fact, fish and shellfish waste streams are still an

important source of relevant ingredients, including fatty acids; natural pigments (carotenoids); different proteins, peptides, amino acids, and enzymes; biopolymers, such as collagen and gelatine, or chitin and chitosan; minerals such as calcium, phosphorus, calcium phosphates, or calcium carbonate; among others[22,24]. Figure 2 illustrates some of the possible valorisation pathways for the different waste streams resulting from fish and shellfish processing, as well as some of the technologies used to obtain each of the bioactives[19].

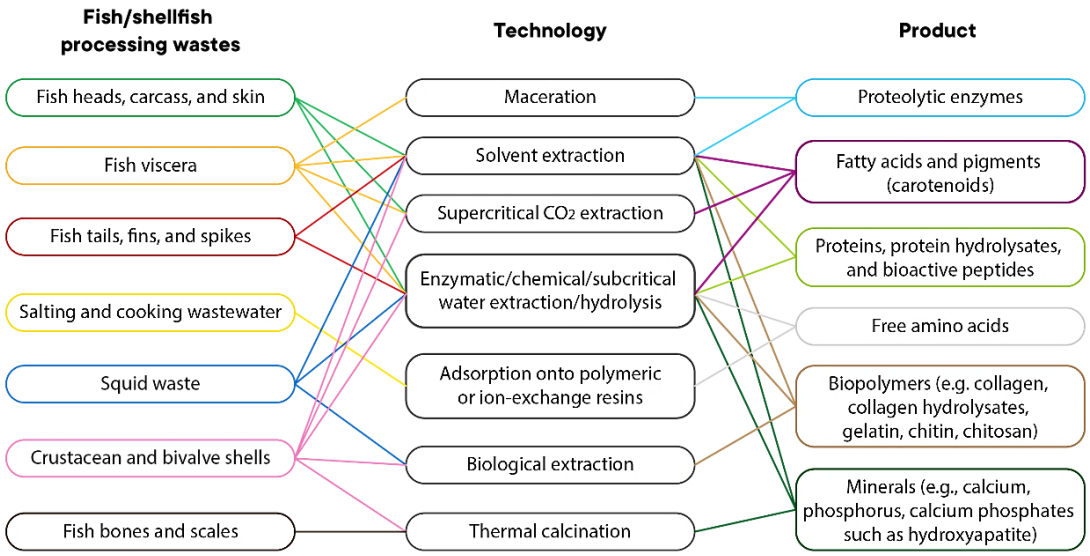

Figure 2. Valorisation pathways for fish and shellfish processing wastes. (Adapted from Caldeira *et al.*)[19,22–24,26].

Depending on the ingredient obtained, these strategies can then lead to their application in a wide range of market segments, including food or feed products and supplements, dietetic products, natural pigments, cleaning products, agrochemicals, pharmaceuticals, biomedical products, nutraceuticals, cosmetics, leather, biodiesel, or biogas production, among others[22]. Therefore, an integrated approach for the valorisation of such wastes, within the context of circular bioeconomy, would not only avoid the disposal or the low-value application of increasing amounts of fish and shellfish residues, but would also prevent resource depletion, while promoting the financial return of fish processing companies, being, on the one hand, an economic opportunity for biorefineries, particularly food waste-based biorefineries, and similar industries; and on the other hand, a solution for the high

costs, legal restrictions, and environmental problems associated to the generation and subsequent disposal of such wastes.

4. High value-added compounds in marine by-products and wastes

As previously mentioned, marine by-products and wastes are an important source of relevant molecules, including fatty acids, carotenoids, proteins and their building blocks, biopolymers, minerals, among others, that can be recovered from those feedstocks and further applied in a wide range of economically interesting markets[22,24].

4.1. Lipids

Lipids are a large group of macro-biomolecules, that includes fatty acids and their derivatives, polar lipids, glyceryl esters, carotenoids and other isoprenoids, fat-soluble vitamins (such as vitamins A, D, E, and K), phenolipids, among others[27], which can still be found in significant quantities (up to 40%) in fish and shellfish waste streams[24,28].

4.1.1. *Fatty acids*

Fatty acids can be classified as carboxylic acids with a variable unbranched aliphatic chain, which can be either saturated (SFA) or unsaturated (*i.e.*, monounsaturated (MUFA) or polyunsaturated (PUFA))[27]. These molecules are widespread in nature, being the major constituents of triglycerides, phospholipids, glycolipids, sphingolipids, sterols, among other complex lipids.

Fatty acids have important biological structural and functional roles in the human body, being the main components of cellular membranes, assuring their fluidity, flexibility, and permeability, as well as the passive transport throughout the membrane[29]. In particular, triglycerides, and consequently fatty acids, are the main contributors to dietary fat in humans, being an important source of energy[30].

Due to their multiple biological roles, including regulating the inflammatory cascade, reducing oxidative stress, and presenting neuro- and cardiovascular protection, essential ω-3 and ω-6 PUFA appear to be the most important fatty acids for human health. The ω-6 fatty acids, being linoleic acid the most important, are the predominant PUFA in all diets. However, in diets highly rich in ω-6 fatty acids, these molecules can be converted into pro-inflammatory prostaglandins. On the other hand, in diets rich in ω-3 fatty acids, the

metabolites formed have recognized anti-inflammatory properties. Therefore, to balance the diet with a healthy ratio of ω-3 and ω-6 fatty acids is particularly important[29].

Seafood products have not only been recognized as an important natural source of different classes of lipids, but have also been regarded as the most prevalent source of long-chain ω-3 PUFA, such as eicosapentaenoic (EPA) or docosahexaenoic acids (DHA)[31]. In particular, fish oil usually consists of fat-soluble vitamins (mainly A and D), sterols, glycerides, free fatty acids, phospholipids, and sterylesters. The typical fatty acid composition of fish oil is usually divided into SFA (mainly myristic, palmitic, stearic, and behenic acids), MUFA (generally myristoleic, palmitoleic, oleic, eicosenoic, gadoleic, erucic, and catoleic acids), and PUFA (including linoleic and α-linolenic acids, EPA, and DHA), whose structures are represented on Figure 3[32].

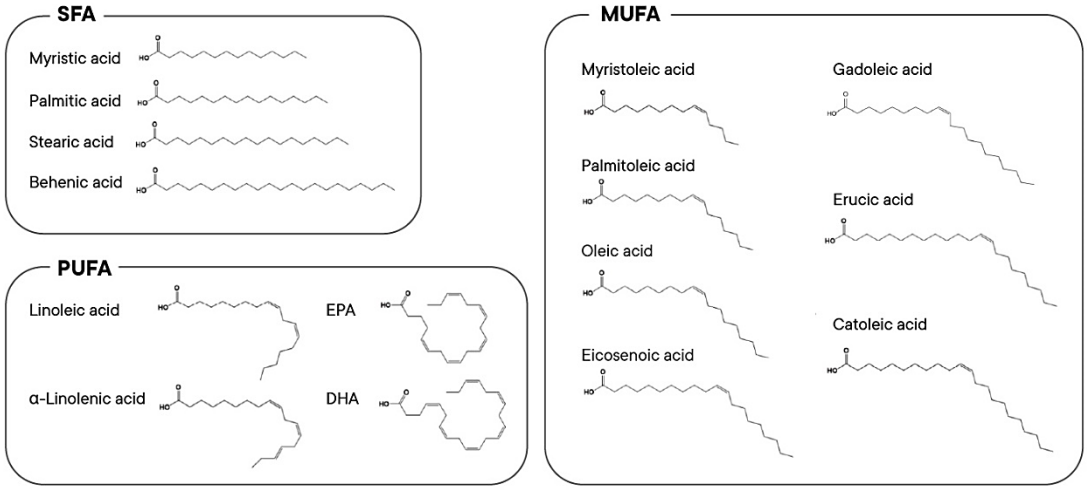

Figure 3. Chemical structures of fish oil-derived fatty acids. (Adapted from Huang *et al.*)[32].

Ever since Dyerberg and co-workers suggested for the first time that an increase in the intake of EPA, whether by dietary change or by supplementation, could reduce the risk of developing thrombosis and atherosclerosis[33], evidence of the importance of ω-3 fatty acids in the prevention and treatment not only of cardiovascular disease, but also diabetes, cancer (*e.g.* colorectal, lung, breast, prostate), inflammatory, neuropsychiatric, neurodegenerative, and skin-related diseases (*e.g.* cancer, wounds, dermatitis), or even in the promotion of visual and neurological development or in maternal and child health, has been accumulating, resulting from numerous pre- and clinical studies[32,34–36].

The global fatty acids market (including SFA, MUFA, PUFA, and *trans* fats) was valued at US$ 31.1 billion in 2018. Forecasts indicate that the market should continue to grow at a compound annual growth rate of 5.2%, reaching a value of US$ 45.8 billion by 2026[37]. In particular, the growth of the market involving ω-3, ω-6, ω-7, and ω-9 fatty acids, whose main applications are the sectors of dietary supplements, infant formulas, pharmaceuticals, food and beverages, animal feed, and cosmetics, should be fostered not only by a rise in awareness of the health benefits associated to unsaturated fatty acids and the consumers' demand for healthier food products, but also by the development of innovative technologies for their production[38].

The countries that have been dominant in the global production of fatty acid concentrates, through the extraction of fish oil, are Peru, USA, and Chile, while Norway, Denmark, and Iceland are the biggest producers in Europe[39]. The production of fish oil has been mainly performed by using the wet pressing method, with the simultaneous production of fish meal. The process comprises the following steps: i) cooking the raw material for protein coagulation, thereby destroying the bound between water and oil, (ii) separation by pressing the cooked material, yielding a solid and a liquid phase, iii) removal of most of the sludge in the liquid phase by centrifugation in a decanter, and iv) subsequent recovery of the oil by centrifugation[40]. This process has been successfully used for many years on fish products or by-products with high-oil content (mainly pelagic species). However, when using species with low-oil content, the results are not so promising. Therefore, since the beginning of the 20th century, numerous patents describing new extraction apparatuses, alternatives to improve the extraction yields, the quality of the oil, or the profitability of the process, have been published. Two technologies that have been highlighted in several of these publications are supercritical fluid extraction (SFE), using CO_2 as a solvent (sc-CO_2), and the extraction with enzymatic (proteolytic) methods, for having demonstrated promising results in terms of oil yield and quality[41].

4.1.2. *Carotenoids*

Carotenoids are isoprenoids widely distributed in both plant- and animal-derived foods and can be classified into carotenes and xanthophylls according to their chemical structure (Figure 4): the carotene carotenoids include, among others, α-carotene, β-carotene, and

lycopene, while the xanthophyll carotenoids include lutein, canthaxanthin, zeaxanthin, violaxanthin, tunaxanthin, β-cryptoxanthin, astaxanthin (AXT), etc.[42,43].

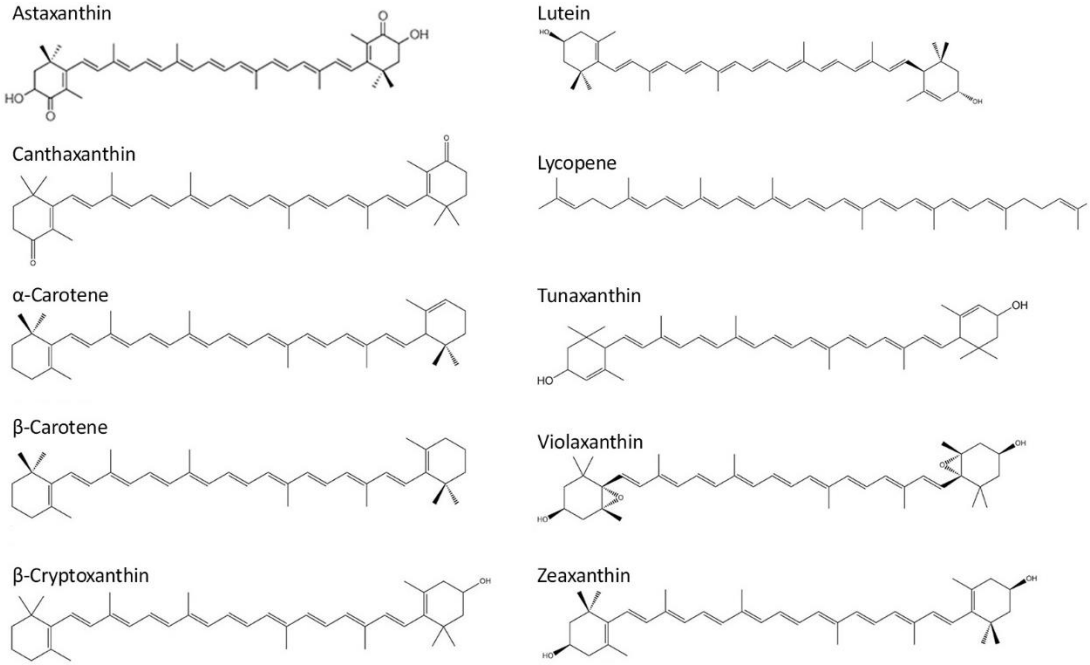

Figure 4. Chemical structure of common carotenoids. (Adapted from Meléndez-Martínez *et al.*)[42].

By-products and wastes resulting from food processing can also be rich sources of carotenoids, including for example tomato waste for lycopene; carrot, apricot, or mango waste for β-carotene; and shellfish shell waste for AXT. Some of these natural pigments can be converted into vitamin A, which is an essential nutrient for humans, being also able to act as health-promoting compounds, as different studies have shown that some carotenoids can reduce the risk of certain types of cancer (cervical, ovarian, colorectal, prostate, or breast), cardiovascular diseases, and bone, skin, or eye disorders. In addition, they can also have a preponderant role in promoting mental and metabolic health, or a healthy pregnancy and early life. Given their bioactivities, in recent years, carotenoids have also attracted interest in the field of functional foods, nutraceuticals, and nutricosmetics[42].

4.1.2.1. Astaxanthin

AXT is a red-orange ketocarotenoid naturally occurring in different living organisms, particularly in the marine environment, being present in a number of microorganisms, some

algae, crustaceans, salmonids, zooplankton, among others[44]. Although AXT can be found in appreciable amounts in these marine organisms, it can only be biosynthesized as a secondary metabolite by microalgae or phytoplankton, accruing subsequently in zooplankton and crustaceans, and only later on in fish[44,45]. Therefore, these organisms owe their characteristic reddish-orange colour to AXT, which accumulates in their skin, muscle, exoskeleton, gonads, etc., from where it is transferred and structurally modified throughout the higher levels of the food chain[42,46]. In many of these aquatic animals, AXT is known to perform numerous essential biological functions including protection against oxidation and ultraviolet light, immune response, pigmentation, communication, reproductive behaviour, and improved reproduction[47].

Similar to marine animals, the vast majority of animals, including humans, cannot biosynthesize AXT or other carotenoids. Therefore, given their nutritional importance, their intake needs to be performed through diet or supplementation[42].

AXT molecule is composed of two terminal rings (β-ionone rings with hydroxyl group) united by a polyene chain (Figure 4)[48], being its red colour attributed to the conjugated double bonds at the centre of the molecule[45]. It exists in the form of stereoisomers, geometric isomers, free, or esterified, all of which can be found in nature, being all-*trans* astaxanthin the dominant isomer[48,49]. Based on its polyene system, AXT acquires a unique molecular structure, as well as distinctive chemical properties and light-absorption features. Additionally, the hydroxyl and keto moieties on each β-ionone ring contribute not only to the more polar configuration of the molecule, which optimizes the rate and extent of its absorption, but also to a higher antioxidant capacity when compared to other widely known antioxidants[44,45,50]. In particular, the polar end groups of the molecule have the ability of quenching free radicals, whereas the conjugated double bonds of the polyene act by donating electrons and by reacting with free radicals. AXT is also able to preserve the integrity of cell membranes by inserting in their bilayers, and to protect the redox state and functional integrity of the mitochondria[44,48]. Unlike other carotenoids, this fat-soluble pigment is not able to convert into retinoids and, therefore, has no vitamin A activity on the human body[48]. However, due to its unique structure and strong antioxidant potential, AXT has been associated to a wide range of additional biological activities, including antimicrobial, anti-inflammatory, anti-lipid peroxidation, antidiabetic, and anticancer

effects, neuro-, cardiovascular, ocular, gastro-, hepato-, and skin photo-protection, muscle endurance, immune response, and fertility improvement, among others[44,45,48,50].

AXT commercial products present, therefore, a variety of health claims, and are currently available in numerous forms, such as biomass, extracts, or different formulations (either isolated or combined to other carotenoids, multivitamins, herbal extracts, ω-3/ω-6 fatty acids, etc.)[48]. Furthermore, AXT has also been extensively used as colouring agent and natural additive for aquaculture (in the cultures of salmon, trout, and shrimp) and animal feed (mainly for poultry), this being the largest application segment[45,51]. Moreover, other market segments such as pharmaceuticals and nutraceuticals, food and beverages, and cosmetics and personal care products, have prompted interest in the last years. In particular, among all AXT market segments, nutraceuticals are estimated to achieve the largest growth over the next years[51].

The global AXT market size was estimated at over US$ 583 million in 2019. Estimates point to a market expansion, driven by a growing preference towards natural therapeutic options, eventually reaching a value of US$ 2.3 billion by the end of 2030[52].

Although AXT can be found and recovered from numerous natural sources, including yeasts and crustaceans, the green microalgae *Haematococcus pluvialis* has been considered to have the highest capacity to accumulate this carotenoid[45,51], and has become, therefore, the primary source of AXT used for human-related applications[48]. In fact, natural, as opposed to synthetic AXT produced from petrochemicals, has held the largest market share in 2019. Accordingly, and owing to its great demand and the safety issues regarding the use of synthetic AXT for human consumption, the growth of natural AXT market is expected to accelerate in the coming years[51].

The strong bond between carotenoids and other macromolecules, such as proteins or fatty acids, has posed a challenge in their extraction from natural sources. For this reason, carotenoid extraction often involves a pre-treatment step using physical (*e.g.*, cooking, drying, milling, ultrasound, osmotic shock, freeze-thaw, cryogenic grinding), chemical (*e.g.*, acid, base, surfactants, liquid nitrogen), enzymatic, or biological methods, in order to increase the extraction efficiency[53,54]. Additionally, due to their highly unsaturated structure, carotenoids are susceptible to oxidation, which limits their exposure to high temperatures, light, acid media, or long extraction times[54].

Given their hydrophobic nature, carotenoids have been traditionally extracted using organic solvents, including hexane, petroleum ether, or tetrahydrofuran, in what concerns non-polar carotenes or esterified xanthophylls; while acetone, ethanol, and ethyl acetate have been applied for the recovery of more polar carotenoids, such as AXT[54]. However, the efficiency of this extraction method relies mostly on the high temperatures and extraction times applied, as well as on solvent/solute interactions. Although organic solvent extraction is a technology easily scalable, problems have arisen related not only to the toxicity of the solvents used, but also to the high energy costs associated to the recovery of the solvent after extraction[53,55]. Recently, different authors have proposed the application of alternative solvents to overcome some of these disadvantages, namely vegetable oils, surfactants, deep eutectic systems (DES), ionic liquids (IL), and pressurized or supercritical fluids. There are some alternative extraction technologies that have also gained some interest in recent years, namely ultrasound-, microwave-, enzyme-, and pulsed or moderate electric field-assisted extraction[54,56–58]. Therefore, although the extraction methods for carotenoid, and in particular AXT recovery, have been rapidly emerging, not only through the application of non- or low-toxicity solvents, but also through the development of more sustainable technologies that allow faster and more effective extractions (in terms of both yield and cost), it remains a main challenge for the industry. Accordingly, further methodological and technological advancements, as well as their consolidation, are desirable.

4.2. Proteins, peptides, and amino acids

Proteins are complex biopolymers formed by long chains of amino acids, linked by peptide bonds. These macromolecules are not only species-specific, but also organ-specific[59]. In particular, fish, molluscs, and crustaceans' muscle are very similar in structure, with a protein content that can range from 17 to 22% for vertebrates and from 7 to 23% for invertebrates (in a fresh weight basis), being mainly composed of myofibrillar, sarcoplasmic, and stroma or connective-tissue proteins. Myofibrillar proteins (soluble in high-salt solutions), such as myosin, actin, tropomyosin, troponin, actinin, desmin, nebulin, other C and M proteins, or paramyosin (only found on invertebrate muscle), can account for up to 75% of the total protein contained in fish and shellfish muscles; sarcoplasmic proteins (soluble in water or low-salt solutions), including myoglobin, haemoglobin, cytochrome

proteins, and endogenous enzymes, represent up to 35% of the total muscle tissue protein; and stroma proteins, which are mainly composed of collagen, elastin, and gelatine, are present in lower concentrations in the muscle (up to 3%), although in some fish (such as shark, ray, or skate) they can account for up to 10% of total muscle protein[60,61]. Additionally, apart from muscle protein, marine arthropods also contain a significant fraction of protein in their exoskeleton that perform important functions such as biomineral growth, maintenance, or repair[62].

In recent years, marine processing wastes, including fish and shellfish, have been recognized as an important source of high-quality protein, which can be found in quantities up to 20% of the total protein content, thus representing a strategy for value creation through mining of proteins, peptides, and amino acids[61].

Depending on the target molecule, different methods can be used for the isolation of proteins and their building blocks, including solvent extraction, enzyme- or microwave-assisted extraction, chemical or enzymatic hydrolysis, microbial fermentation, among others, which may significantly affect not only the process specificity or selectivity, but also the biological activities and bioavailability of the isolated molecules[63–65]. A typical procedure performed at commercial scale involves a first extraction step, in which proteins are isolated from the feedstock using solvents such as methanol or ethyl acetate. The volatile organic solvents are then concentrated, and the proteins or peptides partitioned with hexane, carbon tetrachloride or dichloromethane. However, this method as revealed to be time-consuming, expensive, and environmentally unfriendly. Therefore, there is a need to develop suitable, cost-effective, and safe technologies for the recovery of proteins from food-derived products[66]. When the aim is to obtain specific peptides or amino acids, the protein crude extract can be further processed and subjected to chemical or enzymatic hydrolysis, being the enzymatic hydrolysis preferred in the nutraceutical and pharmaceutical industries, as it avoids harsh chemical or physical treatments, while preserving the functionality and nutritive value of the isolated molecules. Alternatively, the feedstock can also be directly hydrolysed without prior extraction of proteins[60,66]. The resulting extract or hydrolysate can then be purified by membrane separation, such as nanofiltration, ultrafiltration, or electro-membrane filtration, and through different chromatographic methods[64,66].

Introduction

Given their high structural diversity, in addition to the known nutritional and functional properties, dietary proteins, including those found in fish and shellfish, have also been associated with different health promoting activities[60]. However, due to an increased absorption capacity and bioavailability, in most cases, protein hydrolysates or peptides have a higher bioactive potential when compared to their parent proteins[67]. Although it may vary in certain cases, these peptides usually range in size from 2 to 20 amino acid residues, being their bioactivities mainly related to their structure, *i.e.*, the amino acid composition and sequence, the type of N- and C-terminal amino acids, the charge character of the amino acids, the hydrophobic/hydrophilic nature of the amino acids, the length of the chain, among others[68–70].

The first food-derived bioactive peptides were reported by Mellander in 1950, which described the power of casein phosphopeptides to enhance bone calcification in rachitic infants, without the concurrent administration of vitamin D[71]. Since then, a growing body of scientific evidence has demonstrated the potential of food-derived, and in particular marine-derived peptides, to be applied for their multifunctional properties, including antioxidant, anti-inflammatory, antimicrobial, cardioprotective (antihypertensive, anti-atherosclerotic, and anticoagulant), anticancer, antiviral, antidiabetic, analgesic, opioid-like, mineral-binding, immunomodulatory, neuroprotective, photo-protective and anti-photo aging, amongst others[66,70,72].

The global protein ingredients market is valued at over US$ 42 billion, being expected to expand and to reach US$ 85.5 billion by 2028[73]. The food and beverages segment holds the largest revenue share of the market, which includes dietary or weight management supplements, snacks, sports nutrition, among others. Other important applications include animal feed, infant formulations, and clinical nutrition[67]. In particular, the growing of research and development in protein ingredient-based products for application in clinical nutrition has resulted in an increased number of products entering the market after being designed by nutraceutical and biopharmaceutical industries[66,73]. While the nutraceutical products tend to use protein hydrolysates in their formulations, the production of pharmaceutical drugs is usually performed using isolated and pure peptides[66]. Some examples of successful pharmaceutical, nutraceutical, and cosmetic/personal care products are summarized on Table 1.

Table 1. Examples of commercialized marine-derived peptide-based products (Adapted from Cheung *et al.*)[66].

Product designation	Type of product	Compound and source	Application
Ziconotide	Drug	ω-Conotoxin (neurotoxic peptide) isolated from the venom of *Conus magus*	Analgesic
Brentuximab vedotin	Drug	Dolastatin 10 isolated from *Dolabella auricularia*	Cancer treatment (*e.g.*, Hodgkin's lymphoma)
Katsuobushi oligopeptide	Nutraceutical	Pentapeptide isolated from a bonito hydrolysate	Antihypertensive
Dermochlorella®	Cosmetic/personal care	Oligopeptide extract from *Chlorella vulgaris*	Skin toner and firmer
Fish gelatine	Nutraceutical	Fish hydrolysates (including collagen and gelatine)	Nutrient supplements and bone health
Gabolysat PC60® Stabilium® Protizen® Procalm®	Nutraceutical	Fish hydrolysates	Anxiolytic
Seacure®	Nutraceutical	Fish hydrolysates	Intestinal health
Nutripeptin® Hydro MN Peptide®	Nutraceutical	Fish hydrolysates	Postprandial blood glucose control

4.3. Chitin

Chitin is a structural linear polysaccharide and the second most abundant biopolymer on Earth, only preceded by cellulose. It plays a supportive and protective role on the exoskeleton of arthropods (invertebrates such as crustaceans, arachnids, insects, etc.), on the beaks and endoskeleton of molluscs, on the cell walls of bacteria, fungi, and yeasts, on the spines of diatoms, on the eggshells of nematodes, among others[74,75].

The physicochemical properties of chitin are highly dependent on its source and on the isolation method. This polymer can be found in nature in three different crystalline allomorphs, *i.e.*, α- (the most common form, found in fungi, yeasts, krill, lobsters, crabs, shrimps, and insects), β- (found in squid pens), and γ-chitin (found in *Ptinus* beetles, Loligo squids, and mushrooms), which differ from one another in the orientation of the polysaccharide chains[74,76].

In particular, due to the great availability of by-products and wastes resulting from the seafood processing industries, crustacean shells, such as crab, lobster, or shrimp shells, are the most important chitin source for commercial purposes[75].

The exoskeleton of arthropods is a biological nanocomposite material, which is comprised by a complex fibrous structure formed by three fundamental elements, namely chitin, minerals, and proteins, as schematically represented on Figure 5 [77]. While chitin acts as a skeleton, minerals (mainly in the form of inorganic carbonate salts, such as calcium carbonate or calcium phosphate) provide the required strength to the shells, and proteins form the living tissue[78]. Although the extensive covalent and hydrogen bonding between these three components contributes to the mechanical strength that protects the soft body of crustaceans, this complex network also poses difficulties in separating the different elements that comprise the exoskeleton[79].

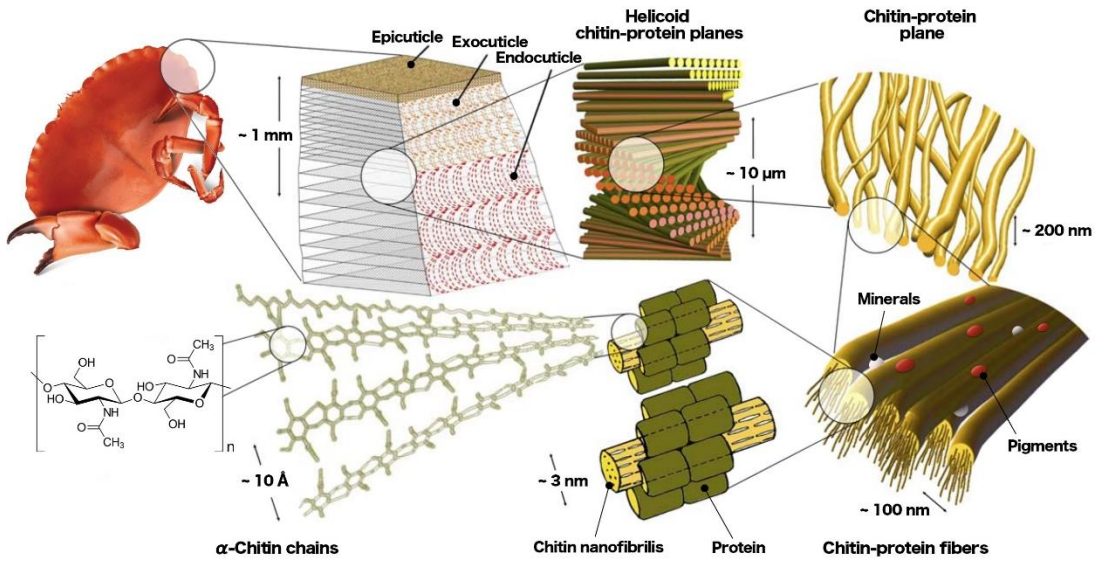

Figure 5. Main structural levels and microstructure elements of the crustaceans' exoskeleton material. (Adapted from Raabe *et al.*)[77].

Owing to its unique properties, chitin and its derivatives (*e.g.*, chitosan, glucosamine) have found many applications in a wide range of different sectors, as summarized on Table 2, including healthcare, biotechnology, animal feed, agriculture, food and beverages, and water treatment, representing a growing market valued at US$ 4.2 billion[80]. Furthermore, when compared to cellulose, chitin has an extra advantage due to the biologically fixed

nitrogen in its side chains, which allows the direct production of nitrogen-containing compounds, thereby overcoming the disadvantages associated to their traditional production from ammonia[81].

Table 2. Application examples of chitin and its derivatives. (Adapted from Pighinelli *et al.*)[76,81,82].

Sector	Application examples
Agriculture	Soil modifier, fungicide, seed coatings, fertilizer release, pest management
Catalysis	Green catalyst, scaffolds to support other catalysts
Bioengineering	Reduced susceptibility to echinocandin with elevated chitin levels in *C. albicans* cells
Biomedical	Enzyme immobilization and purification chelator, emulsifier, flocculent, blood cholesterol control, lectin affinity chromatography, biosensor immobilization of antibody, haemostatic agents
Materials	Packaging, film and sponge sheet materials, hydrogels
Chemicals	Furan derivatives, organic acids, amino sugars and amino sugar ethers, amine and amide polyols, heterocyclic pyrazines
Cosmetic	Lotions, hair additives, body creams, skin delivery formulations, periodontal gels
Energy production	Clostridium paraputrificum M-21 for hydrogen gas production
Food/feed and nutrition	Stabilizing agent, dietary supplements, antioxidant, emulsifying agent, food preservation (edible films), weight loss agents, food and feed additives, food packaging
Medical	Fibres, membranes, artificial organs and skin, surgical sutures, bone and cartilage regeneration, wound healing and dressings, cancer diagnosis, aid in cataract surgery, periodontal disease treatment, collagen synthesis, contact lenses, tumour therapy, stem cell technology, scaffolds, composite membranes, antibacterial nanofibrous mats
Paper industry	Paper wet strength improvement, protective coatings
Pharmaceutical	Manganese supplement complex, drug release, gene delivery
Pollutants removal	Copper removal, capability to obtain more stable diesel oil
Textile industry	Medical textiles, tear strength and antimicrobial activity improvement
Water treatment	Dye removal, absorbent for heavy and radioactive metals

Traditionally, the methodology for obtaining pure and colourless chitin from crustacean shell biomass involves a first demineralization step, followed by deproteination and subsequent

decolouration. Due to the extremely limited solubility of calcium carbonate in water and its low accessibility in the chitin-protein network, it is necessary to impose harsh conditions in order to recover it by using strong acids such as hydrochloric, sulfuric, nitric, acetic or formic acids, high temperatures (up to 100 °C) and long reaction times (up to 2 days). Subsequently, proteins and lipids are removed from the demineralized biomass through extraction with strong bases, such as sodium hydroxide or potassium hydroxide, at high temperatures (up to 100 °C) and for long times (up to 3 days). Finally, a step of oxidative bleaching to eliminate pigments is performed if a colourless chitin is desired[79,83,84]. Although this chemical method is known to be harmful for the environment, economically disadvantageous, and to negatively influence the physicochemical properties of native chitin, it still remains the most commonly used at industrial scale due to its high efficiency[83]. As a consequence, the price of chitin is relatively high and, in most countries, its production is often limited by environmental regulations, thus hindering its applications[79]. Therefore, there has been a growing interest in developing and exploring new technologies for chitin isolation, namely biological methods (including enzymatic deproteination and fermentation using microorganisms), the application of alternative solvents, such as IL or DES, among others. However, further research is needed to make these processes competitive at commercial scales[79,83].

5. Alternative solvents for functional ingredients extraction and isolation

In most industrial processes designed for the extraction of natural products, large quantities of petrochemical or volatile organic solvents are still used, which limits their economic and environmental viability[85]. Most of these solvents are flammable, volatile, and often toxic, being responsible for environmental pollution and greenhouse effect. Nowadays, and according to the regulations that have been enforced, manufacturers that use these type of solvents are under increased pressure, as they are bound to demonstrate the absence of risk during extraction and to prove that the ingredients are safe[86]. Therefore, in recent years, there has been an increasing interest in developing sustainable extraction methods that can either minimize de use or replace the solvents that are commonly applied, from food ingredients to fine chemicals industrial production, while enabling process intensification and a cost-effective production of high-quality extracts[87]. Accordingly, the concept of green

extraction has emerged to describe "the discovery and design of extraction processes that reduce energy consumption, allow the use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract/product"[86]. Some of these processes include enzyme-, microwave-, or ultrasound-assisted extraction; high pressure technologies, such as pressurized, subcritical, and SFE; moderate or pulsed electric fields; among others. A number of these processes can be performed without the use of solvents, while others use alternative solvents such as pressurized water, dense carbon dioxide, IL, DES, ethanol, glycerol, fats and oils, etc.[85–87].

5.1. Supercritical fluids

Supercritical fluids have been studied since the XIX century, after the concept of "critical point" of a substance has been defined for the first time[88]. The term supercritical fluid is used to describe a substance above its critical pressure and temperature, as schematically described on Figure 6.

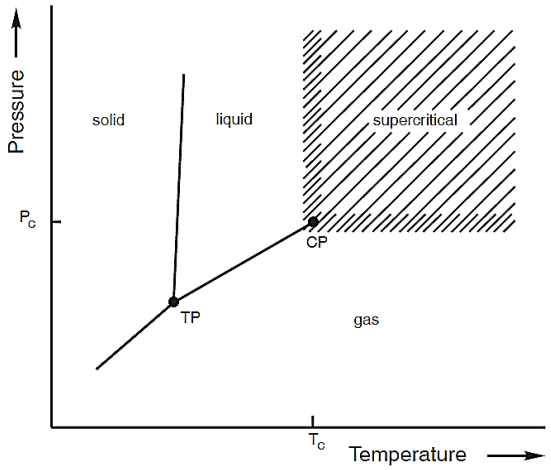

Figure 6. Typical phase diagram of a pure component. CP, critical point; P_c, critical pressure; T_c, critical temperature; TP, triple point[89].

By definition, and for a pure fluid, the gas and liquid phases become undistinguishable beyond its critical point[90]. Therefore, under such conditions, the fluid assumes properties of both gas and liquid, *i.e.*, the density is similar to a liquid, the viscosity and surface tension are similar to a gas, while the diffusivity is intermediate between the two states (Table 3). Furthermore, in this region, the solvent power of the fluid reaches its maximum capacity,

and large variations in the solvent's properties can be achieved through slight changes in temperature and pressure conditions[91].

Table 3. Range values of some physical properties of gases, liquids and supercritical fluids. P, pressure; T, temperature; P_c, critical pressure; T_c, critical temperature. (Adapted from Herrero *et al.*)[92].

State of the Fluid	Density (g/cm^3)	Diffusivity (cm^2/s)	Viscosity ($g \cdot s/cm$)
Gases (P = 1 atm; T = 21 °C)	10^{-3}	10^{-1}	10^{-4}
Liquids (P = 1 atm; T = 15 – 30 °C)	1	$< 10^{-5}$	10^{-2}
Supercritical fluids ($P = P_c$; $T = T_c$)	0.3 – 0.8	$10^{-3} – 10^{-4}$	$10^{-4} – 10^{-3}$

To successfully optimize a SFE process, there are several important factors that should be studied, including the sample pre-treatment, the choice of the solvent and eventual modifiers, and the extraction conditions, such as temperature, pressure, flow rate, or extraction time. The most relevant process parameter is perhaps the density of the fluid (controlled through pressure and temperature conditions), as it can be used to adjust the extraction selectivity, *i.e.*, the higher the density, the greater the solvent power, the lower the selectivity[93].

The most widely used fluid is carbon dioxide because it is relatively inert, nontoxic, non-flammable, inexpensive, widely available, and easily recyclable. Although CO_2 is a greenhouse gas, it is usually considered to be an environmentally friendly solvent, since it is captured, used in a process, and then recirculated, thus not contributing to increasing the greenhouse effect. Its moderate critical temperature (31.1 °C) and pressure (73.8 bar) make it an ideal solvent for extracting thermolabile natural compounds, while avoiding their degradation[88].

Sc-CO_2 is a non-polar solvent presenting a dielectric constant similar to solvents like hexane. For this reason, it is suitable mainly for the extraction of lipophilic compounds. Nevertheless, this drawback can be solved by the addition of a co-solvent or modifier, thus increasing the solubility of more polar molecules in sc-CO_2[94].

Some general advantages and disadvantages of using sc-CO_2 in extraction processes are summarized on Table 4. The most important advantage is probably its flexibility. On the one hand, its low viscosity and intermediate diffusivity enable a much faster mass transfer than in liquids; on the other hand, its solvent power can be easily tuneable with slight adjustments

in pressure and temperature conditions. However, there is a major drawback to its application in industry related to the high investment costs. Nevertheless, the general agreement amongst the scientific community is that this disadvantage can be outweighed by a superior product quality, lower operating costs, and/or integration of several technological steps[95].

Table 4. Advantages and disadvantages of sc-CO_2 extraction processes. (Adapted from Cvjetko Bubalo *et al.*)[85].

Advantages	Disadvantages
Moderate operating temperatures allowing the preservation of compounds with low thermal stability	High pressures
Highly concentrated solvent-free extracts	High investment costs
Environmentally friendly (when recirculated in closed loop), physiologically harmless, germicidal, and non-flammable	Complex solvent/solute phase equilibrium, which can hinder the extractions' design
Generally recognized as safe	Highly polar substances (*e.g.*, sugars, amino acids, inorganic salts, proteins) are insoluble without addition of co-solvent
Inexpensive	
Faster extraction rates (as a result of lower viscosity and higher diffusivity than liquids)	
Fragrances and aromas remain unchanged	
Selective extraction and fractionated separation	
Pure extracts with fewer process steps	
Tuneable solvating power	
High solubility for non- or low-polarity substances	
Possibility of direct coupling with analytical chromatographic techniques	

Although the first reference to supercritical fluids appeared in the 1820s[88], it was not until the 1970s that it reached commercial scales, with the first patented process for green coffee decaffeination, using sc-CO_2 as solvent[96]. Nowadays, supercritical fluids are widely applied in different industrial processes, including the extraction of hop constituents, essential oils, oleoresins, flavouring compounds from herbs and spices, and high value bioactive compounds from different natural matrices; the extraction and fractionation of edible oils; the decaffeination of coffee beans and black tea leaves; and the removal of pesticides from

plant materials[87,88]. In particular, in parallel to this PhD work, the author has helped to design and implement an industrial supercritical fractionation column in a Spanish biotechnology company, intended for separation of fish oil components.

5.2. Subcritical water

The application of subcritical water (scW) as extraction solvent was described for the first time in the literature by Hawthorne and co-workers in 1994, aiming at extracting organic pollutants from environmental solids. At that time, the authors concluded that their results had demonstrated that water was a potential extraction solvent for the entire range of organics found in environmental solids, from polar to nonpolar compounds, as it was possible to drastically reduce water's dielectric constant by simply increasing temperature[97]. Since then, and due to its unique properties and a wide range of possible applications, scW has attracted significant attention within the scientific community, not only as alternative solvent, but also as catalyst and reactant for hydrolytic conversions and extractions[85,98].

The term scW is used not to designate a physically defined state, but rather the region of the condensed phase (above water's vapour pressure) between water's boiling (100 °C) and critical temperatures (374 °C)[99], as shown on Figure 7.

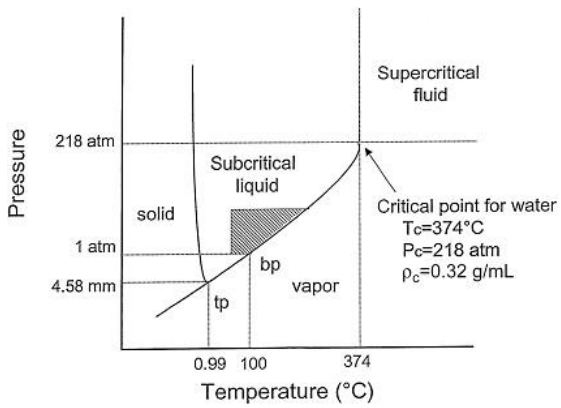

Figure 7. Phase diagram of water. tp, triple point; bp, boiling point; T_c, critical temperature; P_c, critical pressure; ρ_c, critical density[100].

The principle of scW extraction is based on the molecular structure and thermodynamic properties of water. The position of the slightly negative and positive charges grants important properties to water such as polarity, cohesion, surface tension, hydrogen bonding, dipole moment, among others[98].

One of the most important properties to consider when designing an extraction process is the variation of the dielectric constant with temperature[92]. At normal temperature and pressure conditions, water has one of the highest dielectric constants amongst non-metallic liquids, making it only suitable for the extraction of high-polarity compounds. However, this value decreases significantly with increasing temperature, while applying the appropriate pressure to maintain water in the liquid state, to values within the range of organic solvents such as methanol, ethanol, acetone, or acetonitrile. Therefore, by reaching lower dielectric constants at elevated temperatures, scW can be a promising technology for the efficient extraction of a wide range of low- and moderate-polarity compounds[85,92,98]. Another important property to consider is the ionic product of water, which increases with increasing temperature. Because of the increased H^+ and OH^- ions concentration, water becomes a more reactive medium, capable of acting as an acid or alkaline catalyst, making it an ideal solvent for the hydrolysis of biomass[101].

As shown on Table 5, apart from the dielectric constant and the ionic product, there are also other important properties of water that are modified under subcritical conditions, thus promoting its capacity as extraction solvent.

Table 5. Modification of physico- and electrochemical properties of water with increasing conditions of temperature and pressure. (Adapted from Gbashi *et al.*)[98].

Decreases	Increases
Adhesion and cohesion	Collision frequency
Density	Compressibility
Dielectric constant	Diffusivity
Hydrogen bonding	Electrical conductivity
Surface tension	Extraction rate
Viscosity	Ionic product
	Miscibility
	Reaction rate (reactivity)
	Solvent power

Although temperature is probably one of the most important parameters to control during scW extraction, the process efficiency may also be affected by other factors, including for example the particle size of the material, the solvent flow rate, or the addition of co-solvents, being the study of these parameters also important for an appropriate design and optimisation of the process[98].

Water behaves very differently from other solvents and presents many unusual properties, mostly due to the very strong hydrogen bonds that exist in each molecule[98,101]. Its unique characteristics combined to the fact that it is a readily available, safe, low cost, non-toxic, non-flammable, and environmentally friendly solvent, were the basis that led to numerous studies that explored scW applied to the extraction of natural compounds from plant materials, biomass conversion, waste treatment, and recycling[100]. In recent years, scW extraction has gained particular importance within the food and pharmaceutical industries for the recovery of a wide range of compounds from different raw materials, including phenols and flavonoids, essential oils, alkaloids, oils and fatty acids, carotenoids, proteins and respective building blocks, carbohydrates, among others, since the final products have no trace of toxic organic solvents[100,102]. Further advantages and some of the drawbacks of using scW as solvent are summarized on Table 6.

Table 6. Advantages and disadvantages of scW extraction processes. (Adapted from Cvjetko Bubalo *et al.*)[85].

Advantages	Disadvantages
Low cost, easily available, safe, non-toxic, non-flammable and environment friendly solvent	High investment costs
Safe extracts, without trace of toxic solvents	Increased risk of unwanted reactions or formation of toxic compounds at high temperatures
Higher diffusion into the matrix and increased mass transfer properties	Increased risk of clogging induced by caramelization reactions
Higher extraction rates	Increased risk of hydrolysis reactions
Extraction of low-polar and non-polar compounds	Increased risk of degradation of thermolabile compounds
Uncomplicated equipment	Extractions can be less selective due to an increase of solubility of other matrix compounds with increasing temperature

5.3. Deep eutectic systems

Even though the concept of eutectic mixtures is not new, as these mixtures have been used since the 1950s to lower the melting point of salts by forming metal-halide complexes[103,104], the term DES is relatively recent. It was firstly reported by Abbott *et al.* in a study that described the unusual solvent properties shown by eutectic mixtures of urea with different quaternary ammonium salts, properties which revealed to be strongly influenced by hydrogen bonding[105]. In recent years, DES have been gaining increased interest within the

scientific community, as different authors started to explore their potential for a wide range of applications[106].

Due to certain similar properties, such as generally high thermal stabilities, low vapour pressures, and tuneable polarity, DES have been referred to as the fourth generation of IL, even though they might be formed by compounds without ionic character[107]. However, unlike IL, DES can be prepared sustainably and in a simple way, even at higher scales, at low cost, and with acceptable properties concerning toxicity and biodegradability by mixing two or more compounds, which will establish intermolecular hydrogen bonding between the hydrogen bond acceptor (HBA) and the hydrogen bond donor (HBD)[106,108]. The charge delocalization that occur through hydrogen bonding is thought to be responsible for reducing drastically the melting point of the system, when compared to its individual components[109], as schematically illustrated on Figure 8. It should be noted that these systems are formed at any composition above the liquidus line represented on the diagram, and not only at the composition of the eutectic point.

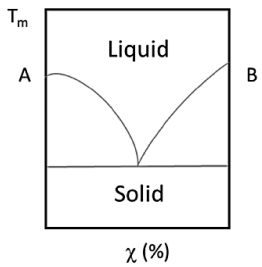

Figure 8. Phase diagram (melting temperature, T_m, as a function of composition, χ) of a fictitious binary eutectic mixture between components A and B[106].

The possibility of a high number of different combinations is a key feature of DES. This characteristic has a direct impact on DES physicochemical properties, including phase behaviour, viscosity, density, polarity, pH, ionic conductivity, surface tension, vapour pressure, refractive index, hydrogen bonding, etc., as well as on their toxicological or biodegradable profile. Therefore, the possibility of fine-tune these properties has opened up new perspectives for the application of DES, as it enables their design in large scale, at low cost, with the required physicochemical properties, and with acceptable features considering toxicity, biodegradability, and biocompatibility[110]. Additional advantages and

some disadvantages of using DES applied specifically in extraction processes are summarized on Table 7.

Table 7. Advantages and disadvantages of DES extraction processes. (Adapted from Cvjetko Bubalo *et al.*)[85,110,111].

Advantages	Disadvantages
Simple preparation	High viscosities can limit their application due to lower extraction efficiencies or higher energy required for stirring and pumping
Use of natural compounds such as organic acids, amino acids, sugars, etc.	Separation of the target compounds from the DES can be difficult
Sustainable production with 100% atom economy (theoretically with no waste generation)	Problems with DES re-use/recycling
Tailor-made systems with a high number of possible combinations between HBAs and HBDs (estimated at 10^6 to 10^8)	DES application at industrial scale remains viable only when the extract is directly used without expensive downstream processing steps
Fine-tuned physicochemical properties for a specific application	
Wide polarity range	
Low toxicity	
High biocompatibility and biodegradability	
Low-volatility and low-flammability	
Low cost (comparable to conventional solvents)	
Some DES are bioactive being able to complement the bioactivity of the extracted compounds	
If biocompatible, DES can be used as active solvents (*i.e.*, act as vehicles/agents of the compound/extract in the final product or as final formulation)	

The majority of the DES that have been prepared can be categorized in different types, from *I* to *V*, as represented on Table 8, being *Type V* the most recent class of DES, while the properties and applications of *Type III* DES have been the most widely studied. However, there are still some systems that do not fit into any of these categories, such as mixtures between some Brønsted-Lowry acids and bases, thus suggesting that other types of DES might be yet to be discovered[107].

Table 8. Traditional classification of DES[107].

Type I	Combine quaternary ammonium salts and metal chlorides
Type II	Combine quaternary ammonium salts and metal chloride hydrates
Type III	Combine quaternary ammonium salts and HBDs (typically organic molecular components such as amides, carboxylic acids, or polyols)
Type IV	Combine metal chloride hydrates and HBDs
Type V	Combine non-ionic molecular HBAs and HBDs

Although significant progress has been made at lab scale, as DES are a rather recent technology, their applications in industry are still very limited[106]. Nevertheless, due to their versatility, these systems have huge potential to be scaled-up and implemented as clean solvents, to replace or complement some of the already existing industrial processes, including in areas such as metallurgy and electrodeposition; separations and gas capture; power systems and battery technologies; (bio)catalysis and organic chemistry; biomass processing; biomolecular structure, folding, and stability; genomics and fundamentals of nucleic acids; pharmaceuticals and medical research; materials and nanomaterials science; among others[106,107,110]. In fact, some DES have already been adopted by the beauty/health care and natural ingredient industries for the extraction of functional active molecules from plants, to be used in cosmetic formulations[112,113].

As DES become more relevant for industrial applications, new scientific developments are expected, particularly in what concerns the characterization of the fundamental properties of these systems, including not only their physicochemical properties, but also detailed toxicity studies that provide evidence of their safety, for humans and for the environment; and the understanding of the phase behaviour and the interactions established between the different components of the mixture[106,110,114,115].

6. Objectives

Considering the aforementioned aspects, the main goal of this dissertation was to develop an integrated and sustainable strategy for the efficient valorisation and exploitation of by-products and wastes resulting from the fish canning and shellfish processing industries, within the biorefinery and circular bioeconomy concepts.

To accomplish this goal, a holistic approach was explored comprising the following steps: (i) selection of the feedstocks (*i.e.*, sardine heads and offal and brown crab shells) and

identification of the target molecules (*i.e.*, fatty acids, AXT, protein derivatives and hydrolysates, and chitin); (ii) biomass fractionation using alternative technologies, including sc-CO_2, scW, and DES extraction; (iii) chemical characterization of the resulting bio-based products (*i.e.*, identification and quantification of the target molecules); (vi) evaluation of the bioactive potential of the most promising bioproducts obtained, using chemical, cell-based, and antimicrobial susceptibility *in vitro* assays; (v) evaluation of the viability (*i.e.*, through an experimental scale-up and integration of the different processes) and the environmental impacts (*i.e.*, through life cycle assessment) of the most promising processes and products.

Overall, the innovative processes developed during this thesis and the resulting high value products, can contribute not only to the valorisation of currently undervalued marine-based waste streams, but also to tackle waste disposal and resource depletion, which might be an advance towards the implementation of an integrated seafood waste-based biorefinery and the establishment of a circular bioeconomy.

7. Outline

This dissertation is divided into five chapters, as schematically illustrated on Figure 9. The thesis layout followed the different steps involved in the research work developed during the PhD project.

The present chapter (Chapter 1) provides a general overview of the state-of-the-art, background, and important concepts that fall within the scope of this thesis, as well as the motivation and the main objectives of the PhD project.

Chapter 2 is divided into two parts and describes the development of bioactive ingredients from sardine residues resulting from the Portuguese fish canning industry. Part I concerns the recovery of fatty acids from the fish biomass. The extracts obtained through sc-CO_2 extraction were characterised in terms of fatty acid profile, and then screened for bioactive activity, targeting as end points their antiproliferative, antioxidant, and anti-inflammatory effects. Part II focuses on the extraction of protein derivatives using betaine/polyol-based DES. This part provides a detailed physicochemical and toxicological analysis of DES, as well as the study of the extraction conditions to improve the protein yields, the determination of

the amino acid profile of the extracts, and the evaluation of their antioxidant and antimicrobial potential.

Chapter 3 reports the isolation of bioactive ingredients from shell residues resulting from a Portuguese brown crab processing company and is divided into three parts, targeting three different molecules. Part I comprises the work developed aiming at obtaining AXT-rich extracts using terpene/fatty acid-based DES, including a detailed physicochemical and toxicological analysis of the DES, the study of the extraction conditions, and the characterization of the extracts, as well as the evaluation of both DES and extracts antiproliferative and antimicrobial activity. Furthermore, Part I also contains a proof of concept using biomasses other than crab, including *H. pluvialis*, which is the most important natural source of AXT. Part II includes the extraction of protein hydrolysates with scW, the characterization of the extracts obtained under different operating conditions in terms of total protein yield, Maillard reaction products formed, and free amino acid profile, and the evaluation of the antioxidant potential of the protein hydrolysate-rich extracts. Part III consists in the recovery of chitin by applying choline chloride/organic acid-based DES. It includes not only the investigation of the most favourable extraction conditions, but also a phytotoxic profile of the DES used, as well as a detailed characterization of the structure and properties of the chitin obtained, using methods such as thermogravimetric analysis, Fourier transform infrared spectroscopy, X-ray diffraction, and scanning electron microscopy.

Chapter 4 describes the experimental scale-up and integration of the most promising processes developed for the valorisation of both biomasses, where the obtained products were evaluated taking into consideration different characterization and bioactivity endpoints. Additionally, it also discusses the environmental impact of the integrated processes, through life cycle assessment, providing a quantification of their environmental burdens, as well as the identification of the processes' hotspots.

In Chapter 5, an overview of the most important findings is provided. The summarised results are discussed in an integrated manner in order to highlight the main advances and conclusions. Additionally, some perspectives for future developments are also presented and discussed.

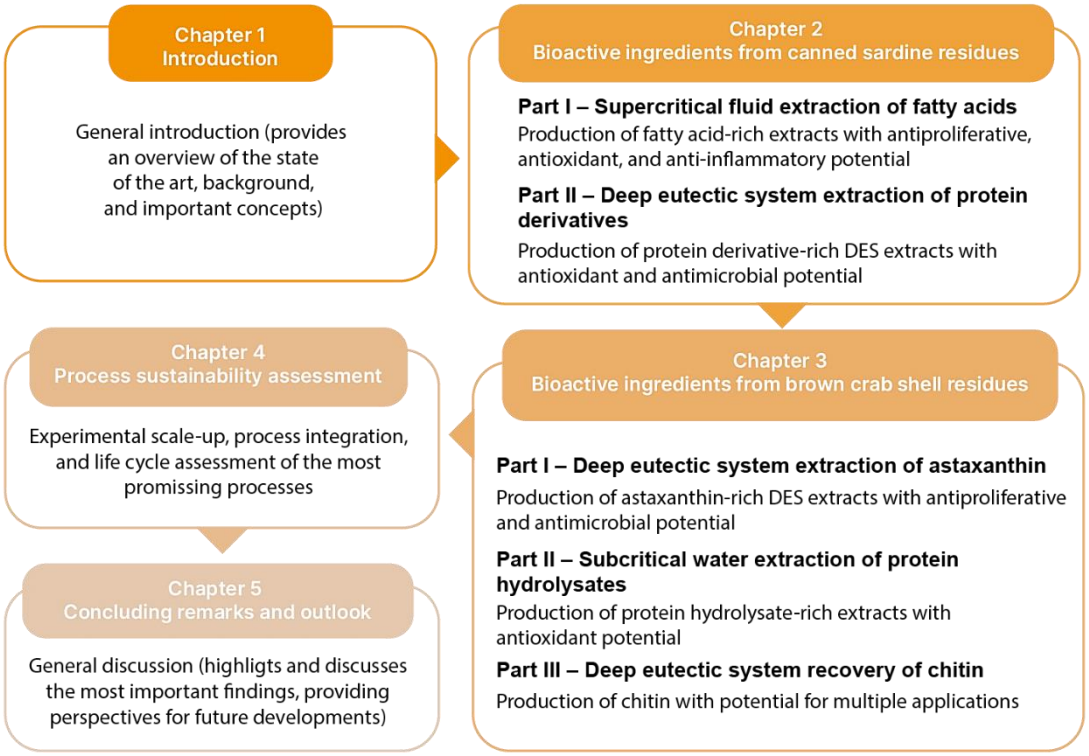

Figure 9. Schematic representation of the thesis layout.

Under the terms of Regulation n.º 377/2013, published in Diário da República on October 2, 2013, and given that this PhD thesis was designed based on scientific articles that were published or are being prepared for publication, each main chapter or chapter part (from Chapter 2 to Chapter 4) was prepared in article format, *i.e.*, including an abstract that summarizes the work performed, an introduction that briefly reviews the state-of-the-art, a materials and methods section, a results and discussion section, the key conclusions of that particular work, and the corresponding acknowledgements and references.

It should also be noted that this thesis does not contain a general list of figures, tables, or abbreviations, since all figure and table captions, as well as abbreviations and symbols, are carefully identified and described throughout each chapter. In particular, abbreviations are defined in parentheses the first time they appear in the abstract as well as in the main text of each chapter or chapter part and used consistently thereafter.

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CHAPTER 2

Bioactive ingredients from canned sardine residues

CHAPTER 2

Part I – Supercritical fluid extraction of fatty acids

Contents

Adapted from:

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The author was involved in the conceptualization and design of all experiments and performed all experimental work except for the characterization of extracts by GC-FID (acquired at INIAV by A. M. C. Partidário), their *in vitro* bioactivity evaluation (performed at iBET by C. V. Pereira), and the statistical analysis (performed at FFUL by L. F. Gouveia). Data processing and interpretation, and results discussion were also performed by the author, as well as the preparation of the original manuscript.

1. Abstract

The importance of fish oil and seafood-derived lipid concentrates on human health and nutrition is well known. In part I of this chapter, supercritical carbon dioxide (sc-CO_2) extraction was explored to recover bioactive lipids from canned sardine residues, with special focus on triglycerides. Aiming at enhancing the recovery of the target molecules, different extraction conditions were applied, including operating pressure (300, 425, and 550 bar), temperature (35, 55, and 75 °C), and CO_2 flow rate (5, 15, and 25 g/min). The performance of sc-CO_2 extractions was compared to a conventional Bligh and Dyer extraction, and the resulting samples were characterized in terms of global yield and fatty acid profile. To evaluate their potential application as bioactive ingredients, extracts were screened for cytotoxicity, oxidative damage, and antiproliferative and anti-inflammatory activities on human intestinal cell lines. Results have shown that sc-CO_2 extractions were able not only to produce extracts highly concentrated in triglycerides (up to 89%) with antiproliferative, antioxidant and anti-inflammatory properties, which were modulated by the operating conditions applied.

2. Introduction

The canning process is regarded as one of the largest sources of fish-processing wastes and by-products[1]. Nevertheless, after the fish is processed, the different resulting waste streams still harbour significant amounts of interesting chemicals. In the case of sardines, one of the main species caught in Portuguese waters and one of the most commonly used by the canning industry, the resulting solid wastes are still a good source of lipids (44%), proteins (39%), and minerals (15%). Therefore, these waste streams represent an opportunity to maximize their economic potential through the production of high value ingredients[2,3].

In particular, seafood are known to contain in their composition a wide diversity of lipids, including free fatty acids, triglycerides, sterols, and phospholipids, being regarded as the most important natural source of long-chain ω-3 polyunsaturated fatty acids (PUFA), such as the essential eicosapentaenoic (EPA) and docosahexaenoic acids (DHA)[4]. Palmitic acid is the most common saturated fatty acid (SFA) in fish, with myristic and stearic acids usually occurring in lower concentrations; while palmitoleic and oleic acids are the most abundant monounsaturated fatty acids (MUFA)[5].

Since Dyerberg *et al.* suggested for the first time, in 1978, the existence of a link between the intake of EPA and a reduced risk of cardiovascular diseases[6], numerous preclinical *in vitro* and *in vivo* studies have focused on the importance of fish oil and seafood-derived MUFA and PUFA concentrates on human health and nutrition. These studies included their potential for prevention and treatment of inflammatory diseases, some types of cancer (mostly colorectal, mammary, and prostatic cancer), among many others[7–9].

The fact that chronic inflammation, such as inflammatory bowel diseases, is closely related to an increased risk of colorectal cancer, is also well known by the scientific community[10]. Inflammation can be triggered by microbial components, tissue damage or metabolic stress. Yet, regardless of the trigger, a common set of cellular pathways is initiated. In response, several mediators are released, including reactive oxygen (ROS) or nitrogen (RNS) species, peptide mediators (*e.g.*, cytokines, chemokines), among others, which can be used and investigated as biomarkers of inflammation[11,12].

Different types of fatty acids are currently applied in a long list of end-user industries, including dietary supplements, infant formulas, animal feed, pharma, food and beverage, and cosmetic industries, representing a growing market, valued at over US$ 31 billion (data from 2018)[13,14].

Overall, traditional industrial processes to produce fish oil involve two phases: the extraction of oil and its refining. The most common process to obtain crude fish oil from fresh fish is the wet pressing method, following the procedure described by the Food and Agriculture Organization of the United Nations in 1986[15]. Nowadays, several companies that produce fish oil in Europe still follow this process, mainly in Iceland, Denmark, and Spain. However, this process only leads to good results when using fish products or by-products with high-oil content. Therefore, in the last years there has been some interest in updating this multistep process and to develop new technologies to obtain fatty acid concentrates for pharmaceutical or nutritional purposes, including for example the application of supercritical fluid extraction (SFE) using supercritical CO_2 (sc-CO_2) as solvent[16].

The main goal of this work was, therefore, to explore the potential of sc-CO_2 extraction to convert by-products and wastes resulting from the Portuguese canning industry into bioactive fatty acid concentrates for nutraceutical and/or pharmaceutical applications. Accordingly, different bioactivity assays were selected to evaluate the potential of fatty acid-

rich extracts, namely cytotoxicity on a human intestinal epithelial cell model (Caco-2), antiproliferative effect on a colorectal cancer cell line (HT-29), and their modulatory effect on oxidative stress biomarkers (ROS and RNS), mitochondrial damage related to the oxidation/inflammation process, and anti-inflammatory activity through the evaluation of inflammatory mediators (interleukin-6, IL-6, and interleukin-8, IL-8) on Caco-2 cells.

3. Materials and methods

3.1. Biomass

Sardine (*Sardina pilchardus*) heads and offal, resulting from one of the first steps of the canning process (removal of heads and viscera after immersion of the fresh fish in a concentrated salt solution), were kindly provided by Fábrica de Conservas A Poveira, Portugal, in July 2018, and stored at -20 °C upon arrival. The biomass was dehydrated using a Coolsafe Superior Touch 55-80 freeze dryer (Scanvac, Labogene, Bjarkesvej, Denmark) at -55 °C for approximately 72 h. The residues were then milled using a cutter-emulsifier CKE-8 (Sammic, Azkoitia, Gipuzkoa, Spain) and stored at -20 °C, in a nitrogen atmosphere.

3.2. Solid-liquid/fluid extractions

3.2.1. *Conventional Bligh and Dyer (B&D) extraction of lipids*

Total lipid content in sardine residues was determined following the methodology described by B&D[17], with slight modifications. Extractions carried out at day 0 were only performed for comparison with the extracts obtained at day 35 (section 4.2). Any other allusions to B&D extracts refer to extracts obtained at day 35.

Briefly, 2 g of freeze-dried residue were homogenized, using a vortex, with a mixture of 2 mL of chloroform (Fisher Chemical, Loughborough, UK) and 2 mL of methanol (Fisher Chemical, Loughborough, UK). After 3 min of homogenization, 2 mL of chloroform were added to the mixture. The mixture was further homogenized for 45 s, after which 2 mL of distilled water were added, and homogenization continued for further 45 s. The resulting extract was filtrated under vacuum, and the residue recovered and re-extracted with 4 mL of chloroform for 45 s. The extract was filtrated once more and left until complete phase separation and clarification. The chloroform layer was then recovered and concentrated under a nitrogen (≥ 99.999%, ALPHAGAZ™ 1, Air Liquide, Algés, Portugal) stream, and the global extraction

yield determined. Samples were stored at -20 °C, in the absence of light, until further analyses. Experiments were performed in duplicates and results were expressed as $g_{extract}/100\ g_{dry\ residue}$.

3.2.2. *Sc-CO_2 extraction of lipids*

Sc-CO_2 extractions were carried out between day 33 and 48 after biomass processing in a SFE system (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50) comprising a 500 mL cylinder extraction cell and two different separators, each of them with 500 mL of capacity, with independent control of temperature and pressure (Figure 1).

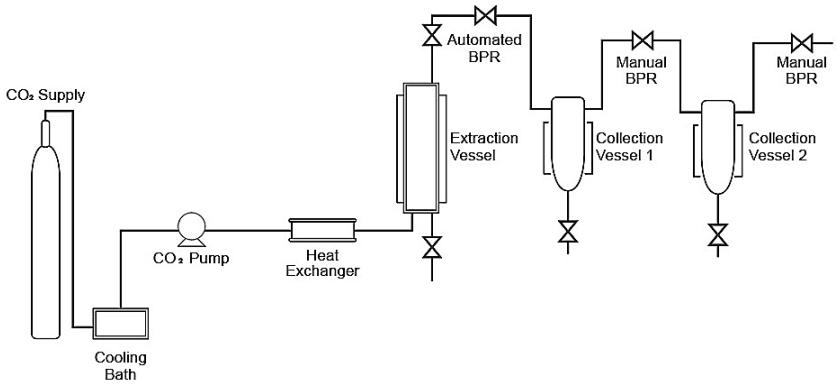

Figure 1. Schematic illustration of the high-pressure plant.

Briefly, 10 g of freeze-dried residue were placed on the extraction vessel packed with laboratory glass beads. CO_2 (99.999%, ALPHAGAZ™ 1, Air Liquide, Algés, Portugal) was delivered to the extraction vessel using a TharSFC P-50 high pressure pump (Thar Technology, Pittsburgh, PA, USA) and the pressure was kept constant by an automated back pressure regulator (TharSFC ABPR, Thar Technology, Pittsburgh, PA, USA). Taking into consideration the work developed by Létisse and co-workers concerning the extraction of EPA and DHA from sardine[18], sc-CO_2 extractions were performed by varying conditions of pressure (300, 425, and 550 bar) and temperature (35, 55, and 75 °C) (Table 1). The impact of CO_2 flow rate (5, 15, and 25 g_{CO2}/min) was also tested at 550 bar and 35 °C. The CO_2 flow stream exiting the extraction vessel was expanded into the first fraction collector, set at 60 bar and 35 °C, and extracts were recovered at different time points during 135 min. At the end of the experiment, the system was depressurized and the extract remaining in both

separators was recovered using acetone (Fisher Chemical, Loughborough, UK). Extracts were concentrated under a nitrogen stream and the global extraction yield of the resulting samples determined. Results were expressed as $g_{extract}/100\ g_{dry\ residue}$. Samples were stored at -20 °C, in the absence of light, until further analyses.

Table 1. Sc-CO_2 extraction conditions applied to canned sardine residues.

Run	Temperature (°C)	Pressure (bar)	CO_2 density (g/mL)
sc-CO_2 1		300	0.93
sc-CO_2 2	35	425	0.98
sc-CO_2 3		550	1.02
sc-CO_2 4		300	0.85
sc-CO_2 5	55	425	0.92
sc-CO_2 6		550	0.97
sc-CO_2 7		300	0.77
sc-CO_2 8	75	425	0.85
sc-CO_2 9		550	0.91

3.3. Fatty acid profile determination

Triglycerides contained in the extracts were converted to methyl esters following the transesterification method described by ISO 5509[19]. Briefly, after solubilisation of extracts in isooctane (99.8%, Merck, Darmstadt, Germany), a solution of potassium hydroxide in methanol (2 N) was added to the mixture, which was then vigorously agitated for 30 s. After complete phase separation and clarification, 2 µL were collected from the upper phase and injected into the gas chromatograph.

Gas chromatography-flame ionization detection (GC-FID) analyses were carried out using a ThermoQuest Trace GC 2000 (CE Instruments, Ltd.) gas chromatograph coupled with a flame ionization detector. The separation of fatty acid methyl esters (FAMES) was achieved using a J&W DB-23 capillary column (Agilent Technologies, Inc., Santa Clara, CA, USA), 60 m × 0.25 mm I.D. and 0.25 µm phase thickness, using helium as carrier gas. The oven temperature program was set as follows: 70 to 195 °C at a rate of 5 °C/min, 30 min isotherm at 195 °C, 195 to 220 °C at 5 °C/min, 65 min an isotherm at 220 °C. The injector and detector temperatures were maintained at 220 and 280 °C, respectively. FAMES were identified in the samples by comparison of relative retention times to palmitic acid with those obtained

in a standard mixture of 52 FAMES (Nu-Chek-Prep, Inc., Elysian, MN, USA). Results were expressed as $mg_{fatty\ acid}/g_{extract}$ or $mg_{fatty\ acid}/100\ g_{dry\ residue}$.

3.4. *In vitro* bioactivity evaluation

3.4.1. *Cell culture*

Human intestinal Caco-2 and HT-29 cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and American Type Culture Collection (Manassas, VA, USA), respectively. Both cell lines were cultured in RPMI 1640 medium supplemented with 10% of heat-inactivated foetal bovine serum (FBS), and 1% penicillin-streptomycin (in the case of Caco-2 cells). Cells were maintained at 37 °C with 5% CO_2 in a humidified incubator and routinely grown as monolayers in 75 cm^2 culture flasks, with culture medium renewal every 48 h. The cell culture medium and supplements were purchased from Invitrogen (Gibco, Paisley, UK).

3.4.2. *Sample Preparation*

Extract stock dispersions were prepared in RPMI 1640 medium supplemented with 0.5% of FBS, immediately before the assays. Dispersions were heated to 37 °C for a few minutes and then thoroughly homogenized using a vortex. To obtain a range of concentrations, samples were further diluted in RPMI 1640 medium supplemented with 0.5% of FBS.

3.4.3. *Cytotoxicity assay*

Cytotoxicity was assessed using confluent and non-differentiated Caco-2 cells, as a model of the human intestinal epithelium, as previously described by Pereira *et al.*[20]. When confluent, Caco-2 cells express some morphological and functional characteristics of mature enterocytes and can be used as a model of the intestinal barrier to assess the potential toxic effects of chemicals, food compounds, and nano- or microparticles[21,22]. Briefly, cells were seeded at a density of 2 x 10^4 cells/well into 96-well plates and cultured for 7 days. At day 7, confluent Caco-2 cells were incubated with different concentrations of extracts. Wells containing cells incubated with culture medium were used as positive control. After 24 h of incubation, the medium was removed, cells were washed with phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) and cell viability was measured using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the

manufacturer's instructions. Absorbances were recorded at 490 nm using a Spark® 10M Multimode Microplate Reader (Tecan Trading AG, Männedorf, Switzerland) and cell viability was expressed as percentage of viable cells in relation to the control. Experiments were performed in triplicates using at least three independent assays. Half maximal effective concentrations (EC50) were obtained from dose-response curves and expressed as $mg_{extract}$/mL.

3.4.4. *Antiproliferative assay*

Antiproliferative effect was evaluated using non-confluent HT-29 cells, as described elsewhere[20]. Briefly, cells were seeded at a density of 1 x 10^4 cells/well into 96-well culture plates. After 24 h, at conditions of exponential growth, cells were incubated with different concentrations of extracts. Wells containing cells incubated only with culture medium were considered as control. After 24 h of incubation, cell viability was determined as previously described for cytotoxicity assay, and EC50 values were calculated. Experiments were performed in triplicates using at least three independent assays.

3.4.5. *Evaluation of intracellular ROS formation*

Intracellular antioxidant activity was assessed according to the method described by Matias *et al.*[23] at two different levels: (i) after incubation with extracts without a stress inducer (basal level) and (ii) after incubation with extracts followed by treatment with a stress inducer (pre-incubation level). Briefly, Caco-2 cells were seeded at a density of 2 x 10^4 cells/well into 96-well culture plates and allowed to grow for 7 days. At day 7, cells were incubated for 1 h with extracts at a non-cytotoxic concentration of 2 mg/mL. Wells containing cells incubated only with culture medium were considered as control. After 1 h incubation, the medium was removed, and cells were washed with PBS. 100 µL of 2',7'-Dichlorofluorescin diacetate (25 µM, Sigma-Aldrich, St. Quentin Fallavier, France) were added and fluorescence was measured using a Microplate Fluorimeter FLx800 (Bioteck Instruments, Winooski, VT, USA) at excitation and emission wavelengths of 485 nm and 528 nm, respectively, as basal level measurement. Cellular stress was then induced using hydrogen peroxide (10 mM, Sigma-Aldrich, St. Quentin Fallavier, France). Wells containing cells incubated with culture medium and stress inducer were considered as positive control.

Fluorescence was measured 1 h later, as pre-incubation level measurement. Three independent experiments were performed in triplicates and results expressed as percentage of fluorescence intensity in relation to the positive control.

3.4.6. *Evaluation of nitric oxide (NO) formation*

NO production was measured through the accumulation of nitrite in cell culture supernatants resulting from the evaluation of intracellular ROS formation at the pre-incubation level, using a colorimetric reaction with Griess reagent, as described by Matias *et al.*[23]. A mixture of 50:50 of Griess reagent (1% sulfanilamide (Sigma-Aldrich, St. Quentin Fallavier, France), 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich, St. Quentin Fallavier, France), and 2% phosphoric acid (Sigma-Aldrich, St. Quentin Fallavier, France)) and cell supernatant was prepared. A mixture of 50:50 of Griess reagent and culture medium were considered as control. Absorbances were determined at 540 nm using a Spark® 10M Multimode Microplate Reader. Three independent experiments were performed in triplicates and NO levels were expressed as percentage in relation to the control.

3.4.7. *Evaluation of mitochondrial integrity*

Assessment of the mitochondrial activity was performed by measuring tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma-Aldrich, Schnelldorf, Germany) inclusion, according to Da Silva *et al.*[24]. Briefly, Caco-2 cells were seeded at a density of 2 x 10^4 cells/well into 96-well plates and allowed to grow for 7 days. At day 7, cells were incubated with extracts (at 2 mg/mL) for 24 h. Wells containing cells incubated only with culture medium were considered as control. After incubation, cells were washed with Hank's Balanced Salt Solution (HBSS, Gibco, Life Technologies, Carlsbad, CA, USA) and 100 µL of TMRE (2 µM) were added. After 30 min, the medium was replaced by HBSS and fluorescence was measured using a Microplate Fluorimeter FLx800 at excitation and emission wavelengths of 560 nm and 590 nm, respectively. Three independent experiments were performed in triplicates and results expressed as percentage of fluorescence intensity in relation to the control.

3.4.8. *Evaluation of IL-6 and IL-8 secretion*

Experiments were performed as previously described by Matias *et al.*[23]. Briefly, Caco-2 cells were seeded at a density of 2.24 x 10^5 cells/mL into 12-well plates and allowed to reach confluence and differentiation for 21 days, under 5% CO_2 humidified atmosphere at 37 °C [25]. Cells were stimulated with a pro-inflammatory cocktail (50 ng/mL of tumour necrosis factor alpha (TNF-α, Peprotech, Rocky Hill, NJ, USA), 25 ng/mL of interleukin-1 beta (IL-1β, Sino Biological, Beijing, China) and 10 µg/mL of lipopolysaccharides from *Escherichia coli* (LPS, Sigma-Aldrich, Jerusalem, Israel)) and co-incubated with fatty acid-rich extracts (2 mg/mL) for 48 h. Wells containing cells incubated with and without pro-inflammatory stimuli were used as positive and negative control, respectively. Supernatants were collected, centrifuged for 10 min at 2000 *g* and stored at -80 °C until further analysis. IL-6 and IL-8 secretion was quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's guidelines and results were expressed as percentage of secretion in relation to the positive control. Pro-inflammatory cocktail and ELISA kits were purchased from PeproTech (London, UK).

3.5. Statistical analysis

GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to estimate the cytotoxicity and antiproliferative activity, as well as the statistical significance of average differences. Statistical significances were calculated either by one-way analysis of variance followed by the Tukey test, or by using an unpaired *t*-test. An alfa error of 5% was accepted in the hypothesis testing to decide for a significant effect. The Principal Component Analysis (PCA) was performed using Unscrambler X software (Camo, Sweden). Data was reported as mean ± standard deviation values.

4. Results and discussion

4.1. Lipid extractions

The method proposed by B&D is the most commonly used methodology for the characterization of total lipids, which may include not only neutral lipids, but also polar lipids, phospholipids, and lipids bound to different components of cellular membranes[17]. Accordingly, to determine the total lipid content of canned sardine residues and to evaluate

the performance of sc-CO_2 extractions, a B&D extraction was performed on freeze-dried residues.

Results presented on Table 2 show that a yield of 31.00 ± 2.39 g/100 g could be obtained by B&D extraction. Bandarra *et al.* studied the seasonal changes in lipid composition of *S. pilchardus* and concluded that, depending on the time of year, the lipid content of sardines could vary between 3.6 and 55.8%, in a dry basis[26]. Therefore, in the work reported herein, the yield obtained by B&D extraction was found to be within this range.

Table 2. Global extraction yields of sc-CO_2 and B&D extracts. For sc-CO_2 extractions, different lowercase letters represent a statistically significant difference. The statistically significant difference between sc-CO_2 extractions and B&D extraction is represented by an asterisk (*). $P < 0.05$ was accepted as statistically significant in all cases.

Run	Temperature (°C)	Pressure (bar)	Global extraction yield ($g_{extract}$/100 $g_{dry\ residue}$)
sc-CO_2 1	35	300	21.16 ^{f} *
sc-CO_2 2		425	23.11 ^{b,d} *
sc-CO_2 3		550	25.59 ^{a} *
sc-CO_2 4	55	300	21.64 ^{e,f} *
sc-CO_2 5		425	22.94 ^{c,d,e} *
sc-CO_2 6		550	23.25 ^{b,c} *
sc-CO_2 7	75	300	20.93 ^{f} *
sc-CO_2 8		425	24.69 ^{a} *
sc-CO_2 9		550	24.36 ^{a,b} *
B&D			31.00 ± 2.39

Coefficient of variation of sc-CO_2 extracts ≤ 2.2%

In order to obtain natural extracts rich in bioactive lipids with potential health-promoting effects, sc-CO_2 extraction was explored and applied to freeze-dried canned sardine residues. As a first approach, the impact of pressure and temperature for a fixed CO_2 flow rate (15 g/min) on the global extraction yield and on the composition of each extract was studied. Table 2 shows the global extraction yields obtained after each experiment. Yields ranged from 20.93 g/100 g (at 75 °C, 300 bar, CO_2 density of 0.77 g/mL) to 25.59 g/100 g (at 35 °C, 550 bar, CO_2 density of 1.02 g/mL). As it would be expected, when comparing these yields with the results obtained for B&D extraction, it can be concluded that sc-CO_2 extraction yields were not as high as the B&D extraction yield, since the latter has the ability to extract different classes of lipids, including more polar lipids, while sc-CO_2 is mainly capable of the extraction of nonpolar lipids[27].

The influence of pressure and temperature in SFE is well-known. When increasing pressure for a fixed temperature, the density of the fluid increases as well. Consequently, the solubility of the solute is expected to increase, resulting in higher extraction yields. The effects of temperature, however, may not be so obvious, since increasing temperature for a fixed pressure decreases the density of the fluid, but also increases the solute vapour pressure for a fixed fluid density[28]. Results summarized on Table 2 show that generally, for a fixed temperature, an increase in pressure resulted in a significant increase in yields. However, increasing temperature for a fixed pressure, did not show a significant effect on extraction yields, although there was a tendency for yields to increase with increasing temperature at similar CO_2 densities. An example are the significant increasing yields obtained at 35 °C and CO_2 density of 0.93 g/mL (21.16 ± 0.46 g/100 g), 55 °C and CO_2 density of 0.92 g/mL (22.94 ± 0.50 g/100 g), and 75 °C and CO_2 density of 0.91 g/mL (24.36 ± 0.53 g/100 g). The work developed by Létisse *et al.* concerning the SFE of oil from sardine heads showed similar findings regarding the impact of pressure on the global extraction yield: the highest the pressure, the highest the yield. Additionally, the authors have also reported that increasing temperature at constant pressure, did not increase extraction yields[18].

Given the highest yield was obtained at 35 °C, 550 bar, and the highest CO_2 density (1.02 g/mL), these operating conditions were kept constant in order to determine the impact of CO_2 flow rate on the extraction yield. Accordingly, three different flow rates were tested: 5, 15, and 25 g/min (Figure 2).

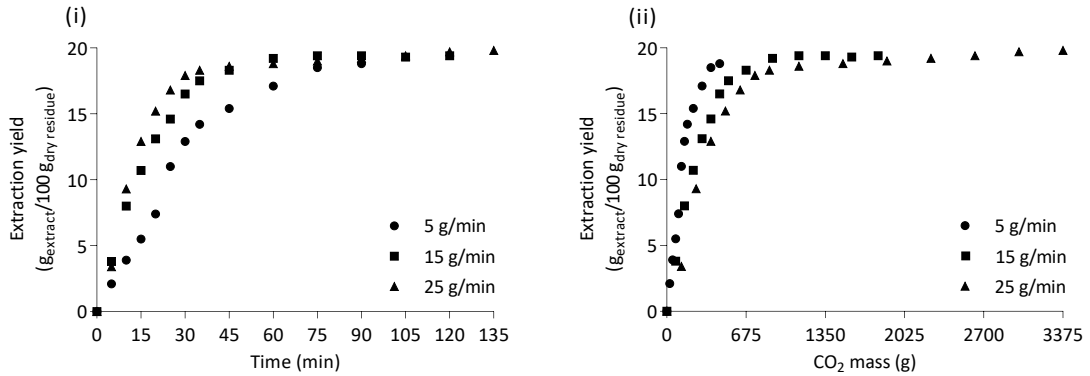

Figure 2. Effect of CO_2 flow rate on the extraction yield as a function of (i) time and (ii) CO_2 mass, obtained at 35 °C and 550 bar.

It is possible to distinguish the two different mechanisms that acted during extraction in the curves representing the cumulative extraction yield as a function of time (Figure 2i). As extraction started, lipids that were readily available at the surface of sardine particles, were extracted at a rapid and constant rate, since the extraction rate was controlled by solubility and external mass transfer resistance. As extraction progressed, lipids from deeper regions of the solid matrix started to be extracted by CO_2, being the internal mass transfer resistance the controlling factor, thus resulting in a much lower extraction rate[29]. Although similar yields were obtained after 135 min of extraction, it is possible to conclude from Figure 2i that the extraction rate was highly influenced by the CO_2 flow rate applied. At 35 °C and 550 bar, it would be possible to reduce the extraction time from 105 min at 5 g/min (corresponding to an overall mass of CO_2 used per assay of 525 g, or to 291 $g_{CO2}/g_{extract}$), to 45 min at 15 g/min (equivalent to an overall mass of CO_2 used of 675 g, or to 344 $g_{CO2}/g_{extract}$, pump energy increase by 22%), or to 30 min at 25 g/min (equivalent to an overall mass of CO_2 used of 750 g, or to 384 $g_{CO2}/g_{extract}$, pump energy increase by 30%). However, as shown on Figure 2ii, even though the extraction rate was slower at 5 g/min, a lower CO_2 mass was required to achieve the same yield. Therefore, from an energy perspective, it would be more favourable to work at 5 g/min.

4.2. Fatty acid composition of extracts and stability of sardine residues

A summary of the major compounds found in both B&D and sc-CO_2 extracts is shown on Table 3, while a more complete fatty acid profile is summarized on Appendix A, Tables A1 – A3.

Overall, extracts were found to be mainly composed of triglycerides (88 to 89%), being myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, gadoleic acid, EPA, cetoleic acid, and DHA the most relevant, corresponding to 73 to 76% of the extracts. These results are in accordance with the work of De Leonardis and Macciola, in which the lipid composition of *S. pilchardus* was investigated[30].

Sc-CO_2 extraction performed at 35 °C and 300 bar was able to yield a higher PUFA content, including EPA and DHA, when compared to the remaining sc-CO_2 extraction conditions or to the B&D extraction, resulting in up to 1.5-fold increase in PUFA content and up to 1.2-fold decrease in SFA content. PCA analysis (Appendix B, Figure B1) suggests that the composition

of the B&D extract differs from sc-CO_2 extracts mainly for its high concentration in stearic acid (C18:0) and low concentration in total PUFA, EPA (C20:5 (7, 10, 13, 16, 19)), palmitoleic (C16:1 (9)), and myristic (C14:0) acids.

Table 3. Major fatty acids in sardine extracts quantified by GC-FID. Results were expressed as $mg_{fatty\ acid}/g_{extract}$.

		35 °C			55 °C			75 °C		
Fatty acid	**B&D**	**300 bar**	**425 bar**	**550 bar**	**300 bar**	**425 bar**	**550 bar**	**300 bar**	**425 bar**	**550 bar**
C14:0 Myristic acid	75.1	71.5	78.9	112.7	67.6	92.9	114.8	88.2	86.0	84.3
C16:0 Palmitic acid	238.2	225.4	226.3	255.0	217.2	242.6	253.4	238.2	226.9	232.0
C16:1 (9) Palmitoleic acid	38.7	60.0	60.0	69.9	55.4	66.3	69.8	62.8	59.2	61.5
C18:0 Stearic acid	63.7	37.3	36.5	33.7	38.5	35.4	37.0	36.2	36.6	37.3
C18:1 (9) Oleic acid	143.3	100.2	95.8	83.6	101.2	92.4	83.9	93.4	95.1	94.4
C18:1 (11) cis-Vaccenic acid	17.1	21.3	21.6	18.5	23.7	19.0	17.7	21.6	21.3	21.8
C18:4 (6, 9, 12, 15) Stearidonic acid	10.1	17.6	18.7	16.9	17.1	17.8	14.4	15.6	16.7	16.4
C20:1 (9) Gadoleic acid	34.9	54.9	52.6	36.0	59.3	38.1	38.5	46.5	50.8	52.2
C20:5 (5, 8, 11, 14, 17) EPA	27.8	76.9	67.5	38.1	66.2	65.4	50.5	53.6	57.1	64.0
C22:1 (11) Cetoleic acid	51.7	62.7	68.2	66.8	78.9	50.0	57.8	68.7	72.2	65.5
C22:6 (4, 7, 10, 13, 16, 19) DHA	60.1	59.2	57.4	39.9	56.6	46.4	49.5	45.8	52.5	52.8
Other fatty acids	115.2	104.3	111.3	106.2	102.8	112.4	92.9	107.4	108.2	103.4
SFA	411.9	364.4	375.3	424.3	346.5	404.4	434.2	390.4	383.2	379.5
MUFA	321.0	319.1	319.6	303.6	340.1	294.6	291.7	319.5	320.1	318.3
PUFA	143.0	207.9	199.9	149.4	197.9	179.7	154.2	168.2	179.2	187.8
TOTAL fatty acids	876.0	891.4	894.8	877.2	884.5	878.7	880.1	878.1	882.4	885.7

The global fatty acid profile of sc-CO_2 extracts slightly varied with pressure and temperature conditions, mainly on PUFA and SFA total contents, while MUFA composition remained approximately constant, regardless of the SFE conditions applied (Table 3). Some examples can be found in the literature showing that the selectivity and purity of sc-CO_2 fatty acid-rich extracts can be tuned by the conditions of pressure and temperature applied[31–34]. Table 3 shows that, in general, there was a slight increase in SFA and a decrease in PUFA with increasing pressure at a fixed temperature. However, at 75 °C, total concentrations were quite stable regardless of the pressure applied. Additionally, for a fixed pressure, it was also noticeable a slight decrease in PUFA concentrations with increasing temperature. However, this trend was reversed at 550 bar, at which PUFA concentrations slightly increased. Results

from PCA analysis (Appendix B, Figure B2) suggest that sc-CO_2 extracts differ from one another mainly due to different concentrations of myristic acid (C14:0), EPA (C20:5 (5, 8, 11, 14, 17)), gadoleic acid (C20:1 (9)), and the total content of ω-6 PUFA.

To evaluate the stability of the processed residue (after freeze-drying and milling), B&D extractions were performed immediately after processing, at day 0, and after 35 days of storage at -20 °C under a nitrogen atmosphere. Results summarized on Appendix A, Table A3 show that the fatty acid profiles changed considerably over time and that the processed residue was not stable, even when stored at -20 °C under a nitrogen atmosphere. Overall, the SFA relative concentration increased while the relative concentration of PUFA decreased, possibly due to PUFA oxidative degradation. The fatty acids that contributed the most for the increment of SFA relative concentration were palmitic (C16:0), stearic (C18:0), and myristic (C14:0) acids, although minor SFA, such as C15:0 or iC17:0, were the most affected by oxidation of unsaturated fatty acids, increasing 2.3- and 3.2-fold, respectively, within 35 days of storage. Regarding PUFA, the ω-3 fatty acids were the most affected by oxidation, namely EPA (C20:5 (5, 8, 11, 14, 17)), DHA (C22:6 (4, 7, 10, 13, 16, 19)), docosapentaenoic acid (DPA, C22:5 (7, 10, 13, 16, 19)), and stearidonic acid (C18:4 (6, 9, 12, 15)), resulting in a 4.6-, 1.9-, 4.2-, and 2.1-fold decrease in relative concentrations, respectively, within 35 days of storage. In fact, ω-3 PUFA are known to be highly prone to oxidation due to their large number of double bonds, as well as their position within the fatty acid chain. Additionally, long chain PUFA, such as EPA, DPA, and DHA, have a higher number of bisallylic carbons, being especially vulnerable to oxidative degradation when compared to short chain ω-3 PUFA (*e.g.*, α-linolenic acid) or most ω-6 PUFA[35,36]. In this way, if the original fatty acid composition of the residue is to be preserved, it should be processed before extraction, thus avoiding a period of storage after freeze-drying and milling.

4.3. *In vitro* bioactivity evaluation

4.3.1. *Cytotoxicity and antiproliferative activity*

The cytotoxic effect of fatty acid-rich extracts was assessed using confluent and non-differentiated Caco-2 cell monolayers. EC50 values summarized on Table 4 show that both B&D and sc-CO_2 extracts presented similar cytotoxicity, with values ranging from 7.008 to 7.200 mg/mL.

Table 4. EC50 values (mg/mL) obtained for fatty acid-rich extracts evaluated on Caco-2 and HT-29 cells, after an incubation period of 24 h. (sc-CO_2 1) 35 °C, 300 bar; (sc-CO_2 2) 35 °C, 425 bar; (sc-CO_2 3) 35 °C, 550 bar; (sc-CO_2 4) 55 °C, 300 bar; (sc-CO_2 5) 55 °C, 425 bar; (sc-CO_2 6) 55 °C, 550 bar; (sc-CO_2 7) 75 °C, 300 bar; (sc-CO_2 8) 75 °C, 425 bar; (sc-CO_2 9) 75 °C, 550 bar.

Run	Cytotoxicity (Caco-2 cells)	Antiproliferative effect (HT-29 cells)
sc-CO_2 1	7.128 ± 0.099	1.146 ± 0.152
sc-CO_2 2	7.166 ± 0.135	1.684 ± 0.208
sc-CO_2 3	7.160 ± 0.061	1.173 ± 0.155
sc-CO_2 4	7.072 ± 0.043	0.934 ± 0.237
sc-CO_2 5	7.182 ± 0.105	1.362 ± 0.291
sc-CO_2 6	7.146 ± 0.123	1.239 ± 0.383
sc-CO_2 7	7.018 ± 0.039	0.841 ± 0.114
sc-CO_2 8	7.008 ± 0.144	1.202 ± 0.473
sc-CO_2 9	7.053 ± 0.070	1.075 ± 0.254
B&D	7.200 [a]	0.826 [b]

[a] It was not possible to calculate an $CI_{95\%}$

[b] $CI_{95\%}$ [0.784, 0.903]

In this way, non-cytotoxic concentrations of each extract were tested in a human colorectal adenocarcinoma cell line (HT-29). Results showed that both sc-CO_2 and B&D extracts had the ability of decreasing HT-29 cell viability in a dose-dependent manner (data not shown), with EC50 values ranging from 0.826 and 0.841 for B&D and sc-CO_2 7 extracts, respectively, to 1.684 mg/mL for sc-CO_2 2 extract (Table 4). However, given the data obtained, it was not possible to establish significant differences among the EC50 values calculated.

Dietary fatty acids, and in particular MUFA and ω-3 PUFA, have been shown to play an important role on the prevention and/or treatment of cancer. Specifically, a growing body of epidemiological, clinical, and experimental evidence suggests a protective role of these fatty acids against colorectal cancer[37]. The work developed by Sarotra *et al.* indicated that the administration of fish and corn oil has a dose- and time-dependent chemopreventive effect in experimental colon cancer and that this effect is probably mediated by modulation of oxidative stress and apoptosis[38]. To the best of the author's knowledge, this was the first time that an oil obtained from *S. pilchardus* was tested on human cancer cell lines. Nevertheless, both oil and PUFA-rich extracts from other sardine species, namely *Sardinella longiceps* and *Sardinella fimbriata*, have already shown antiproliferative effect against different cancer cell lines, including breast (MCF-7), prostate (DU-145)[39], lung (A549), and colon (HCT 15)[40]. In particular, the oil obtained from *S. longiceps* by acetone extraction have shown EC50 values of approximately 8 mg/mL in colon cancer cells (HCT 15). Therefore, it is

possible to conclude that the oil extracted from *S. pilchardus* by sc-CO_2, as reported herein, was more effective in inhibiting colon cancer cells, than the oil obtained by acetone extraction from *S. longiceps*.

Since the different extracts showed similar cytotoxicity and antiproliferative effects, samples obtained with sc-CO_2 extraction at the lowest and highest temperature and pressure conditions (*i.e.*, sc-CO_2 1, 3, 7, and 9 runs), and the B&D extract were selected to be tested for antioxidant and anti-inflammatory activity.

4.3.2. *Antioxidant and anti-inflammatory activity*

Oxidative stress is generally characterized by an overproduction of ROS, which can damage virtually all components of the cell[41]. Intracellular antioxidant capacity of sardine extracts was determined through the detection of ROS generation prior (Figure 3i) and after (Figure 3ii) subjecting cells to oxidative stress.

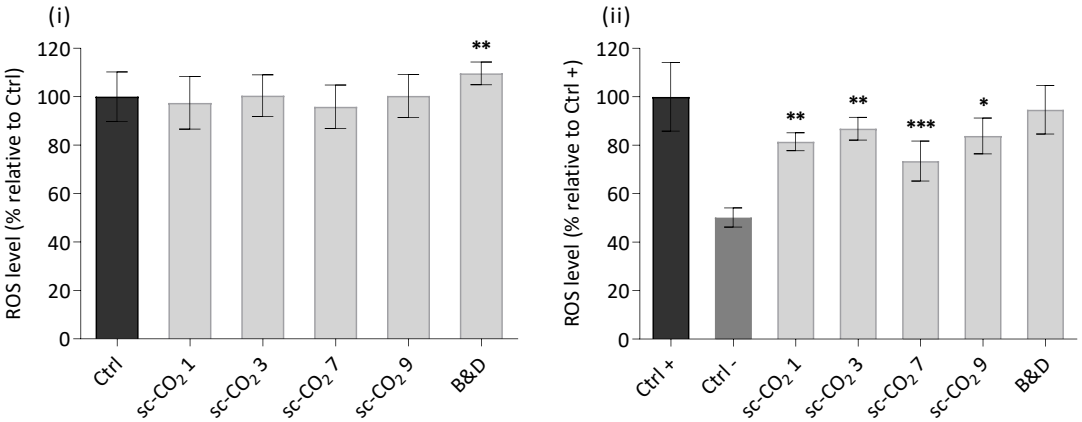

Figure 3. ROS levels measured after 1 h of incubation of Caco-2 cells with extracts (i) before and (ii) after incubation with a stress inducer. (sc-CO_2 1) 35 °C, 300 bar; (sc-CO_2 3) 35 °C, 550 bar; (sc-CO_2 7) 75 °C, 300 bar; (sc-CO_2 9) 75 °C, 550 bar. Statistically significant differences are represented by asterisks (*). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

As illustrated on Figure 3i, in which Caco-2 cells were treated with sardine extracts without the presence of a stress inducer, ROS levels remained unchanged, thus suggesting that the sc-CO_2 extracts tested did not induce stress on cells. However, at the concentration tested, the conventional B&D extract showed to significantly increase intracellular stress ($P < 0.01$). A stress inducer was then added to Caco-2 cells, after treatment with sardine extracts for 1 h. Results summarized on Figure 3ii show that all sc-CO_2 extracts had a preventive effect and

significantly reduced the ROS generated, being sc-CO_2 7 (75 °C, 300 bar) the most effective antioxidant, while the conventional B&D extract did not significantly affect the ROS levels. It has been hypothesized that fatty acids are one of the physiological factors involved in the control of oxidative stress by interacting at the mitochondrion level, and more specifically, on the electron transport and respiratory chain[42]. There are several studies that point unsaturated fatty acids, including, EPA, DHA, arachidonic acid, α-linolenic acid, linoleic acid, among others, as being capable of decreasing oxidative stress in different cell types[43,44], *Caenorhabditis elegans*[45], and also in patients with ulcerative colitis[46]. In this way, the behaviour displayed by sc-CO_2 extracts is in accordance with the literature.

The level of RNS can also be used as an oxidation marker[47]. Therefore, changes in RNS levels were measured indirectly by the accumulation of nitrites, the end products of NO metabolism. As represented on Figure 4, all extracts stimulated the production of NO, when compared to the control.

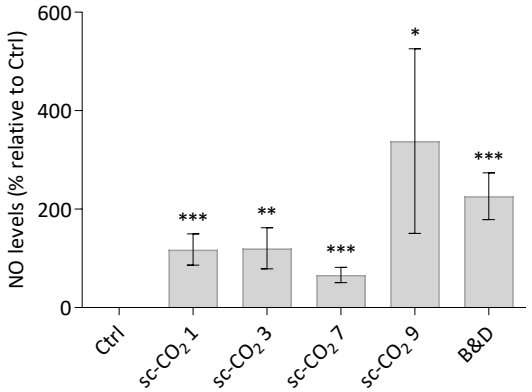

Figure 4. NO levels measured after 1 h incubation of Caco-2 cells with extracts in the presence of a stress inducer. (sc-CO_2 1) 35 °C, 300 bar; (sc-CO_2 3) 35 °C, 550 bar; (sc-CO_2 7) 75 °C, 300 bar; (sc-CO_2 9) 75 °C, 550 bar. Statistically significant differences are represented by asterisks (*). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The upregulation of NO by different fatty acids, namely palmitic, stearic, oleic, linoleic, arachidonic acids, DHA, and EPA, has been previously described by de Lima *et al.*. This stimulation occurred at low concentrations, being more prominent at concentrations up to 10 µM. However, when the dose of each fatty acid was increased (up to 200 µM), an inhibitory effect occurred. It was also mentioned by the authors that these findings could explain the disparities found between the results reported in the literature, since in some cases an increase in NO production is described after exposure to fatty acids, and in other

cases, evidences of a decrease were found[48]. In fact, the overproduction of NO at the settings of local or systemic inflammatory responses is of extreme importance and provides the host with an overall survival advantage, being of particular importance in human inflammatory bowel diseases[49]. Additionally, Pierini and Bryan have also described NO as a physiological regulator, being its levels closely related with ROS production or inhibition, mentioning that an increase of ROS led to a reduction of NO levels by chemical inactivation. Oxidative stress plays a significant role in the pathogenesis of hypertension, for example, in part by inactivation of NO[47]. Therefore, since fatty acid-rich extracts did not affect ROS levels (Figure 3i), except for B&D extract which slightly increased ROS concentration, it was expected that NO production would not be inactivated.

Since fatty acids are known to interact at the mitochondrion level, it was important to assess if sardine extracts could cause damage to this organelle. In this way, mitochondrial integrity was assessed by measuring TMRE inclusion. TMRE is a permeable fluorescent dye that specifically stains live mitochondria, and accumulates in proportion to mitochondrial membrane potential[24]. Within this context, it can be concluded by the results illustrated on Figure 5 that most extracts were able to significantly increase the mitochondrial membrane potential in comparison to the control. The only exception was extract sc-CO_2 7, which did not have a significant impact in TMRE inclusion.

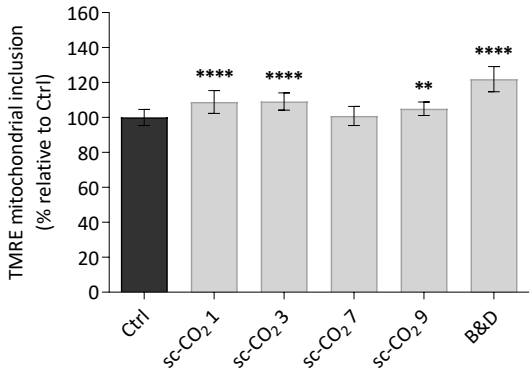

Figure 5. TMRE mitochondrial inclusion measured after 24 h incubation of Caco-2 cells with extracts. (sc-CO_2 1) 35 °C, 300 bar; (sc-CO_2 3) 35 °C, 550 bar; (sc-CO_2 7) 75 °C, 300 bar; (sc-CO_2 9) 75 °C, 550 bar. Statistically significant differences are represented by asterisks (*). ** $P < 0.01$; **** $P < 0.0001$.

Cytokines and chemokines are known for modulating the immune response in case of infection or inflammation and for regulating inflammation itself[50]. The pro-inflammatory

biomarker IL-6, a disease-perpetuating cytokine, is one of the most commonly measured, although other cytokines, chemokines, adhesion molecules, and leucocyte numbers can also be used[11]. Regarding inflammatory bowel disease, IL-8, a pro-inflammatory chemokine, has also been consistently reported as an important biomarker[51]. Accordingly, the impact of fatty acid-rich extracts was evaluated on the secretion of IL-6 and IL-8 upon pro-inflammatory stimulus. As illustrated on Figure 6, all extracts tested were able to decrease both IL-6 and IL-8 levels. Additionally, sc-CO_2 9 extract (75 °C, 550 bar) showed to be the most promising in inhibiting IL-6 secretion, while all sc-CO_2 extracts tested showed to be more effective in inhibiting IL-8 secretion than the B&D extract. It is also important to highlight that these results corroborate the upregulation of NO (Figure 4), which has been associated with a decrease in inflammatory cytokine levels[52].

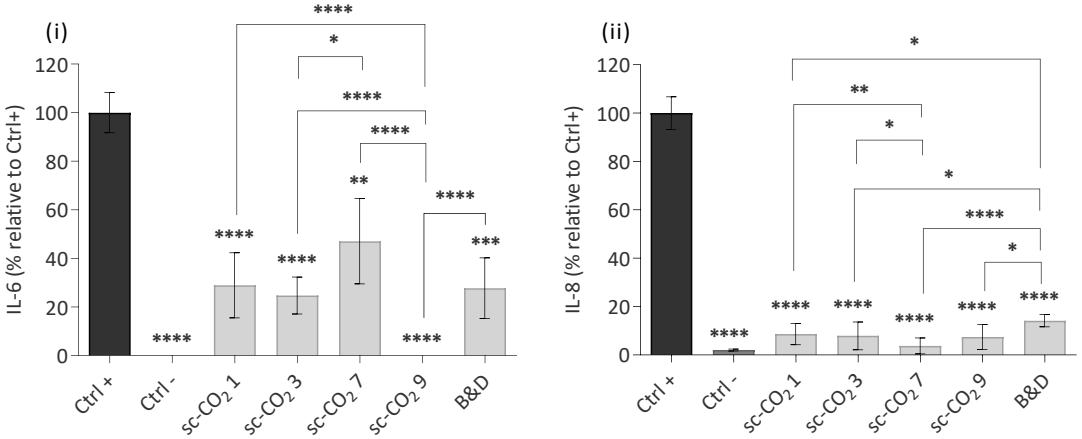

Figure 6. Modulation of inflammatory mediators (i) IL-6 and (ii) IL-8 by sardine extracts after 48 h incubation of differentiated Caco-2 cells. (sc-CO_2 1) 35 °C, 300 bar; (sc-CO_2 3) 35 °C, 550 bar; (sc-CO_2 7) 75 °C, 300 bar; (sc-CO_2 9) 75 °C, 550 bar. Statistically significant differences are represented by asterisks (*). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Several studies have already shown the anti-inflammatory potential of short and long chain fatty acids[53], in particular by decreasing cytokine, chemokine, and transcription factor levels, including IL-6 and IL-8 in TNF-α-stimulated human intestinal Caco-2 cells[54]; IL-1β and TNF-α in LPS-stimulated macrophages[55]; and IL-6, IL-8, interleukin-1 receptor type 1, and the nuclear factor NFkβ1 in IL-1β-stimulated human foetal intestinal epithelial H4 cells[56], among others. Additionally, marine-derived lipid extracts are already commercially available to relieve symptoms of chronic inflammation. An example is Lyprinol®, a stabilized lipid extract

of the New Zealand green-lipped mussel (*Perna canaliculus*), used in cases of arthritis. This mussel oil is a complex mixture of triglycerides, sterol esters, sterols, polar lipids, and free fatty acids, and has been shown to have the potential to decrease cytokine levels associated with inflammation and to increase interleukin-10 levels, an anti-inflammatory cytokine[57,58].

5. Conclusions

The work herein reported highlights the potential of sc-CO_2 extraction for the isolation of bioactive fatty acids from canned sardine waste streams, which have shown antiproliferative, antioxidant, and anti-inflammatory potential.

Extractions performed with sc-CO_2 were able to yield extracts highly concentrated in fatty acids, presenting concentrations similar to those obtained by B&D extraction (*ca.* 88 – 89%). GC-FID results showed that it was possible to tune the selectivity of extractions, mainly for SFA and PUFA, by varying the operating conditions, being the extraction performed at 35 °C and 300 bar the one that allowed to increase the most PUFA concentrations, while decreasing SFA concentrations. On the other hand, the extract obtained at 35 °C and 550 bar presented the lowest PUFA concentrations and the highest SFA concentrations, similar to those obtained by B&D extraction. Although all extracts revealed to have the potential for inhibiting colorectal cancer cells, while reducing the secretion of both IL-6 and IL-8, the operating conditions applied during sc-CO_2 extractions have also shown a potential to modulate the anti-inflammatory activities of sardine extracts. Although not significant in all cases, the results suggest that the extract obtained at 75 °C and 300 bar was the most promising in inhibiting colorectal cancer cells, in reducing ROS formation, and in decreasing IL-8 secretion; while the extract obtained at 75 °C and 550 bar showed to increase the most NO levels and presented the highest potential for reducing IL-6 secretion.

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CHAPTER 2

Part II – Deep eutectic system extraction of protein derivatives

Contents

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The DES phytotoxicity results were obtained during a visit to the Laboratory for Cell Culture Technology and Biotransformations, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia, within the scope of COST Action GREENERING (CA18224), under the supervision of Prof. I. R. Redovniković.

The author was involved in the conceptualization and design of the experiments and performed all experimental work, except for the evaluation of DES' cytotoxicity (performed at iBET by M. Cardeira), and the antimicrobial activity of DES and extracts (performed at iBET by I. C. Leonardo under the supervision of F. B. Gaspar). Data processing and interpretation, and results discussion were also performed by the author, as well as the preparation of the original manuscripts.

1. Abstract

In addition to their nutritional and functional value, fish proteins and peptides are known to exert different biological activities. In the work reported herein, the application of betaine/polyol-based deep eutectic systems (DES) was explored for the recovery of bioactive protein derivative-rich extracts contained in sardine processing waste streams. Different mixtures of betaine with glycerol, propylene glycol, and ethylene glycol, as well as their mixtures with water, were prepared and characterized in terms of physicochemical properties. Additionally, the potential toxic effect of DES was also evaluated on a human intestinal epithelial cell model (Caco-2) and on wheat (*Triticum aestivum*) seeds. Aiming at maximizing the extraction of target compounds, the impact of operating temperature (25, 45, and 80 °C), extraction time (6 and 18 h), and solid-liquid ratio (1:8, 1:16, 1:40, and 1:80) on the extraction performance was evaluated. Extractions using DES as alternative solvents were compared to a conventional solid-liquid extraction with water. The antioxidant activity of sardine extracts was assessed using the oxygen radical absorbance capacity assay, while their antimicrobial potential, as well as of DES, was evaluated towards *Staphylococcus aureus* and *Escherichia coli*. Results have shown that the physicochemical properties of the DES tested could be tuned by different parameters, namely the hydrogen bond donor applied, temperature, or water content. Additionally, although DES presented different responses towards different organisms, generally, these systems presented a low toxicity profile. As regards extraction results, the protein maximum extraction yields obtained with DES have shown to be similar to those achieved with water (162.2 ± 20.4 compared to 145.7 ± 2.9 $mg_{protein}/g_{dry\ residue}$). Nevertheless, most DES extracts obtained at 80 °C were able to surpass the antioxidant and antimicrobial potential of water extracts, with an increase in activity of up to 3-fold and more than 250-fold, respectively, for the most promising extracts.

2. Introduction

The nutritional and functional properties of fish proteins are well known. However, the interest in investigating protein, peptide, and amino acid bioactive properties in order to expand their applications as functional ingredients is rather recent[1,2]. Fish-derived protein hydrolysates, peptides, and amino acids have in fact been associated with a wide range of biological activities, either specific or multi-functional, including antioxidant, antimicrobial,

anti-inflammatory, anti-hypertensive, anticoagulant, immunomodulatory, among many others[3–5]. Due to their lower molecular weights, these molecules are known to be more bioactive than parent proteins, as they have a higher probability for an increased rate of intestinal absorption and to enter the cells[6].

In the last two decades, fish processing waste streams and underutilized fish have been explored as relevant sources of commercially valuable ingredients. In particular, proteins and their bioactive building blocks, such as small peptides (usually ranging in size from 2 to 20 amino acid residues) or functional amino acids (such as arginine (Arg), cystine, leucine (Leu), methionine (Met), tryptophan (Trp), tyrosine (Tyr), aspartic (Asp) and glutamic (Glu) acids, glycine (Gly), proline (Pro), and taurine (Tau)) can be found in these waste streams in quantities up to 23% of the total protein contained in the harvested fish. These discards represent, therefore, an interesting source for biofunctional ingredients mining, within the context of a circular bioeconomy based on zero waste[7–9].

The broad spectrum of bioactivities linked to protein-derived ingredients has enabled their utilization by the food and beverage, animal feed/nutrition, infant formulation, personal care and cosmetic, nutraceutical, and pharmaceutical industries, representing an expanding market, valued at US$ 42.5 billion[10,11]. In particular, there are a few examples of marine-derived peptide pharmaceutical products, with analgesic and anticancer properties, already approved by the U.S. Food and Drug Administration, while a large number of other peptides and amino acids continue being evaluated in different phases of clinical and preclinical trials, both in the U.S. and Europe. Concerning the nutraceutical and cosmetic/personal care segments, marine peptides and derivatives have already reached the market as nutrient supplements targeting bone health, hypertension, anxiety, skin care, blood glucose, intestinal health, among others[10].

The isolation of bioactive protein derivatives from marine sources can be performed by different methods, including extraction, chemical or enzymatic hydrolysis, microbial fermentation, or precipitation and salting out, which may ultimately affect their biological activities[5,12]. Typically, their isolation is performed either by extraction of protein and posterior hydrolysis or by direct hydrolysis of the muscle without prior extraction. At industrial scale, volatile organic solvents, such as methanol or ethyl acetate, are usually used for extraction. The resulting extract is then concentrated and partitioned with other organic

solvents, including hexane, tetra- or dichloromethane. Therefore, in order to avoid the use of harmful solvents, there has been a growing interest in finding safer and more sustainable extraction methods for the recovery of bioactive protein-derived ingredients from marine sources, including fish waste biomass[7,10].

Deep eutectic systems (DES) have emerged as a viable alternative to conventional organic solvents, for the extraction of a wide range of bioactive compounds, including proteins, peptides or amino acids[13]. Importantly, DES have already been used as preservation/stabilization media[14–17], or for the processing of biomacromolecules, including for their solubilization[16,18], functionalization[19], partitioning/purification[20–24], and extraction[25–30].

Typically, the most commonly used DES are based on choline chloride (ChCl)[31]. However, ChCl-based DES present some limitations in their application, particularly in the cosmetic industry, where the use of choline salts and their esters, including ChCl, is prohibited[32]. On the other hand, betaine has started to be widely used as hydrogen bond acceptor (HBA) in the formation of DES. Specifically, DES based on betaine and different polyols have already been used for the extraction of proteins[20] or polyphenols[33], as non-aqueous media for enzymatic reactions[34], in the purification of gasoline[35], in carbon dioxide absorption[36], among others.

Within this context, this work aimed at exploring the potential of different DES formed by mixtures of betaine with glycerol, propylene glycol, ethylene glycol, and their aqueous mixtures, for the recovery of bioactive protein derivative-rich extracts from by-products and wastes resulting from the sardine canning industry. Firstly, DES, as well as their aqueous mixtures, were characterized in terms of density, viscosity, and polarity. Additionally, to evaluate the safety of the prepared systems, their *in vitro* cytotoxicity was evaluated on a human intestinal epithelial cell model (Caco-2 cells) and their phytotoxicity was determined on wheat (*Triticum aestivum*) seeds. Different operating conditions were then applied aiming at improving the yield and bioactive quality of the extracts, namely temperature, extraction time, and solid-liquid ratio. Extracts were then characterized in terms of total protein yield and amino acid profile, after which were evaluated for their antioxidant activity through oxygen radical absorbance capacity (ORAC) assay and antimicrobial potential towards a Gram-positive bacterium (*Staphylococcus aureus* ATCC 6538) and a Gram-

negative bacterium (*Escherichia coli* ATCC 8739), which are considered to be relevant bacterial targets to substantiate health claims on functional ingredients to be applied as preservatives in pharmaceutical, nutraceutical, or cosmetic/personal care products.

3. Materials and methods

3.1. Biomass

Sardine (*Sardina pilchardus*) heads and offal resulting from the canning process were kindly provided by Fábrica de Conservas A Poveira, Portugal, in January 2017, and stored at -20 °C upon arrival. The biomass was freeze-dried and then milled, as previously described on Chapter 2, Part I, section 3.1.. After processing, the residues were protected from light and stored at room temperature, in a low moisture environment.

3.2. DES preparation

Chemicals used for DES preparation were: betaine (trimethylglycine, ref. B2629) and glycerol (ref. G8773) from Sigma-Aldrich (products from Finland and Germany, respectively), propylene glycol (ref. 31296) from Fragon (Spain), and ethylene glycol (ref. 121316) from Panreac AppliChem (Barcelona, Spain). The mixture of two (betaine:polyol) or three (betaine:polyol:water) components was heated to 80 °C under constant stirring, until a clear liquid was formed.

3.3. DES physicochemical characterization

3.3.1. *Water content determination*

The water content of the DES prepared without water addition was determined by Karl Fischer titration, using an 831 KF Coulometer with generator electrode without diaphragm (Metrohm, Herisau, Switzerland). HYDRANAL™ - Coulomat AG (Honeywell, Charlotte, NC, USA) was used as anolyte solution. The water content of the DES in which water was added as third component was estimated based on the water contained in the original DES (obtained by Karl Fischer titration) and on the mass of water added to each system. Results were expressed as mass percentage, as a mean of three measurements.

3.3.2. *Density and viscosity measurements*

Density and viscosity of DES were measured using an Anton Paar viscometer/densimeter (SVM 3001, Graz, Austria), using a range of temperatures between 20 °C and 80 °C. The temperature reproducibility was 0.03 °C, the repeat deviation value for density was 0.0010 g/cm^3 and for viscosity was 2.5%. Density and viscosity results were expressed as $g_{\text{DES or individual component}}$/cm^3 and mPa·s, respectively, as a mean of three measurements.

3.3.3. *Polarity measurements*

Relative polarity of DES was measured using the solvatochromic dye Nile red (Sigma-Aldrich, India) as probe, as previously described by Craveiro *et al.*[37]. Briefly, a stock solution of Nile red was prepared in ethanol absolute (Fisher Chemical, Loughborough, UK) at a concentration of 1 mg/mL. 5 µL of Nile red stock solution were added to a 1 cm^3 quartz cuvette containing 1 mL of DES. Ethanol was evaporated under a nitrogen stream, and the UV spectra acquired immediately after using a Genesys 10UV UV-vis spectrophotometer (Thermo Spectronic). The maximum wavelength ($\lambda_{max,NR}$) was determined for each system, and the E_{NR} parameter was calculated as follows:

$$E_{NR} = \frac{28591}{\lambda_{max,NR}} \qquad \textbf{Equation 1}$$

Results were expressed as kcal/mol, as a mean of three measurements.

3.4. DES *in vitro* cytotoxicity determination

3.4.1. *Cell culture*

Human intestinal Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of heat-inactivated foetal bovine serum (FBS), 1% of non-essential amino acids (NEAA), and 1% penicillin-streptomycin (Gibco, Invitrogen, Paisley, UK). Cells were maintained as monolayers as previously described on Chapter 2, Part I, section 3.4.1..

3.4.2. *Sample preparation*

DES or their isolated component stock solutions were prepared in DMEM supplemented with 0.5% of FBS and 1% of NEAA, immediately before the assays. To obtain a range of concentrations, samples were further diluted in DMEM supplemented with 0.5% of FBS and 1% of NEAA.

3.4.3. *Cytotoxicity assay*

Cytotoxicity assays were performed by incubating confluent Caco-2 cells with different concentrations of DES, or respective isolated compounds for 24 h, as described on Chapter 2, Part I, section 3.4.3.. Cell viability was evaluated using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, according to the manufacturer's instructions. Absorbances were measured at 490 nm and cell viability was expressed as percentage of viable cells relative to the control. Three independent assays were performed in triplicate and the half maximal effective concentrations (EC50) were calculated from dose-response curves and results were expressed as $mg_{DES\ or\ individual\ component}$/mL and converted to $M_{DES\ or\ individual\ component}$ to simplify the comparison with literature results.

3.5. DES *in vitro* phytotoxicity determination

3.5.1. *Sample preparation*

DES stock solutions were prepared in distilled water immediately before the phytotoxicity assay. Samples were then two-fold serially diluted in distilled water to obtain a range of concentrations (0 – 20 mg_{DES}/mL).

3.5.2. *Phytotoxicity assay*

The methodology used for evaluating DES phytotoxicity was previously described by Bubalo *et al.* and Radošević *et al.*[38,39]. Briefly, wheat (*T. aestivum*) seeds were sterilized prior to germination using 1% sodium hypochlorite (Sigma-Aldrich, St. Quentin Fallavier, France) solution for 30 min. After sterilization, seeds were washed with distilled water and incubated for 24 h in the darkness at 23 °C. Petri dishes (Ø=15 cm) were prepared by placing two pieces of filter paper covered with a thin layer of cotton wool. 15 seeds were placed in each Petri dish and moistened with 30 mL of different concentrations of DES. A control was maintained with distilled water. Wheat seedlings were grown for 7 days at 23 ± 1 °C with shift cycles of 14 h/day and 10 h/night. Seeds were re-moistened with DES solutions every 48 h in order to keep concentrations stable. After 7 days of exposure, seedlings were harvested and the effect of DES on germination and early growth of wheat was determined. All experiments were performed in duplicates. Results were expressed as germination inhibition and shoot height inhibition in comparison to controls. The corresponding EC50 values were calculated

from the dose-response curves and expressed as mg_{DES}/mL and converted to mM_{DES} to simplify the comparison with literature results.

3.5.3. *Lipid peroxidation (LPO) measurement*

To determine LPO, approximately 0.2 g of fresh wheat leaves were homogenized with 5 mL of 0.1% trichloroacetic acid (Fisher Scientific, Loughborough, UK), using a mortar and pestle. The levels of LPO in the supernatants were measured through the determination of malondialdehyde (MDA) content, using the thiobarbituric acid (TBA) method[40]. Briefly, mixtures containing 1 mL of supernatant and 4 mL of 0.5% TBA (Acros Organics, Geel, Belgium) in 20% trichloroacetic acid were heated to 95 °C. After 30 min of reaction, the mixtures were immediately cooled in an ice bath and centrifuged (5000 *g* for 20 min). Absorbances were measured at 532 nm using a GENESYS^{TM} 10S UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. MDA content was calculated according to its molar extinction coefficient (ε = 155 $mM^{-1} \cdot cm^{-1}$) and expressed as $nmol_{MDA}/g_{fresh\ leaves}$ as a mean of three measurements.

3.5.4. *Chlorophyll (CHL) content determination*

CHL content was determined by following the method previously described by Arnon[41]. Briefly, approximately 0.1 g of fresh wheat leaves were extracted using 10 mL of 80% acetone (eci, Zagreb, Croatia). Samples were protected from light and maintained at 4 °C, under constant stirring, for 24 h. After extraction, samples were centrifuged (5000 *g* at 4 °C for 15 min) and the absorbance of the supernatants measured at 663 nm and 645 nm. Contents of CHL *a*, CHL *b*, and total CHL were calculated as follows:

$$CHL\ a = 12.25A_{663} - 2.79A_{645} \quad \textbf{Equation 2}$$

$$CHL\ b = 21.50A_{645} - 5.10A_{663} \quad \textbf{Equation 3}$$

$$Total\ CHL = CHL\ a + CHL\ b \quad \textbf{Equation 4}$$

Results were expressed as $\mu g_{CHL}/g_{fresh\ leaves}$.

3.5.5. *Antioxidant enzyme activity determination*

For enzyme analysis, approximately 0.4 g of fresh wheat leaves were homogenized with 0.2 g of hydrated polyvinylpolypyrrolidone (Acros Organics, China) in 4 mL of 100 mM potassium phosphate buffer solution (pH 7.0; potassium phosphate monobasic, Fisher Scientific, Loughborough, UK; potassium phosphate dibasic, Kemika, Zagreb, Croatia), containing 1 mM ethylenediaminetetraacetic acid (EDTA, LKB Bromma, Stockholm, Sweden) and 5 mM L-ascorbic acid (Kemika, Zagreb, Croatia), using a pre-chilled mortar and pestle. The homogenates were centrifuged (5000 *g* at 4 °C for 20 min) and the supernatants were used for both protein content and antioxidant enzyme activity determination.

Total soluble protein contents of each enzyme extract were estimated in duplicates, according to the method proposed by Bradford[42], using bovine serum albumin (Sigma-Aldrich, St. Quentin Fallavier, France) as standard.

Superoxide dismutase (SOD) activity was determined following the method proposed by Beauchamp and Fridovich[43], by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium. Briefly, the reaction mixture was composed of potassium phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), methionine (13 mM, Acros Organics, China), nitro blue tetrazolium chloride (75 mM, Alfa Aesar, Kandel, Germany), riboflavin (2 mM, Carlo Erba Reagents, Val de Reuil, France), and a suitable aliquot of enzyme extract. Test tubes were vortexed and placed 30 cm away from a 30 W fluorescent lamp. The increase in absorbance due to formazan formation was measured immediately after at 560 nm. The activity of one unit of SOD was defined as the amount of enzyme that inhibited the nitroblue tetrazolium photoreduction by 50%. The activity of SOD was expressed as $units_{SOD}/mg_{protein}$.

Guaiacol peroxidase (GPX) activity was estimated by measuring the increase in absorbance of oxiguaiacol at 470 nm (ε = 26.6 $mM^{-1}\cdot cm^{-1}$), as previously reported by Chance and Maehly[44]. The reaction mixture contained phosphate buffer (50 mM, pH 7.0), guaiacol (18 mM, Acros Organics, China), hydrogen peroxide (5 mM, 30%, Gram mol, Zagreb, Croatia), and a suitable aliquot of enzyme extract. Results were expressed as $nmol_{oxidized\ guaiacol}/(mg_{protein}\cdot min)$.

Catalase (CAT) activity was determined by measuring the decomposition of hydrogen peroxide and consequent decrease in absorbance at 240 nm (ε = 40 $mM^{-1}\cdot cm^{-1}$), as previously described by Aebi[45]. The reaction mixture was composed of phosphate buffer (50

mM, pH 7.0), hydrogen peroxide (10 mM), and suitable aliquot of enzyme extract. Results were expressed as $nmol_{decomposed\ H2O2}/(mg_{protein}\cdot min)$.

Ascorbate peroxidase (APX) activity was measured following the decrease in absorbance of ascorbate at 290 nm (ε = 2.8 $mM^{-1}\cdot cm^{-1}$), as previously described[46]. The reaction mixture contained phosphate buffer (50 mM, pH 7.0), EDTA (0.1 mM), L-ascorbic acid (0.5 mM), hydrogen peroxide (0.12 mM), and a suitable aliquot of enzyme extract. Results were expressed as $nmol_{oxidized\ ascorbate}/(mg_{protein}\cdot min)$.

All experiments were performed at least in duplicates.

3.6. Solid-liquid extractions

Briefly, approximately 4 g of water or two- or three-component DES were added to 50 mg of sardine freeze-dried residue. Extractions were performed in silicone baths under constant magnetic stirring (*ca.* 60 rpm) and the influence of operating temperature (25, 45 and 80 °C) and extraction time (6 and 18 h) on total protein yield was studied. To determine the impact of solid-liquid ratio, four different ratios were applied at 80 °C and 18 h, namely 1:8, 1:16, 1:40, 1:80 $g_{dry\ residue}/g_{solvent}$. Extracts were centrifuged at 6 000 rpm for 10 min at room temperature, and the supernatant recovered. Samples were stored at -20 °C in the absence of light, until further analyses. All experiments were performed at least in duplicate.

3.7. Extract characterization

3.7.1. *Lowry method for total protein quantification*

Total protein was measured by UV-vis spectroscopy following the method reported by Lowry *et al.*[47], modified as described by Barbarino and Lourenço[48]. Briefly, 1 mL of a solution 50:0.5:0.5% of sodium carbonate (2%, Panreac, Barcelona, Spain) in sodium hydroxide (0.1 M, Acros Organics, Sweden) with copper(II) sulphate pentahydrate (0.5%, Acros Organics, Italy) and sodium potassium tartrate (1%, Sigma-Aldrich, Spain) was added to 0.2 mL of extract, blank or standard at appropriate concentration. The mixture was vortexed and incubated at room temperature for 10 min. After incubation, 0.1 mL of Folin & Ciocalteu's Phenol Reagent (1 N, Sigma-Aldrich, Switzerland) were added to each tube. Samples were vortexed, protected from light, and incubated for 30 min at room temperature. Absorbances were recorded at 750 nm and total protein concentrations were calculated using bovine

serum albumin (Sigma-Aldrich, USA) as standard. Experiments were performed in duplicate and results were expressed as $mg_{protein}/g_{dry\ residue}$.

3.7.2. *Total amino acid profile determination*

Amino acids in protein-rich extracts were analysed, as previously described by Usydus *et al.*, after acid hydrolysis with 6 M hydrochloric acid (≥ 37%, Sigma-Aldrich, Austria) for 22 h at 110 °C in glass tubes under nitrogen[49]. After hydrolysis, samples were derivatised with an *o*-phthaldialdehyde (OPA, Sigma-Aldrich, India) reagent solution according to the method proposed by Antoine *et al.*, with slight modifications[50]. Briefly, OPA reagent solution was freshly prepared by dissolving 25 mg of OPA in 500 µL of methanol (Fisher Chemical, Loughborough, UK). Then, 5 mL of 0.1 M sodium tetraborate (pH 9.5, Riedel-de Haën, Seelze, Germany) were added, followed by 50 µL of 2-mercaptoethanol (Sigma-Aldrich, Japan). The mixture was vigorously vortexed, stored in a tightly closed dark glass vial, and used throughout the day. Pre-column derivatization was performed by adding 800 µL of OPA reagent to 200 µL of hydrolysed sample or standard and vortexing for 1 min. 100 µL of derivatised sample or standard were injected immediately after on a 2695 Waters Alliance HPLC system, equipped with a 2475 fluorescence detector (Waters, Milford, MA, USA). Separation was achieved with a ReproSil 100 C18 column (5 µm, 250 x 4.6 mm, Dr. Maisch GmbH HPLC, Ammerbuch, Germany), at 25 °C, in gradient mode, with a mobile phase formed by sodium acetate buffer (0.1 M, pH 7.2, Sigma-Aldrich, Germany), methanol, and tetrahydrofuran (Fisher Chemical, Loughborough, UK) (90.5:9.0:0.5%, eluent A); and methanol (100%, eluent B), as described by Dai *et al.*[51]. The elution gradient was set as follows, at a flow rate of 1 mL/min: 100% A (0 – 1 min), 100 – 75% A (1 – 11 min), 75% A (11 – 20 min), 75 – 0% A (20 – 50 min), 0 – 100% A (50 – 60 min). Fluorescence was monitored at excitation and emission wavelengths of 338 and 425 nm, respectively. Results were expressed as relative mass percentage by using a calibration curve prepared with a mixture of the following amino acids: alanine (Ala, Sigma, USA), Arg (Sigma, Japan), Asp (Aldrich, USA), Glu (Sigma, Japan), Gly (Sigma-Aldrich, USA), histidine (His, Sigma, USA), isoleucine (Ile, Sigma-Aldrich, USA), Leu (Sigma, Japan), lysine (Lys, Sigma, China), Met (Sigma-Aldrich, Japan), phenylalanine (Phe, Sigma, Japan), serine (Ser, Sigma, USA), Tau (Sigma, USA), threonine (Thr, Sigma-Aldrich, USA), Tyr (Fluka, Buchs, Switzerland), and valine (Val, Sigma-

Aldrich, USA). Cysteine, Trp, and Pro cannot be detected by this method of analysis. Asparagine and glutamine were quantified along with Asp and Glu, respectively.

3.8. *In vitro* bioactivity evaluation

3.8.1. *ORAC assay*

The antioxidant activity of protein-rich extracts was evaluated by ORAC assay according to the procedure previously reported by Huang *et al.*[52] modified for the FL800 microplate fluorescence reader (BioTek Instruments, Winooski, VT, USA), as described by Feliciano *et al.*[53]. Briefly, 25 µL of sample or respective blank and 150 µL of fluorescein sodium salt (3×10^{-4} mM, Sigma-Aldrich, USA) in phosphate buffered saline (75 mM, pH 7.4; sodium chloride, potassium phosphate monobasic, sodium phosphate dibasic dihydrate, Sigma-Aldrich, USA, Japan, and Germany, respectively; potassium chloride, Merck, Darmstad, Germany) were dispensed in a black 96-well microplate. After incubating the microplate at 37 °C for 10 min, the reaction was started by adding 25 µL of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (153 mM, Sigma-Aldrich, USA) in phosphate buffered saline (75 mM, pH 7.4) to each well. The fluorescence emitted by the reduced form of fluorescein was recorded every minute for 40 min, at an emission wavelength of 530 ± 25 nm and excitation wavelength of 485 ± 20 nm. Samples were analysed in duplicate. (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, Fluka, Germany) was used as standard and results were expressed as $\mu mol_{trolox\ equivalents}/L$.

3.8.2. *Bacterial assays*

3.8.2.1. Bacterial test strains

S. aureus ATCC 6538 and *E. coli* ATCC 8739 strains were selected as representative of Gram-positive and Gram-negative species, respectively.

3.8.2.2. Sample preparation

DES or protein-rich extract stock solutions were prepared in cation-adjusted Mueller Hinton broth (CAMHB, BD Difco, Sparks, NV, USA) immediately before the antimicrobial susceptibility testing assay. Pipetting up and down 20 times was performed to ensure a

homogeneous extract stock solution. Solutions were then 2-fold serially diluted in CAMHB to obtain a range of concentrations.

3.8.2.3. Antimicrobial susceptibility testing assay

Assays were performed following the broth microdilution method of CLSI M07-A10 guidelines[54]. Briefly, different DES or extract concentrations were dispensed in a 96-well round bottom microtiter plate. To achieve a homogenous suspension in saline solution, the growth method was used to prepare the inoculum. The adjusted inoculum was further diluted in CAMHB to ensure that, after inoculation, each well contained approximately 5 × 10^4 colony-forming units. Inoculated microtiter plates were then incubated under aerobic conditions at 37 °C for 16 to 20 h. For each sample tested, a positive control (CAMHB and diluted inoculum), a medium sterility control (uninoculated CAMHB), and a DES or extract sterility control (uninoculated 2-fold DES or extract stock solution in CAMHB) were also analysed. Minimum inhibitory concentrations (MIC) were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited after incubation. When ambiguous MIC values were obtained, MICs were determined using the cell viability reagent PrestoBlue™ (Invitrogen, San Diego, CA USA) according to the manufacturer's instructions. An additional MIC value, denominated MIC*, was also determined and defined as the lowest concentration of an antimicrobial agent at which bacterial growth was visually and differentially affected, when compared to the positive control, thus enabling the identification of the DES or extracts that had the ability to interfere with the fitness of the target microorganisms even when growth inhibition was not fully achieved. MIC and MIC* results were expressed as a median of the values obtained after three biological replicates as μL_{sample}/mL.

3.9. Statistical analysis

The estimation of cytotoxicity and phytotoxicity, as well as the statistical significance of average differences determination, was performed using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance of average differences was assessed by one-way analysis of variance followed by the Tukey test. An alfa

error of 5% was accepted in the hypothesis testing to decide for a significant effect. Data was reported as mean ± standard deviation values.

4. Results and discussion

4.1. DES preparation and physicochemical characterization

DES based on betaine, glycerol/ethylene glycol, and water have already shown potential for the extraction of different protein standards in DES aqueous two-phase systems[20]. Therefore, in the work reported herein, DES based on betaine and different polyols, including glycerol, propylene glycol, and ethylene glycol, were explored for an efficient recovery of bioactive protein derivative-rich extracts from sardine residues, which are known to be an interesting source of biofunctional ingredients. Betaine was selected as HBA through the oxygen atoms present in its structure, while the polyols were selected as hydrogen bond donors (HBD) through their hydroxyl groups. Additionally, in order to obtain a wider range of physical properties, namely density, viscosity, and polarity, in some cases water was also added to DES as third component (Table 1). All DES resulted in clear and transparent liquids, which maintained this visual aspect even when cooled to room temperature.

Table 1. Summary of the DES prepared, respective molar ratios, water content, and denomination used.

Component A	Component B	Component C	Molar ratio [a] (A:B:C)	Water content (mass %)	Denomination
Betaine	Glycerol	-	1:2:0.2	1.1 ± 0.03	B:G (1:2)
		Water	1:2:1.2	6.7 ± 0.20	B:G:W (1:2:1)
			1:2:5.2	24.1 ± 0.72	B:G:W (1:2:5)
			1:2:10.2	38.5 ± 1.16	B:G:W (1:2:10)
	Propylene glycol	-	1:3:0.6	3.3 ± 0.02	B:PG (1:3)
		Water	1:3:1.6	8.2 ± 0.06	B:PG:W (1:3:1)
			1:3:5.6	24.0 ± 0.17	B:PG:W (1:3:5)
			1:3:10.6	37.6 ± 0.26	B:PG:W (1:3:10)
	Ethylene glycol	-	1:3:0.2	1.2 ± 0.03	B:EG (1:3)
		Water	1:3:1.2	6.8 ± 0.15	B:EG:W (1:3:1)
			1:3:5.2	24.1 ± 0.52	B:EG:W (1:3:5)
			1:3:10.2	38.5 ± 0.83	B:EG:W (1:3:10)

[a] Molar ratios take into account the water content of each system

To evaluate the potential of betaine/polyol-based DES as green extraction solvents, important properties of each system, including water content, density, viscosity, and polarity, were evaluated.

Since the water contained in a DES can significantly modify its physical properties, including density, viscosity, and polarity[55,56], it was important to study this parameter. Results summarized on Table 1 show that the water content varied between 1.066 and 3.278% for two-component systems, and between 6.708 and 38.488% for the systems in which water was added as the third component of the mixture. The work developed by Dai *et al.* revealed that a progressive rupture of hydrogen bonds in a DES can occur with the addition of increasing amounts of water. Dai *et al.*'s results on ChCl:propylene glycol:water (1:1:1) showed that the supermolecular complex structures of the DES were preserved at a content of water below 50% (v/v). However, further dilution resulted in a solution of the free forms of the individual components in water[55]. Therefore, in the work reported herein, special care was taken not to exceed this volume of water in the different three-component DES prepared.

The study of DES density and its temperature dependence is important so a proper design and operation of separation processes can be achieved[57]. Accordingly, betaine/polyol-based DES densities were studied as a function of temperature (Figures 1i, iii, v).

Densities of two-component DES increased as follows: B:PG (1:3) < B:EG (1:3) < B:G (1:2), ranging from 1.087 to 1.219 g/cm^3 at 20 °C and from 1.047 to 1.184 g/cm^3 at 80 °C. These values showed to be higher than the density of the isolated polyols, except for B:G (1:2) DES that presented a lower density than glycerol. Additionally, the density of two-component DES also showed to increase with increasing number of hydroxyl groups in the polyol, which is in accordance to the work developed by Zahrina *et al.*[58]. When comparing these three systems, the distinct density values suggest that there was a considerable difference in packing, as previously hypothesized for ionic liquids (IL). In fact, the hole theory speculates that it is possible that IL, or in this case DES, contain empty vacancies or holes, the size of which will determine the systems' density[59].

DES densities tended to decrease not only with increasing temperature, but also with increasing water content, as it would be expected[55]. As most of the DES described in the literature[60], betaine/polyol-based DES densities revealed to be higher than water (ranging from 1.069 to 1.219 g/cm^3 at 20 °C and from 1.028 to 1.184 g/cm^3 at 80 °C), being within the same range of what was previously reported for other systems based on betaine[58,61].

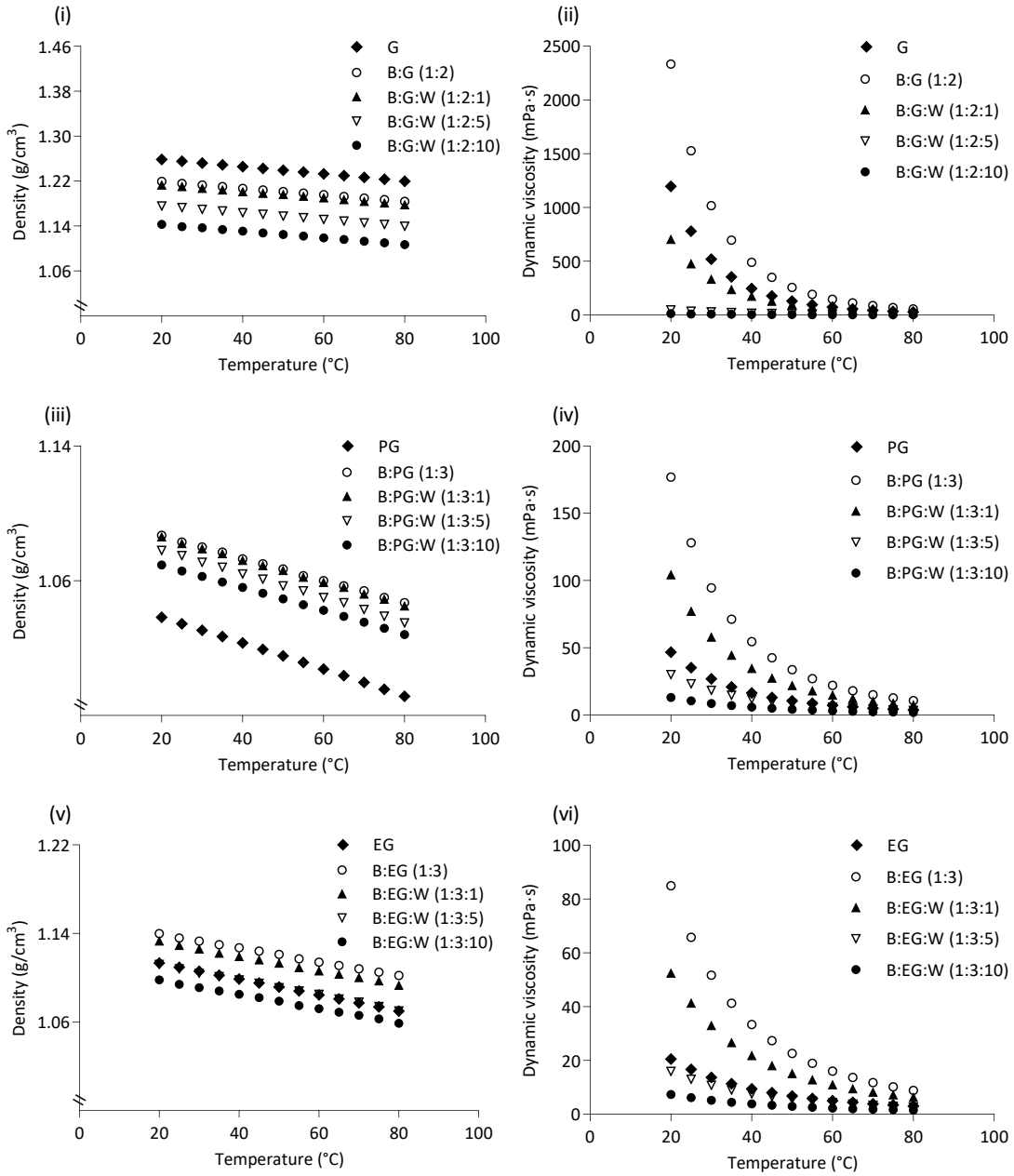

Figure 1. Variation of the density of (i) glycerol and B:G-based DES, (iii) propylene glycol and B:PG-based DES, and (v) ethylene glycol and B:EG-based DES; and the viscosity of (ii) glycerol and B:G-based DES, (iv) propylene glycol and B:PG-based DES, and (vi) ethylene glycol and B:EG-based DES; as a function of temperature.

Viscosity is another important parameter that should be taken into account when selecting an extraction solvent, especially when working with DES, since a high viscosity is usually an inherent characteristic of these systems[56,57]. Their high viscosity is usually associated with

extensive hydrogen-bonding, van der Waals, and/or electrostatic interactions between the components of the mixture, which can strongly limit mass transfer and consequently hamper the extraction yield[55,62].

Figures 1ii, iv, vi show the viscosity of betaine/polyol-based DES as a function of temperature. Viscosities of two-component DES increased as follows: B:EG (1:3) < B:PG (1:3) < B:G (1:2), ranging from 84.9 to 2333.7 mPa·s at 20 °C and from 8.9 to 57.0 mPa·s at 80 °C. Similar to what could be found for density, viscosity values appear to be related to the HBD, increasing with increasing number of hydroxyl groups in the polyol molecule, which is in accordance with what was previously described by Zahrina and co-workers[58]. Additionally, according to the hole theory[59], the high density of the B:G (1:2) system (Figure 1i) suggests a reduction in the average hole radius and consequently a decrease in mass transport properties, which may have led to an increased viscosity of this system (Figure 1ii).

All two-component DES showed an increased viscosity when compared to the isolated polyols, possibly due to the thickening potential of betaine, which has been explored mainly by the cosmetic industry[63].

As it would be expected, results showed a pronounced decrease of viscosities with increasing temperature (up to 40.9-fold) and water content (up to 185.7-fold). As described by Liu *et al.*, temperature has the ability of regulating the intermolecular cohesive forces in a DES, which in turn contribute to their viscosity, *i.e.*, higher temperatures contribute to overcoming the strength of intermolecular forces and consequently decrease DES viscosity[64]. Regarding three-component DES, the water content also contributed to weaken hydrogen-bonding interactions between the components[55], reducing viscosity up to 185.7-fold for B:G, 13.5-fold for B:PG, and 11.6-fold for B:EG.

The extraction efficiency can also be deeply influenced by the polarity of the DES, since this is a physical property that directly affects the solubility of a given solute in a solvent. Different studies have used solvatochromic dyes, such as Nile red, to estimate the polarity of numerous solvents, including a wide range of molecular and switchable solvents, IL[65], and DES[37,66,67]. In the presence of higher polarity solvents, the maximum wavelength of the dye shifts to higher wavelengths, resulting in lower E_{NR} values, according to Equation 1. By measuring these shifts and comparing them with the values obtained for a solvent of reference, it is possible to estimate the relative polarity of a given solvent. Figure 2

summarizes the E_{NR} values obtained for the betaine/polyol-based DES prepared and compares these values to the E_{NR} of water and methanol, which were considered as solvents of reference[65]. DES E_{NR} values ranged from 48.71 (for B:G:W (1:2:10)) to 51.15 kcal/mol (for B:PG (1:3)). Regarding specifically the relative polarity of two-component DES, it increased as follows: B:PG (1:3) < B:EG (1:3) < B:G (1:2). Figure 2 suggests that the different DES prepared covered a wide range of polarities, from polarities similar to water (B:G:W (1:2:10) and B:EG:W (1:3:10)) to polarities close to methanol (B:PG (1:3)). As it would be expected, the water content revealed to have a strong impact on tailoring the polarity of DES[55,66], resulting in solvents with a polarity similar to water when higher water contents were applied (B:G:W (1:2:10), B:EG:W (1:3:10), B:PG:W (1:3:10), and B:G:W (1:2:5)).

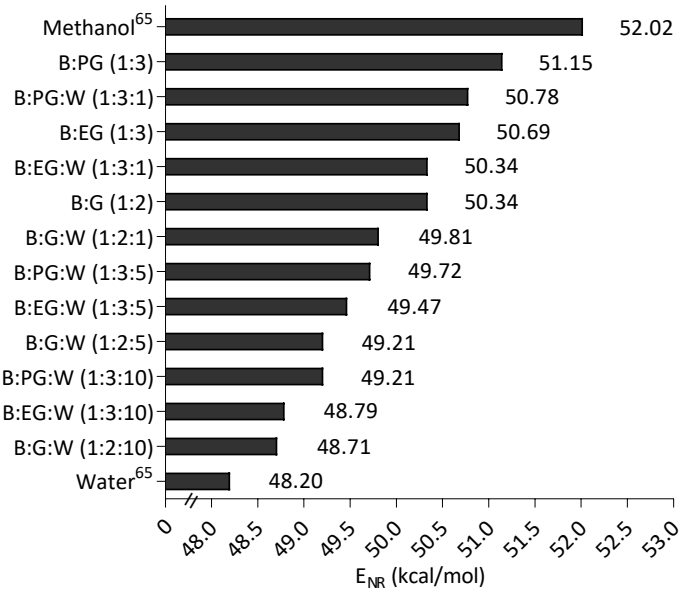

Figure 2. E_{NR} values obtained for each DES. Water and methanol E_{NR} values were included as solvents of reference. Coefficient of variation ≤ 0.25%.

4.2. DES *in vitro* toxicity

Different authors have described DES as being non-toxic, green, and environmentally friendly, mainly based on the existing toxicity data for their individual components. However, this assumption does not take into account the possible synergistic, additive, or antagonistic effects of a DES as a system, which could significantly influence their toxicity, as well as their biological properties[39,68,69]. Additionally, it has also been hypothesized that the charge delocalization that occur through hydrogen bonding when the DES is formed can

possibly lead to a more toxic system, when compared to their individual components[68]. In this way, it is of the utmost importance to evaluate the safety of a DES, by studying their toxicity at different trophic levels, while determining their potential for a particular application.

4.2.1. *DES in vitro cytotoxicity*

The possible cytotoxic effects of betaine/polyol-based DES as well as their individual components were evaluated on Caco-2 cells. Table 2 summarizes the EC50 values obtained from dose-response curves, after a 24 h treatment of Caco-2 cells with betaine, polyols, and two- and three-component DES. All DES showed to have a cytotoxic effect on Caco-2 cells with very distinct EC50, ranging from 103.5 to 259.6 mg/mL, or from 1.2 to 7.5 M. It is interesting to note that all DES were less toxic for Caco-2 cells than betaine (up to 11.4-fold for EC50 expressed in M), although the toxicity of two-component DES was either similar or higher than the isolated polyol (up to 1.6-fold for EC50 expressed in M). Several authors have already commented on how important the HBD can be for the toxicity of a given system[70,71]. These results further support this hypothesis, since distinct toxicity values were obtained by simply modifying the polyol in each system. Toxicity effects were lower for the mixtures with ethylene glycol, intermediate for the systems with glycerol, and higher for DES prepared with propylene glycol. The same tendency could be observed for the cytotoxicity of the isolated polyols (when looking at the results expressed in M). Two-component DES were generally more toxic. However, their toxicity greatly decreased with increasing water content in three-component DES (up to 4.1-fold for EC50 expressed in M), which is in accordance with what was previously described by Hayyan and co-workers for ChCl-based systems[72].

Different *in vitro* studies have shown that the toxicity of DES greatly depends on their composition, molar ratio of their individual components, as well as on the model organism tested. When comparing the results obtained in this work with previous studies involving the analysis of DES toxicity on different cell models, including ChCl/organic acid-based (1.6 < EC50 < 35 mM)[39,71,72]; tetrabutylammonium chloride-based (34 < EC50 < 497 µg/mL)[73]; ChCl/polyol or amide-based (9 < EC50 < 129 µg/mL)[70]; or ChCl/sugar or organic acid-based (EC50 < 25 mg/mL)[74], it is possible to conclude that the toxicity demonstrated by the DES

reported herein was quite low. In the last years there has been an effort to try to classify DES in different levels of toxicity. Some works have followed the recommendations of the UFT Merck ILs Biological Effects Database for IL that classifies EC50 values obtained on IPC-81 cell line into four levels of toxicity: (i) very high toxicity, (EC50 < 1 μM), (ii) high toxicity (1 < EC50 < 100 μM), (iii) moderate toxicity (100 < EC50 < 5000 μM), and (iv) low toxicity (EC50 > 5000 μM)[39,75]. Overall, and according to this rating scale, it is possible to further support the conclusion that the betaine/polyol-based DES under study showed low cytotoxicity (EC50 > 5 mM) on Caco-2 cells. This opens the possibility for these systems to be considered for applications in pharmaceutical, nutraceutical, or cosmetic/personal care products.

Table 2. EC50 values obtained for betaine/polyol-based DES and respective individual components, on Caco-2 cells, after an incubation period of 24 h.

Samples	EC50 (mg/mL)	EC50 (M)
Individual components		
Betaine	76.9 ± 6.6	0.7 ± 0.06
Glycerol	224.4 ± 10.9	2.4 ± 0.12
Propylene glycol	88.32 ± 6.3	1.2 ± 0.08
Ethylene glycol	164.8 ± 7.3	2.7 ± 0.12
DES		
B:G (1:2)	151.4 ± 11.9	1.5 ± 0.12
B:G:W (1:2:1)	167.3 ± 5.3	2.1 ± 0.07
B:G:W (1:2:5)	226.8 ± 31.3	4.6 ± 0.64
B:G:W (1:2:10)	231.4 ± 34.9	6.2 ± 0.94
B:PG (1:3)	103.5 ± 5.1	1.2 ± 0.06
B:PG:W (1:3:1)	106.6 ± 4.8	1.5 ± 0.07
B:PG:W (1:3:5)	135.9 ± 27.2	2.8 ± 0.56
B:PG:W (1:3:10)	155.5 ± 9.1	4.1 ± 0.24
B:EG (1:3)	181.8 ± 13.8	2.4 ± 0.18
B:EG:W (1:3:1)	153.4 ± 7.3	2.4 ± 0.11
B:EG:W (1:3:5)	167.1 ± 23.6	3.8 ± 0.54
B:EG:W (1:3:10)	259.6 ± 15.1	7.5 ± 0.44

Currently, there is still a knowledge gap regarding the mechanisms involved in the toxic effects of a DES or their individual components. However, it has been postulated that these systems may interact with the biological membranes, or even cause membrane damage due to oxidative stress imbalance that cannot be hold by the antioxidant defence of the organism[70,76]. Even so, further studies are necessary to provide better insight of such mechanisms.

4.2.2. *DES in vitro phytotoxicity*

So far, the toxicity of DES has been mainly assessed on microorganisms and cell models. However, after being used in an industrial process, DES can be released into soil, air, and water, being of great importance to know their effects at higher trophic levels (such as plants), since these effects cannot be extrapolated from lower levels of biological organization[77]. In this way, phytotoxicity assays have been commonly used to determine the capacity of a compound to cause temporary or long-lasting damage to plants[78] and can, therefore, help determine the environmental impact of a given system on terrestrial plants and crops. Wheat (*T. aestivum*) is a relevant crop (one of the most important economic crop plants worldwide), which has been frequently used as a reliable eco-toxicological indicator and model for phytotoxicity evaluation of environmental contaminants, pharmaceutical compounds, or nanomaterials[79].

Within this context, wheat was selected as model organism to evaluate the phytotoxicity of betaine/polyol-based two-component DES. After treating wheat seeds for 7 days with different concentrations of DES, growth factors, including germination and shoot height inhibition, were measured. The corresponding EC50, calculated from dose-response curves, are summarized on Table 3 and show that the phytotoxicity of the DES tested was considerably low. Although with some limitations, wheat seeds could still grow and develop in a DES-rich environment. Additionally, results also suggest that the early growth of seedlings was more sensitive to the toxic effects of DES than seed germination. Although there was some inhibition of germination at the highest concentration tested (data not shown), EC50 values were still higher than 20 mg/mL for all systems studied. Regarding early growth of seedlings, both B:G and B:PG inhibited in more than 50% the shoots height at the highest concentration tested, presenting an EC50 of 8.1 and 19.8 mg/mL, or 80.6 and 229.3 mM, respectively. Similarly to what was found for Caco-2 cells, B:EG displayed the lowest toxicity among the three DES studied. It is also important to highlight that shoots grew in an uneven way in seeds treated with DES, when compared to the control, even at the lowest concentrations studied. However, no signs of leaf necrosis were observed (data not shown). Previous studies have evaluated the phytotoxic effects of ChCl-based DES on garlic (*Allium sativum*) cloves[80], on microalgae (*Raphidocelis subcapitata*)[81], and on wheat (*T. aestivum*) seeds[39]. Wen *et al.* used the roots length as a growth marker and evaluated the morphology

of root tip cells, after a treatment with ChCl:glycerol (1:1), ChCl:ethylene glycol (1:1), among others, and their individual components. The authors found that garlic roots growth was affected by the treatment with the DES tested (at a concentration of approximately 1 mg/mL) or by their individual components[80]. Lapeña and co-workers explored the ecotoxicity of DES formed by mixtures of ChCl with glycerol, ethylene glycol, and urea on the microalgae *R. subcapitata* (EC50 values varied from 7.1 to 9.2 mg/mL). Following a classification in which chemicals are classified into several categories of toxicity based on their effective concentrations, Lapeña *et al.* concluded that the DES studied were "relatively harmless" (EC50 > 1 mg/mL, the least toxic category according to this classification system)[81]. In the work of Radošević et al., the authors assessed the phytotoxic effect of ChCl:glycerol (1:2), among other systems, by evaluating the inhibition of germination (EC50 > 20 mg/mL), shoot (EC50 of 3.7 mg/mL), and root growth (EC50 of 3.2 mg/mL) of wheat seeds. Additionally, although there was some inhibition of the early growth of seedlings, seeds were still able to germinate. Similarly to what was reported in the work described herein, Radošević and co-workers have also concluded that the DES tested displayed low toxicity on wheat[39].

Table 3. EC50 values obtained for betaine/polyol-based two-component DES, on wheat seeds, after an incubation period of 7 days.

	EC50 (mg/mL)		EC50 (mM)	
DES	**Germination**	**Shoot height**	**Germination**	**Shoot height**
B:G (1:2)	> 20	8.1 [a]	> 199	80.6 [a]
B:PG (1:3)	> 20	19.8 [a]	> 232	229.3 [a]
B:EG (1:3)	> 20	> 20	> 264	> 264

[a] GraphPad Prism 9 software was not able to calculate a standard deviation

Inhibition of germination and growth is often related to an increased level of reactive oxygen species (ROS) accumulated in plant cells. ROS production is usually induced by environmental stress, and can lead to the disruption of the lipid bilayer membrane inducing LPO[82]. Therefore, the level of LPO has been widely used as an indicator of ROS mediated damage of cell membranes under stress, in particular by measuring the accumulation of MDA, the cytotoxic product of peroxidation of the unsaturated fatty acids existing in phospholipids[39,83]. Figure 3i shows the accumulation of MDA in the leaves harvested from seedlings treated with two different concentrations of DES (5 and 20 mg/mL). In comparison

to untreated wheat seeds, B:G (1:2) (at 20 mg/mL) was the only system that significantly increased the MDA content ($P < 0.01$), which seem to correlate with its higher toxicity to shoot growth (EC50 of 8.1 mg/mL). The treatments at lower concentrations (5 mg/mL) with B:G (1:2) and B:EG (1:3) did not show statistically significant differences, although there was a tendency to increase MDA content in the first case and to decrease it in the second. At 5 mg/mL, B:PG (1:3) was able to significantly decrease MDA levels, as well as B:PG (1:3) and B:EG (1:3) at a concentration of 20 mg/mL. A significant decrease in MDA has already been reported in the roots of poplar (*Populus nigra* var. *italica*) after treatment with heavy metals. The authors hypothesized that this could suggest an adaptive response of the antioxidant mechanisms to the phytotoxic agent[83]. In another work, it was suggested that lower LPO levels in barley seeds (*Hordeum vulgare*) treated with different concentrations of IL could be due to a more efficient ROS scavenging. However, this increased scavenging capacity did not lead to improved seedling performance, which could mean that the growth inhibition caused by the IL tested was induced by damage at some other level, such as on DNA or on photosynthetic activity[38]. These conclusions drawn by Radojčić Redovniković *et al.* and Cvjetko Bubalo *et al.* might offer a possible clarification for the results obtained in the work reported herein.

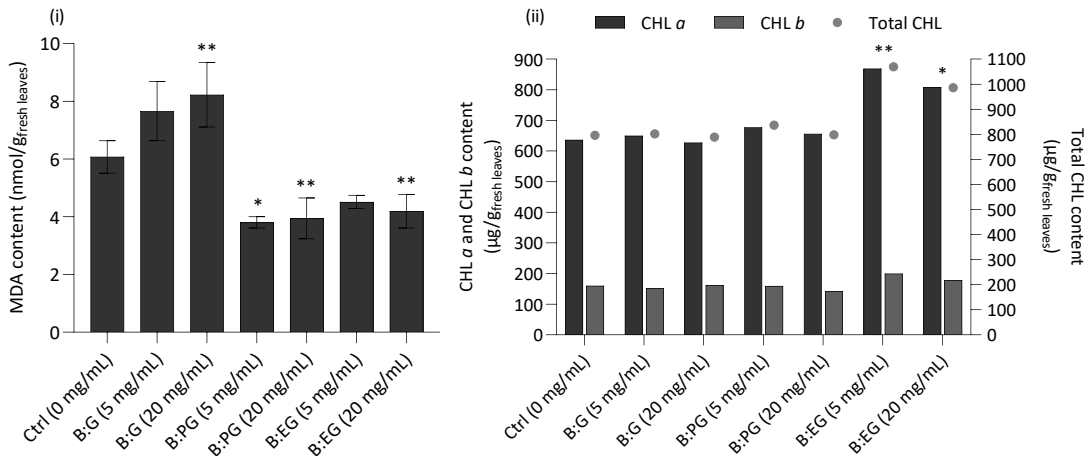

Figure 3. Effect of betaine/polyol-based DES on (i) MDA and (ii) photosynthetic pigments content. Statistically significant differences between the effect of DES and the control are represented by asterisks (*). *$P < 0.05$, **$P < 0.01$.

CHL levels have also been considered by several authors as an abiotic stress biomarker, as well as a good indicator of plant health[38,84]. Accordingly, the content of photosynthetic pigments, including CHL *a*, CHL *b*, and total CHL was measured in leaves. Results summarized on Figure 3ii show that although B:G (1:2) induced LPO on wheat seedlings (Figure 3i), it did not affect significantly the CHL content, as well as B:PG (1:3), for both concentrations tested. However, the CHL content in shoots treated with B:EG (1:3) showed a significant increase for both concentrations, which could be related to the fact that B:EG (1:3) displayed the lowest toxicity among the DES tested (EC50 for both germination and shoot height > 20 mg/mL). Significant decreases in CHL content of plants exposed to increasing concentrations of ChCl/organic acid-based DES[39,81] or different IL[38,84] have been reported by other authors. However, it is important to highlight that, not only plants fine tune their response to different stress factors, but also the severity of stress symptoms are highly dependent on their capacity to tolerate a specific stress inducer[85]. Therefore, an increase in CHL levels may also result from abiotic stress. This has been reported in different plants, particularly in response to drought[86], heavy metals[87], and ammonium stress[88].

ROS scavenging pathways from different cellular compartments are coordinated by a complex antioxidative defence system, constituted by non-enzymatic and enzymatic components. In particular, the enzymatic components of this defence mechanism comprise several antioxidant enzymes, including SOD, GPX, CAT, APX, among others, that maintain cell homeostasis and provide a concerted response to oxidative stress[89]. Therefore, the up- or downregulation of the antioxidant enzymes activity is considered to be an indicator of the oxidative stress level in the plant[90]. Within this context, SOD, GPX, CAT, and APX were selected as biomarkers to determine the oxidative stress caused by DES on the enzymatic antioxidant defence system of wheat. Figure 4 shows that antioxidant enzymes responded differently depending on the DES and in some cases on the concentration tested, indicating that the toxicities of these compounds were affected by their specific characteristics. Overall, all DES showed a trend to either maintain or inhibit the enzymatic activity at the different concentrations tested, except for B:PG at 20 mg/mL, which significantly increased APX activity.

SOD constitutes a frontline in the defence against ROS, and its activity has been reported to increase in plants exposed to different environmental stresses, such as drought, salt, heavy

metals[91], IL[38], and even DES[39] in a number of different crops. However, some authors have also mentioned SOD inhibition with increasing concentration and time of exposure to a phytotoxic agent. An example is the work of Chen *et al.*, in which SOD was inhibited with increasing concentrations of imidazolium-based IL[92]. In the work described herein, SOD activity seems to be concentration-dependent with results suggesting a possible SOD upregulation for higher concentrations of B:PG (1:3) (Figure 4i).

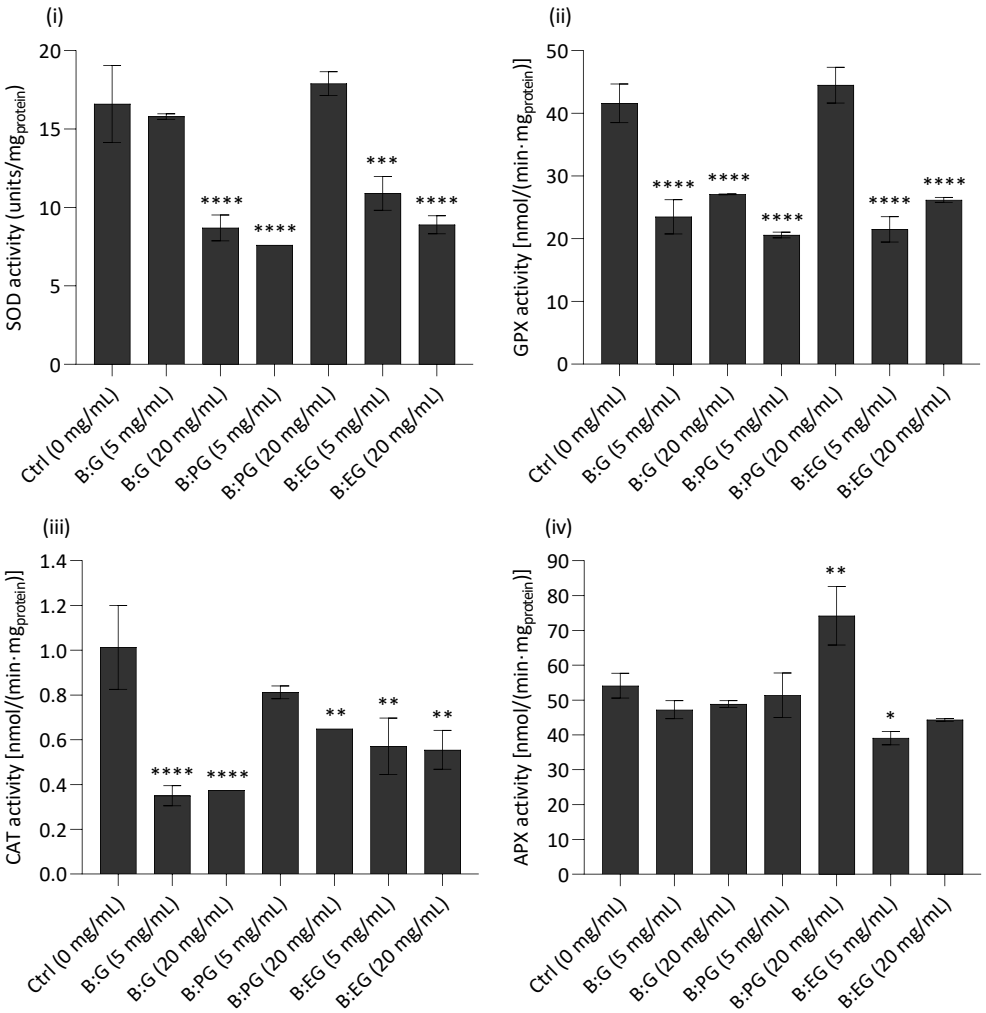

Figure 4. Effect of betaine/polyol-based DES on (i) SOD, (ii) GPX, (iii) CAT, and (iv) APX activities. Statistically significant differences between the effect of DES and the control are represented by asterisks (*).*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

Figures 4ii and iii show the inhibition of both GPX and CAT activities by at least one of the concentrations of the different DES tested. There was a slight tendency for CAT activity to

be further inhibited with increasing concentrations (Figure 4iii), while for GPX higher concentrations tended to lessen the inhibition or to increase the activity (Figure 4ii). Similar profiles have also been reported by Chen *et al.*[92]. APX activity remained mainly unchanged, except for B:EG (1:3) at 5 mg/mL, which slightly inhibited the enzymatic activity, while B:PG (1:3) increased APX activity at 20 mg/mL (Figure 4iv). Radošević *et al.* have described a reverse regulation between CAT and APX activities on wheat treated with a ChCl:oxalic acid DES, with APX activity increasing with increasing DES concentration, while CAT activity decreased until, eventually, the enzymatic activity was inhibited[39].

Overall, the above-mentioned results suggest that specific and distinct antioxidant and redox homeostasis responses could be triggered by DES with different characteristics, which were probably modulated by their HBD, which might have promoted the formation of different free radicals, such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), or hydroxyl radical (OH·). Additionally, these results suggest an ineffective response of wheat defence mechanism, in particular towards B:G (1:2), since this DES inhibited all antioxidant enzymes activity, except for APX, while increasing the MDA content in shoots. This is probably the reason why B:G (1:2) was the DES that inhibited the most the development of seedlings.

4.3. Protein derivative extractions

Aiming at recovering protein derivative-rich extracts from canned sardine residues, as a first approach, the impact of different process parameters on the extraction performance of DES was studied, namely operating temperature (25, 45, and 80 °C) and extraction time (6 and 18 h), for a fixed solid-liquid ratio (1:80). As the ultimate green solvent[93], and having proved to be effective in extracting proteins from macro- and micro-algae[48,94], water was also tested under the same operating conditions. Total protein yields obtained for each extract are represented on Figure 5.

It is well known that higher temperatures have the potential to favour extraction. By increasing temperature, the solubility of the solute increases as well, while the viscosity of the solvent decreases, thus enhancing the diffusion rate. This is a particularly important parameter to control when working with viscous solvents such as DES[95]. However, it is important to note that some molecules are extremely sensitive to thermal shifts and may

degrade with increasing temperature. Proteins are an example of such molecules, considering that temperature may affect their structure stability, and consequently their function, or even intensify their hydrolysis[96]. Nevertheless, smaller peptides (generally ranging from 2 to 20 amino acid residues), protein hydrolysates, or even partially denatured proteins obtained by heat treatment, are thought to be more interesting in terms of bioactive properties than parent proteins[97–99]. Accordingly, in order to promote the extraction of bioactive protein-derived ingredients, in the work reported herein, operating temperatures up to 80 °C were studied.

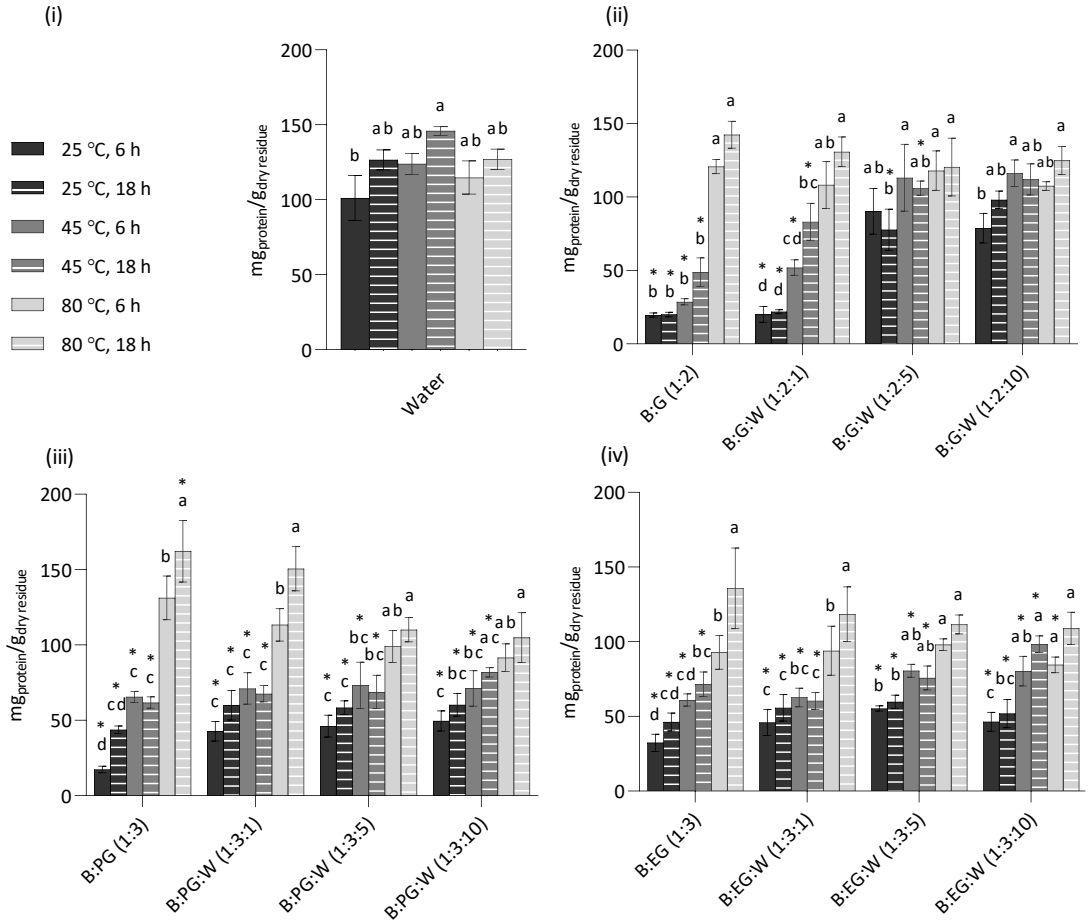

Figure 5. Total protein yields determined by the modified Lowry method[48] in the extracts obtained from sardine residues with (i) water, (ii) B:G, (iii) B:PG, and (iv) B:EG DES at different conditions of temperature and extraction time for a fixed solid-liquid ratio. For each of the applied solvents (water, two-, or three-component DES), the statistically significant differences between the extraction conditions studied are represented by different lowercase letters. The significant differences between the extracts obtained with DES and the extracts obtained with water are represented by an asterisk (*). $P < 0.05$ was accepted as statistically significant in all cases.

Results displayed on Figure 5 show that protein yields greatly varied depending on the conditions of temperature applied when DES were used as solvents (Figures 5ii-iv), tending to increase with increasing temperature. However, the yields obtained by water extraction (Figure 5 i) were similar for all temperatures tested. These results suggest that extractions performed with DES at lower temperatures were hampered by their high viscosities, which limited mass transfer and diffusivity[55]. Nevertheless, as discussed in section 4.1., the viscosity of betaine/polyol-based DES could be reduced by up to 40.9-fold with increasing temperature (up to 80 °C), thus allowing a possible increase in the extraction yields at higher temperatures. The fact that less viscous two- or three-component DES performed better at lower temperatures than highly viscous DES, such as B:G (1:2), also supports this conclusion. As protein yields increased with increasing temperature (at lower DES viscosities) and, tendentially, with extended extraction times, the extraction mechanism was presumably controlled by the diffusion of the DES into the solid matrix.

At 80 °C, the DES with no added water and the DES with 1 molar ratio of water were able to perform in a similar way to water alone, with highlight to B:PG (1:3) that resulted in a significantly higher protein yield (at 80 °C and 18 h). However, an increased water content in DES (up to 5 or 10 molar ratio) led to a substantial decrease in the protein yields, particularly for B:PG- and B:EG-based DES. Several hypotheses can help justifying these results. It was previously demonstrated that the addition of water to the DES could strongly decrease their viscosity while increasing their polarity (section 4.1.). However, a DES with an excessive water content may lead to a decrease in the hydrogen bond interconnection network, and consequently result in a decrease in the extraction yield[58], as could be observed for B:PG- and B:EG-based DES. A different interpretation could be related to the increase of the hydrophobicity of proteins with increasing temperature. Some authors have reported that the solubility of proteins can be affected during heating, since this property is closely related to their structure. In fact, an originally water-soluble protein can become more hydrophobic as temperature increases and its unfolding starts exposing nonpolar amino acids[100,101]. This interpretation is supported by the fact that yields slightly decreased in extractions with water (E_{NR} of 48.02 kcal/mol) at 80 °C, but significantly increased when the least polar DES (B:PG (1:3), E_{NR} of 51.15 kcal/mol) was used as solvent. A third possibility is that the increase of the DES water content might have caused an intensification in

hydrolysis of proteins to smaller peptides or free amino acids and, although it is known that most dipeptides can be detected by the Lowry method, free amino acids do not produce a coloured product with the Lowry reagent, and cannot, therefore, be detected by this method[102,103].

Although extractions performed for 18 h tended to present higher protein yields, the time of extraction was not a significant parameter in most cases. Even so, in order to evaluate the impact of solid-liquid ratios on protein yields, extractions were performed for 18 h at 80 °C using the most promising DES and three additional solid-liquid ratios, namely 1:8, 1:16, and 1:40. Results presented on Figure 6 show that protein yields slightly increased with a decreasing solid-liquid ratio, as it would be expected, reaching a plateau for most solvents between 1:40 and 1:80, with the exception of B:PG (1:3) and B:PG:W (1:3:1), which preformed significantly better at 1:80. Nevertheless, since solvents tended to perform better at a solid-liquid ratio of 1:80, some of the extracts obtained at this ratio were selected to be evaluated for their bioactivity.

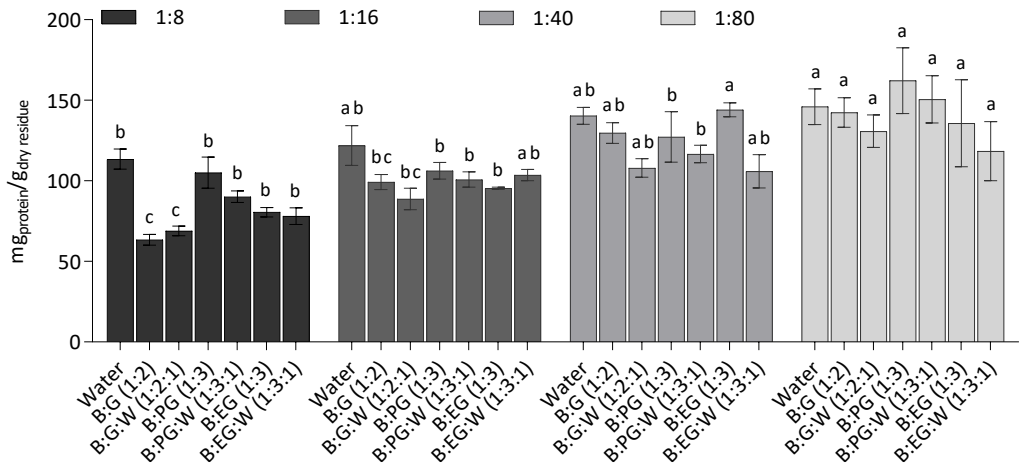

Figure 6. Total protein yields determined by the modified Lowry method[48] in the extracts obtained from sardine residues with water and DES when applying different solid-liquid ratios at 80 °C and 18 h. For each of the applied solvents (water, two-, or three-component DES), the statistically significant differences between the solid-liquid ratios studied are represented by different lowercase letters. $P < 0.05$ was accepted as statistically significant in all cases.

The amino acid profile of some of the extracts obtained at 80 °C for 18 h is shown on Table 4. Results suggest that the total amino acid profile of the extract obtained with water presented some similarities to those already reported in the literature for sardine muscle

hydrolysates. However, there was a pronounced decrease in the relative mass percentage of amino acid residues such as Ile, Leu, Met, Phe, and Tyr, while the relative percentage of Thr increased, when compared to what was previously reported in the literature[104–106].

Table 4. Total amino acid composition (relative mass %) of some of the canned sardine extracts obtained at 80 °C for 18 h and a solid/liquid ratio of 1:80, after acid hydrolysis.

Amino acid	Hydropathy index[107]	Water	B:G (1:2)	B:G:W (1:2:1)	B:G:W (1:2:10)	B:PG (1:3)	B:PG:W (1:3:1)	B:PG:W (1:3:10)	B:EG (1:3)	B:EG:W (1:3:1)	B:EG:W (1:3:10)
Ala	1.8	8.7	10.4	13.1	12.5	13.4	12.4	11.5	7.9	7.4	9.9
Arg	-4.5	6.8	2.5	2.6	5.0	6.5	9.0	8.1	5.7	10.6	11.8
Asp	-3.5	10.3	10.4	11.3	8.4	10.7	10.5	10.3	11.0	10.5	9.7
Glu	-3.5	15.0	16.9	16.2	13.1	16.7	15.1	14.9	16.2	15.8	13.6
Gly	-0.4	4.3	6.0	2.0	1.1	1.8	1.7	1.3	3.3	3.6	4.3
His	-3.2	3.8	3.6	2.2	2.3	2.3	2.6	3.0	3.2	2.8	2.5
Ile	4.5	2.7	0.7	1.5	2.8	2.2	2.5	2.3	5.7	0.9	2.9
Leu	3.8	5.8	8.2	6.5	5.0	6.8	6.7	6.1	5.9	6.5	5.0
Lys	-3.9	9.0	0.0	0.0	5.0	0.0	0.0	3.2	0.0	4.4	7.1
Met	1.9	0.3	0.0	1.9	1.0	1.1	1.5	0.7	1.0	2.2	0.8
Phe	2.8	2.7	4.0	1.5	2.3	2.3	2.6	2.8	3.7	3.1	2.2
Ser	-0.8	4.5	5.2	4.6	4.8	5.3	5.5	5.1	4.7	4.7	4.7
Tau		4.8	3.0	5.8	5.3	3.3	3.7	4.5	6.1	3.4	4.9
Thr	-0.7	14.9	19.8	21.9	24.9	22.4	21.0	21.4	18.8	16.9	14.7
Tyr	-1.3	1.8	1.5	1.0	2.3	0.2	0.2	0.3	0.5	0.6	0.3
Val	4.2	4.7	7.8	8.0	4.2	4.9	5.1	4.4	6.4	6.7	5.5

Coefficient of variation ≤ 17.7%

It is known that the processing methods applied to fish may cause different effects on chemical, physical, and nutritional compositions of the raw material[108]. Specifically, it is important to highlight that the residue used in this research work resulted from one of the first steps of the canning process, *i.e.*, the removal of sardine heads and viscera after immersion of the fresh fish in a concentrated salt solution. Such process might not only have had an impact on protein structure[109], but also on the loss of some of the proteins, peptides, or amino acids contained in the fresh fish[109,110], thus influencing the amino acid yield of each extract. Additionally, as a residue and as a matrix highly concentrated in lipids, particularly long chain polyunsaturated fatty acids, these sardine residues are extremely prone to oxidation (as discussed on Chapter 2, Part I, section 4.2.), which might have led to amino acid destruction[108], consequently changing the amino acid relative mass percentages, when compared to a fresh fish hydrolysate. In fact, amino acid residues such as Met, Phe, or Tyr have been recognized to be more susceptible to oxidation[111]. Furthermore, the selectivity of

water as extraction solvent towards specific amino acid residues might also be related to the composition differences that were mentioned.

Overall, samples resulting from DES extractions were rich in Ala, Arg, Asp, Glu, and Thr, even though the amino acid relative mass percentages varied between extracts. Samples have also revealed to be an interesting source of Tau (3.0 to 5.8%), a free sulfonic acid derived from cysteine, that although not incorporated into proteins, is considered to be conditionally essential for humans, being important not only for the physiological functions it performs, but also for its bioactive potential[7,112].

When compared to the water extract, the extracts obtained with DES presented some considerable differences in amino acid composition. The only exceptions were His, Phe, Ser, and Tyr, whose relative mass percentages have remained constant regardless of the solvent used.

As previously mentioned, the function of a given protein or peptide is closely related to their structure, which can be determined by a wide range of different factors, one of which is the amino acid hydrophobicity. Accordingly, several amino acid hydrophobicity scales have been developed over the years, aiming at giving some insight into this question. The most commonly used hydropathy indexes were proposed by Kyte and Doolittle, and represent the hydrophobic or hydrophilic properties of each amino acid side chain: the larger the index, the more hydrophobic the amino acid is (Table 4)[107,113]. When comparing the DES extracts with the sample obtained with water, it is interesting to note that in relative terms, and according to this hydrophobicity scale, there was a trend for hydrophilic amino acids to decrease, while the relative mass percentage of more hydrophobic amino acids increased, particularly for DES with lower water contents. Some examples are the decrease of Arg in the extracts obtained with B:G (1:2) and B:G:W (1:2:1), or the decrease of Lys in all DES extracts, which was more pronounced for the systems with a lower water content. Regarding hydrophobic amino acids, the increase of Ala in B:G (1:2), B:G:W (1:2:1), B:G:W (1:2:10), B:PG (1:3), B:PG:W (1:3:1), and B:PG:W (1:3:10) extracts, the increase of Ile in the extract obtained with B:EG (1:3), the increase of Leu in B:G (1:2) extract, or the increase of Val in B:G (1:2), B:G:W (1:2:1), B:EG (1:3), and B:EG:W (1:3:1) extracts are also worth highlighting. This decrease of hydrophilic amino acids or increase of hydrophobic amino acids yield in extracts obtained with some of the DES used, particularly those with a lower

water content, was probably related to the lower polarity displayed by these solvents, when compared to water (as discussed in section 4.1.).

4.4. *In vitro* bioactivity evaluation

4.4.1. *Antioxidant activity*

Antioxidant protein-derived ingredients have become attractive due to their multiple sources, such as seafood by-products, and significant antioxidant activity[114]. In this work, the scavenging potential of some selected protein-rich extracts towards peroxyl radicals was evaluated by ORAC assay (Figure 7). The ORAC assay has been widely accepted as an effective tool to measure the antioxidant capacity of different nutraceutical, pharmaceutical, and food products[52,115]. It has been particularly useful in determining the effectiveness of chain-breaking antioxidants, which are the major antioxidants present in foods[115].

Results displayed on Figure 7 show that all extracts tested presented some peroxyl radical scavenging potential, except for the extracts obtained with B:G (1:2) at 25 °C. This was probably due to the high viscosity of B:G (1:2) at room temperature and consequent mass transfer and diffusivity limitations during protein derivatives extraction. Furthermore, apart from water extracts, a statistically significant positive correlation could be found between the total protein yield of each sample and its antioxidant capacity ($0.7956 \leq R^2 \leq 0.9117$, $P \leq 0.0001$), reflecting the scavenging capacity of the protein derivatives extracted with DES against peroxyl radicals. Findings reported by other authors regarding the antioxidant potential of fish proteins corroborate this conclusion, since, as previously mentioned, fish-derived proteins and peptides have found different applications that rely on their antioxidant potential[10]. Therefore, extracts with a higher protein yield, namely those obtained with DES with no added water or with 1 molar ratio of water at the highest operating temperature tested, tended to show an increased peroxyl radical scavenging potential. It is also important to highlight that DES were used as blank during the assay, so the antioxidant activities reflect solely the capacity of each extract.

The mechanism by which marine protein-derived ingredients display antioxidant activity is not yet entirely understood. However, as reviewed by Harnedy and FitzGerald, protein hydrolysates and peptides can act as radical scavengers and as transition metal chelators,

being able to exert antioxidant activities against enzymatic (lipoxygenase mediated) and non-enzymatic peroxidation of lipids and fats, and to induce specific genes encoding for endogenous non-enzymatic antioxidant components and enzymatic systems[7].

The correlation between structure and functional activity of bioactive protein-derived molecules has also not yet been fully established. However, there are some structural features that appear to influence the biological action of these compounds[7]. The overall antioxidant activity of a protein is believed to be enhanced by different factors, including, for example, changes in its physical structure. Although all amino acid residues found in protein or peptide structures have the ability to potentially behave as antioxidants, their antioxidant capacity is often limited by the tertiary structure of the (poly)peptide. Therefore, one approach that has been applied by different authors is a partial denaturation by heat treatment, which potentially increases the accessibility to the amino acid residues with antioxidant capacity[98]. This corroborates the higher antioxidant activities displayed by the extracts obtained with DES at higher temperatures (Figure 7).

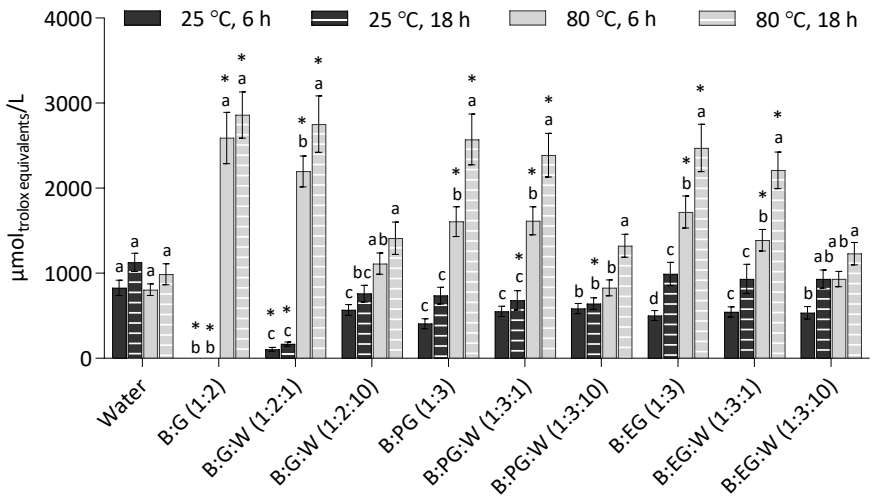

Figure 7. Antioxidant capacity of some of the protein derivative-rich extracts (solid-liquid ratio of 1:80) determined by ORAC assay. For each of the applied solvents (water, two-, or three-component DES), the statistically significant differences between the extraction conditions studied are represented by different lowercase letters. The significant differences between the extracts obtained with DES and the extracts obtained with water are represented by an asterisk (*). $P < 0.05$ was accepted as statistically significant in all cases.

When comparing the antioxidant potential of two-component DES extracts with the results obtained for three-component DES with 1 molar ratio of water, no statistically significant differences could be found. However, a further increase in water content (up to 10 molar

ratio of water) had a negative effect on the antioxidant activity of extracts, with samples performing similar to water extracts. Although peptides are known to increase free radical scavenging activity, metal chelation, or aldehyde adduction activity, in general, free amino acids are not very effective antioxidants[98]. Accordingly, a more pronounced hydrolysis induced by increasing amounts of water could have resulted in a decrease in the antioxidant activity of the tested extracts due to the presence of a greater amount of free amino acids.
The presence of hydrophobic amino acids has also been consistently considered as key in peptide ability to scavenge radicals[113,116,117]. Some of these amino acids are believed to enhance radical scavenging activity by donating protons to electron deficient radicals[7]. As previously highlighted, when compared to water extracts or higher water content DES extracts, DES with lower water contents resulted in extracts richer in hydrophobic amino acids, including Ala, Ile, Leu, and Val, which might present a possible explanation for their enhanced peroxyl radical scavenging potential. Additionally, it is also possible that the protein derivatives extracted hold a higher stability in two-component DES than in water or in three-component DES with higher water contents, as previously demonstrated by other authors[56,118,119].

4.4.2. *Antimicrobial activity*

Marine-derived proteins and in particular peptides are known for their antimicrobial and preservative capacity, being able to target a broad spectrum of microorganisms[4]. Within this context, and based on the results obtained for antioxidant activity, DES and some of the samples resulting from both DES and water extractions at 80 °C, 18 h, and a solid-liquid ratio of 1:80 were selected to be evaluated for their antimicrobial potential against *S. aureus* and *E. coli* bacteria, which are relevant to determine the potential of preservatives for water-based pharmaceutical, nutraceutical, or cosmetic/personal care products[120,121].
MIC* and MIC values are shown on Figure 8. Among the protein derivative-rich extracts tested, the water extracts were the only samples that did not show capacity to inhibit visible growth of both *S. aureus* and *E. coli* (MIC > 500 µL/mL), nor to visually or differentially affect bacterial growth (MIC* > 500 µL/mL). MIC* values for protein derivative-rich extracts obtained with DES ranged from 2 to 500 µL/mL, and MIC from 250 to 500 µL/mL, being the extracts obtained with DES with lower water contents (no added water or 1 molar ratio of

water) the most promising. As other authors have reported for different DES based on betaine[71,122–124], betaine/polyol-based DES have also shown potential to inhibit bacterial growth, with MIC* values ranging from 62.5 to 250 µL/mL, and MIC from 125 to 500 µL/mL. It is important to highlight that for most DES and respective extracts the minimum concentration required to affect the fitness of the microorganisms (MIC*) was lower than the concentration required to inhibit their visible growth (MIC). This suggests the presence or accumulation of compounds in the tested samples that had the ability of interfering with the growth of the target bacteria at lower concentrations, even without reaching full visible growth inhibition.

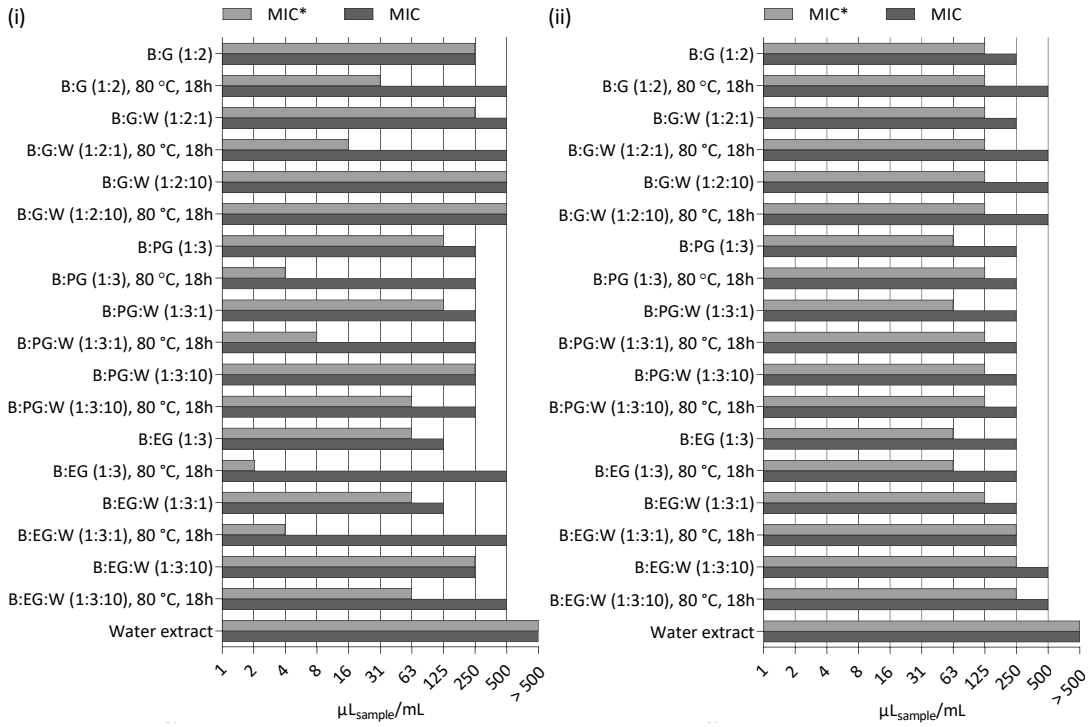

Figure 8. MIC* and MIC values of some of the DES and protein derivative-rich extracts obtained at 80 °C, 18h, and solid-liquid ratio of 1:80 in the bacterial targets: (i) *S. aureus* ATCC 6538 and (ii) *E. coli* ATCC 8739.

Generally, for both DES and protein derivative-rich extract treatment, *E. coli* showed to be more resistant than *S. aureus*. The mechanism by which DES affect bacteria is still unknown, although it is believed to be related to their interaction or permeation into the microorganisms membranes[80]. These interactions could be dependent on their permeability, pH, osmolality, potential for membrane bound divalent cations chelation, or

cell wall disruption through hydrogen bonding or electrostatic interaction with polysaccharide chains[80,125]. In addition, since Gram-negative bacteria possess an extra outer lipopolysaccharide membrane on the cell wall, therefore being less permeable, it has been hypothesized that this may be the reason why DES tend to inhibit *E.coli* to a smaller extent than *S. aureus*[71]. Nevertheless, further studies would be important for a deeper understanding of the mechanism of action and interaction of DES with the bacterial membranes.

It is important to note that extracts showed a higher potential to affect *S. aureus* growth than DES alone, presenting lower MIC* values, being the extract obtained with B:G:W (1:2:10) the only exception. It is known that the bioactivity of an extract can be strongly influenced by synergistic, additive, and antagonistic effects of its different constituents[126,127]. For protein derivative-rich extracts obtained with DES, a synergic and/or additive antimicrobial effect of the extracts' components with the solvents might be presumed. This behaviour suggests that it would be of interest not to separate the extracts from the solvents, in order to obtain a combined bioactivity. In addition, DES have been reported to improve the stability of proteins[56,118,119], which may be important to maintain the extracts' bioactivity. In this work, the DES used were prepared using known osmolytes (both betaine[71] and polyols[128]), which further supports this hypothesis. Therefore, to use the DES in the final product formulation would allow not only an improved bioactivity and stability of the extract, but also a reduction of the number of unit operations of the overall process and, consequently, a reduction of the total energy required, thus contributing to the sustainability of the proposed method.

Another aspect to highlight is that, generally, for *S. aureus*, the antimicrobial potential of an extract was directly proportional to its antioxidant capacity, which decreased with increasing water content. In fact, antioxidant peptides are often multifunctional and may exhibit other bioactivities, such as antimicrobial effects, being therefore attractive ingredients to be applied in different industrial applications[114].

Although several marine peptides have shown antimicrobial potential in a number of studies, their pharmacokinetics is still poorly understood. Nevertheless, antimicrobial peptides are thought to act either by attaching to membrane lipids of microorganisms and forming trans-membrane pores, or by interacting with intracellular targets, including DNA,

RNA, or proteins, eventually leading to cell death[99,129]. It is interesting to note that the most promising antimicrobial extracts in both bacteria were obtained with less polar DES. As previously mentioned, it has been reported that the functional and bioactive properties of peptides correlate directly with their structure. Features such as the presence of a hydrophobic core or a net positive charge have been considered to be crucial on facilitating the interaction of antimicrobial peptides with cell walls and membranes of microorganisms[99,113,117]. Within this context, it can be hypothesized that in the case of water extractions, or even in the extractions with three-component DES with a higher water content, there was a lack of selectivity for the recovery of antimicrobial ingredients, which according to the reported literature, usually present a more hydrophobic character.

5. Conclusions

Overall, this study demonstrated the potential of betaine/polyol-based DES not only as extraction solvents for the recovery of multifunctional protein derivative-rich extracts with antioxidant and antimicrobial potential, but also to be used as vehicles of the extracts in the final product.

Results have shown that the physicochemical properties of the DES prepared, such as density and viscosity, could be tuned by the HBD used to form the system, as well as by temperature and water content. The HBD and water content have also shown to affect the DES polarity, thus enabling a wide range of polarities, from values similar to water to values close to methanol, by simply changing the HBD or by increasing the water content. Additionally, DES exhibited low toxicity towards a human intestinal epithelial cell line (Caco-2 cells), as well as towards wheat (*T. aestivum*) seeds, which could be further decreased by increasing DES water content (when evaluated on Caco-2 cells).

Although in most cases DES extractions were not able to perform significantly better than water in terms of total protein yield (except for B:PG (1:3) at 80 °C for 18 h and a solid-liquid ratio of 1:80), it is possible that the lower polarity of the DES used, namely B:G (1:2), B:PG (1:3), B:EG (1:3), or their mixtures with water (up to 1 molar ratio), has enabled the extraction of protein derivatives with a more hydrophobic character, which are known to have an enhanced bioactive potential when compared to more hydrophilic protein-derived molecules. Additionally, since the results suggested a synergistic and/or additive

antimicrobial effect between the solutes and the DES, the extract as a whole could be envisioned as the final product/formulation, to be potentially used as functional ingredient or natural preservative in different products, without further separation steps. Furthermore, the fact that these systems have demonstrated a low toxicity profile towards the tested living organisms, makes them safer and less hazardous alternatives for applications in the pharmaceutical, nutraceutical or cosmetic industries.

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CHAPTER 3

Bioactive ingredients from brown crab shell residues

CHAPTER 3

Part I – Deep eutectic system extraction of astaxanthin

Contents

Adapted from:

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The DES phytotoxicity results were obtained during a visit to the Laboratory for Cell Culture Technology and Biotransformations, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia, within the scope of COST Action GREENERING (CA18224), under the supervision of Prof. I. R. Redovniković.

The author was involved in the conceptualization and design of all experiments and performed all experimental work except for the characterization of DES by NMR (acquired at ITQB NOVA by N. Fernández and P. Lamosa), the *in vitro* evaluation of cytotoxicity and antiproliferative effect of DES and extracts (performed at iBET by C. V. Pereira), and the antimicrobial activity of DES and extracts (performed at iBET by I. C. Leonardo under the supervision of F. B. Gaspar). Data processing and interpretation, and results discussion were also performed by the author, as well as the preparation of the original manuscript.

1. Abstract

The recognized antioxidant properties of astaxanthin (AXT), which have been reported to surpass those of β-carotene and α-tocopherol, has prompted an interest in further studying its potential benefits to human health and nutrition. In this work, the potential of deep eutectic systems (DES) to efficiently obtain AXT-rich extracts from crab shell residues was evaluated. Therefore, different terpene/fatty acid-based DES were prepared and their physicochemical and toxicological properties determined. Aiming at maximizing the AXT recovery, the effect of operating temperature (30, 45, and 60 °C) and extraction time (2, 6, and 24 h) on the extraction performance was evaluated. As a proof of concept, the potential of DES for AXT extraction from shrimp shells, mussels, and *Haematococcus pluvialis* was also highlighted. The biological potential of AXT-rich extracts, AXT standard, DES, their individual components, and equivalent physical mixtures was evaluated, namely cytotoxicity, antiproliferative effect on human colorectal cancer cells, and antimicrobial activity towards *Staphylococcus aureus* and *Escherichia coli*. Results have shown that terpene/fatty acid-based DES presented some toxicity in both human cells and wheat, with half maximal effective concentrations (EC50) up to 0.89 and 0.038 mg/mL, respectively. Concerning extractions, menthol:myristic acid (8:1) (60 °C, 2h) was able to match the AXT extraction yield obtained by a 6 h Soxhlet extraction with acetone. Additionally, when using the same DES to recover AXT from the other biomasses under study, there was a 3- to 657-fold increase in yields, when compared to the Soxhlet extraction. Furthermore, terpene/fatty acid-based DES have shown potential to inhibit colorectal cancer cell proliferation, while both DES and AXT-rich extracts obtained with DES were able to inhibit the growth of both *S. aureus* and *E. coli*.

2. Introduction

Every year, 6 to 8 million tons of crustacean shell waste are produced globally[1]. These shell residues are mainly constituted by calcium carbonate (20 – 50%), proteins (20 – 40%), and chitin (15 – 40%), along with some minor compounds that include lipids and pigments[2]. In recent years the need to find new solutions for an efficient valorisation of such residues, within the concept of an ocean-based biorefinery, has arisen[3].

Deep eutectic system extraction of astaxanthin

Astaxanthin (AXT), a lipid-soluble carotenoid, is one of the high value molecules that can be found on crustacean shells[4]. Although AXT is mainly known for its outstanding antioxidant capacity[5], the potential benefits of this carotenoid to human health and nutrition are extensive and include anticancer, anti-lipid peroxidant, anti-inflammatory, anti-diabetic, immuno-modulator, skin photo-protection, among many others[4,5]. Additionally, AXT derived from marine organisms is known to be a valuable ingredient with cosmeceutical effects, in particular due to its antimicrobial and preservative potential[6,7].

Currently, AXT finds applications in different market segments. The natural pigment derived from *Haematococcus pluvialis* microalgae has been mainly used on human-targeted applications, including dietary supplements, nutraceuticals, personal care/cosmetics, pharmaceuticals, and food and beverages. In 2019, the global market of both synthetic and natural AXT was valued at close to US$ 600 million. The value projections point to a rapid market growth that should reach a value of over US$ 2 billion by 2030 [8]. Within this context, despite being a minor component of crustacean shells, there is potential for recovering this pigment, as an integral part of the valorisation of such residues. Additionally, there is also an interest in finding new AXT sources in order to guarantee its supply according to the market demand.

Natural AXT recovery has been performed using a wide range of volatile organic and neoteric solvents[9–11]. Saini and Keum concluded that Soxhlet extraction is the conventional method that allows the most efficient recovery of carotenoids. However, this is a time-consuming methodology that requires the use of high amounts of volatile organic solvents at temperatures that, most of the times, are not suitable for the recovery of thermosensitive molecules and can cause *cis-trans* isomerization of carotenoids. Additionally, some of the solvents that are commonly used, are highly toxic and are not allowed for food, nutraceuticals, or cosmetics processing. Unconventional methods that have been applied for carotenoid recovery include, supercritical fluid or pressurized liquid, pulsed electric fields, ultrasound-, microwave-, and enzyme-assisted extraction. According to the same authors, the most suitable environmentally friendly method for carotenoid recovery is supercritical carbon dioxide extraction using ethanol as co-solvent, providing high purity extracts and avoiding carotenoid thermal degradation. This extraction technology offers several advantages over conventional methods, including high selectivity and the possible

application of the resulting extracts in food and cosmetic products. However, the yield of more polar carotenoids, such as xanthophylls, is usually low, and implementation and energy costs are high[9]. Therefore, there is an interest in developing new, safe and cost-effective technologies and methodologies for the recovery of these high-value pigments.

Deep eutectic systems (DES) have already shown potential to be used in a number of applications, including in the extraction of carotenoids from natural sources or waste biomass[12–15], as inexpensive solvents with easily tuneable physicochemical properties[16].

The aim of part I of this chapter was, therefore, to explore the potential of new terpene/fatty acid-based DES for the efficient recovery of AXT-rich extracts from marine-derived residues, the bioactive potential of the extracts, as well as the potential of using DES as bioactivity enhancers, targeting as end-users the nutraceutical, pharmaceutical or cosmetic/personal care industries. Accordingly, two toxicity assays and two bioactivity assays were selected to evaluate AXT-rich extracts, AXT standard, DES, their individual components, and equivalent physical mixtures: cytotoxicity on an intestinal epithelial cell model (Caco-2 cells) and antiproliferative effect on a colorectal cancer cell line (HT-29), which are relevant to substantiate health claims on functional ingredients; phytotoxicity (only for DES) on wheat (*Triticum aestivum*) seeds; and antimicrobial potential towards a Gram-positive (*Staphylococcus aureus* ATCC 6538) and a Gram-negative bacterium (*Escherichia coli* ATCC 8739), which are important when evaluating preservatives applied in water-based pharmaceutical, nutraceutical or cosmetic/personal care products.

3. Materials and methods

3.1. Biomasses

Brown crab (*Cancer pagurus*) and shrimp (*Penaeus vannamei*) shells were kindly provided by Tejo Ribeirinho, Portugal, in November 2017; mussels (*Mytilus galloprovincialis*) by Testa & Cunhas, Portugal, in March 2019; and *H. pluvialis* by Buggypower, Spain, in April 2019. Biomasses were stored at -20 °C upon arrival, except for *H. pluvialis*, which was provided as dry biomass. The remaining feedstocks were freeze-dried and milled as previously described (Chapter 2, Part I, section 3.1.). The particle size of the ground material was determined using an AS 200 basic vertical vibratory sieve shaker (Retsch, Haan, Germany), with a

measuring range between 250 mm and 710 µm. After processing, residues were protected from light and stored at room temperature, in a low moisture environment.

3.2. DES preparation

Chemicals used for DES preparation were: (*S*)-(−)-perillyl alcohol (PA, ref. 218391), (±)-camphor (CA, ref. 148075), and eucalyptol (EU, ref. C80601) from Sigma-Aldrich (St. Quentin Fallavier, France); DL-Menthol (ME, ref. W266507) from Sigma-Aldrich (St. Louis, MO, USA); and myristic acid (MA, ref. 156962500) from ACROS Organics (Geel, Belgium). Systems were prepared by heating the mixture of two components to 55 °C under constant stirring, until a clear liquid was formed.

3.3. DES physicochemical characterization

3.3.1. *Differential scanning calorimetry (DSC) analysis*

DSC measurements were carried out on a DSC TA instruments Q200 (module MDSC, TA instruments, New Castle, DE, USA) under anhydrous high purity nitrogen, at a flow rate of 50 mL/min. Approximately 6 mg of DES were placed in aluminium pans and sealed. Samples were equilibrated at 20 °C, cooled to -90 °C, at a cooling rate of 10 °C/min; followed by an isothermal period of 2 min, and heating to 80 °C, at a heating rate of 10 °C/min.

3.3.2. *Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectroscopy*

FTIR-ATR analyses were carried out on a Thermo Scientific FTIR spectrometer (Class 1 Laser Product Nicolet 6100, San Jose, CA, USA). The equipment included ATR accessories with a diamond crystal of 42^{o} for solids and a zinc selenide crystal of 45^{o} for liquids. Spectra acquisition was performed using OMNIC 7.3 software (Thermo Electron Corporation). A background spectrum was collected before acquisition and used as reference. Spectra were recorded at room temperature between 4000 – 650 cm^{-1} by placing each sample in the corresponding ATR crystal. The final spectrum corresponds to the average of 32 individual scans, obtained with a resolution of 4 cm^{-1}.

3.3.3. *Nuclear magnetic resonance (NMR) spectroscopy*

NMR spectra were acquired using an Avance II 500 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 500.23 MHz, equipped with an

indirect detection probe head (BBI-Z) with pulse field gradients. The system was locked using the frequency of deuterium oxide (Sigma-Aldrich, St. Quentin Fallavier, France) present in a capillary inside the NMR tube. Prior to acquisition, samples were allowed to equilibrate for 10 min using a Thermocouple-T to monitor the temperature. ^{1}H NMR spectra were then acquired at a range of temperatures (25 – 50 °C) to determine the existence of intra- and intermolecular bonds, by evaluating the chemical shifts of the H-bonded protons[17].

3.3.4. *Water content determination*

DES water content was determined by Karl Fischer titration, as previously described on Chapter 2, Part II, section 3.3.1.. Experiments were performed in triplicate and results were expressed as mass percentage.

3.3.5. *Density and viscosity measurements*

Densities and viscosities were measured as a function of temperature (ranging from 20 °C to 60 °C) using a viscometer/densimeter, as previously described (Chapter 2, Part II, section 3.3.2.). Results were presented as a mean of three measurements, as g_{DES}/cm^3 for density and mPa·s for viscosity.

3.3.6. *Polarity measurements*

Relative polarity of DES was measured using the solvatochromic dye Nile red as probe[18], as previously described (Chapter 2, Part II, section 3.3.3.). Experiments were performed at least in duplicate. The E_{NR} parameter was calculated, and results were expressed as kcal/mol.

3.4. DES *in vitro* phytotoxicity determination

3.4.1. *Sample preparation*

DES stock dispersions were prepared in distilled water immediately before the phytotoxicity assay. Mixtures were heated to 37 °C, under constant stirring, until a homogeneous dispersion was achieved. Samples were then two-fold serially diluted in distilled water to obtain a range of concentrations (0 – 0.5 mg_{DES}/mL).

3.4.2. *Phytotoxicity assay*

DES phytotoxicity was evaluated as previously described (Chapter 2, Part II, section 3.5.2). Briefly, sterilized wheat (*T. aestivum*) seeds were placed on petri dishes, previously prepared with filter paper and cotton wool, and moistened with the different concentrations of DES. Wheat seedlings were allowed to grow for 7 days and then harvested. The effect of DES on germination and early growth of wheat was determined and the half maximal effective concentrations (EC50) were calculated from dose-response curves. All experiments were performed in duplicates and results were expressed as mg_{DES}/mL.

3.4.3. *Lipid peroxidation (LPO) measurement*

LPO accumulated on wheat leaves was evaluated by measuring the malondialdehyde (MDA) content, as described on Chapter 2, Part II, section 3.5.3.. Absorbances of the supernatants resulting from an extraction of wheat leaves with trichloroacetic acid (0.1%), after reaction with thiobarbituric acid, were read at 532 nm (with subtraction of the unspecific turbidity at 600 nm), as a mean of two independent measurements. Results were expressed as $nmol_{MDA}/g_{fresh\ leaves}$.

3.4.4. *Chlorophyll (CHL) content determination*

CHL content was determined as previously described (Chapter 2, Part II, section 3.5.4.). Absorbances of the supernatants resulting from an extraction of wheat leaves with 80% acetone were measured at 663 nm and 645 nm. Results were expressed as $\mu g_{CHL}/g_{fresh\ leaves}$.

3.4.5. *Antioxidant enzyme activity determination*

Enzyme analysis was performed as described in detail on Chapter 2, Part II, section 3.5.5.. Briefly, wheat leaves were extracted with a potassium phosphate buffer solution. Superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX) activities were then determined by UV-vis, immediately after supernatants were subjected to different specific reactions. Experiments were performed at least in duplicates and results were expressed as $units_{SOD}/mg_{protein}$ for SOD, $nmol_{oxidized\ guaiacol}/(mg_{protein}\cdot min)$ for GPX, $nmol_{decomposed\ H2O2}/(mg_{protein}\cdot min)$ for CAT, and $nmol_{oxidized\ ascorbate}/(mg_{protein}\cdot min)$ for APX.

3.5. Solid-liquid extractions

3.5.1. *Conventional Soxhlet extraction of AXT*

Extractions were performed using acetone as solvent, following the procedure described by Ruen-ngam *et al.* for the extraction of AXT from *H. pluvialis*[19], with slight modifications. Briefly, 6 g of freeze-dried residue were extracted with 200 mL of acetone (Fisher Chemical, Loughborough, UK) for 6 h, using a Soxhlet apparatus. The experiment was replicated and the resulting samples were analysed immediately after extraction by UV-vis as suggested by Scott[20], or by high-performance liquid chromatography (HPLC), in order to determine the AXT content. After quantification, extracts were concentrated in a rotary evaporator at 40 °C, under reduced pressure, and stored at -20 °C in the absence of light.

3.5.2. *DES extraction of AXT*

AXT extractions were performed in silicone oil baths under constant magnetic stirring (c.a. 60 rpm) using the different terpene/fatty acid-based DES as solvents. Briefly, 1 g of DES were added to 0.25 g of freeze-dried crab shell residue. The influence of temperature (30, 45, and 60 °C) and extraction time (2, 6, and 24 h) on total AXT yield was studied. The selection of the solid-liquid ratio was based on preliminary experiments (data not shown) and on the work of Pires *et al.*[21].

As a proof of concept, extractions of brown crab and shrimp shells, mussels, and *H. pluvialis* were also performed by adding 2.50 g of DES to 0.25 g of biomass and stirring for 2 h at 60 °C. It was necessary to adapt the solid-liquid ratio due to the different densities displayed by the four biomasses. Extracts were then centrifuged at 14000 rpm for 10 min at room temperature and the supernatant recovered. Samples were analysed immediately after by UV-vis spectroscopy or HPLC, in order to determine the AXT content. After quantification, the extracts were stored at -20 °C in the absence of light. Experiments were performed at least in duplicates.

3.6. Astaxanthin quantification

The UV-vis method used for AXT quantification was previously validated by HPLC (data not shown). Briefly, an Alliance e2695 HPLC model (Waters, Milford, MA, USA) equipped with a photodiode array detector was used for AXT analysis. Chromatographic separation was

carried out with a YMC 30 Carotenoid Column (Classical Analytical HPLC Column S-5 µm, 250 x 4.6 mm, YMC, Kyoto, Japan) coupled with a Guard Cartridge YMC C30 pre-column (5 µm, 10 x 4.0 mm, YMC, Kyoto, Japan), with a mobile phase of methanol:methyl tert-butyl ether (80:20) (Fisher Chemical, Loughborough, UK, and Honeywell | Riedel-de-Haën Seelze, Germany, respectively) at 1 mL/min and 25 °C for 45 min. Mussels and *H. pluvialis* extracts obtained by Soxhlet with acetone and *H. pluvialis* extract obtained with DES presented several peaks at the maximum wavelength. For this reason, these three samples were quantified using the HPLC method described above. When analysed by HPLC, the remaining extracts presented only one peak corresponding to AXT. Therefore, these samples were quantified by UV-vis spectroscopy immediately after extraction, based on the absorbance obtained at the maximum wavelength (478 nm for acetone; 488 nm for PA:CA and ME:PA; and 484 nm for ME:CA, EU:ME, and ME:MA extracts) and on linear correlation equations. AXT from *Blakeslea trispora* (Sigma-Aldrich, St. Quentin Fallavier, France) was used as standard. Results were expressed as $\mu g_{AXT}/g_{dry\ residue}$.

3.7. *In vitro* bioactivity evaluation

3.7.1. *Human cell-based assays*

3.7.1.1. Cell culture

Human intestinal Caco-2 and HT-29 cell lines were cultured in RPMI 1640 medium supplemented with 10% of heat-inactivated foetal bovine serum (FBS) and 1% penicillin-streptomycin (in the case of Caco-2 cells) (Gibco, Invitrogen, Paisley, UK). Cells were maintained as monolayers as previously described on Chapter 2, Part I, section 3.4.1..

3.7.1.2. Sample preparation

DES, respective isolated compounds and physical mixtures, AXT standard, and extract stock solutions/dispersions were prepared in RPMI 1640 medium supplemented with 0.5% of FBS, immediately before the assays. Physical mixtures were achieved by preparing isolated solutions of each DES individual component, which were then mixed to mimic DES molar ratios. AXT standard and Soxhlet extracts were previously solubilized in dimethyl sulphoxide (DMSO, Carlo Erba, Val-de-Reuil, France) at their solubility limits (1 mg/mL for AXT standard, 25 mg/mL for crab shell, mussel, and *H. pluvialis* extracts, and 8 mg/mL for shrimp shell

extract). Solutions/dispersions were then heated to 37 °C for a few minutes and thoroughly homogenized using a vortex. To obtain a range of concentrations, samples were further diluted in RPMI 1640 medium supplemented with 0.5% of FBS.

3.7.1.3. Cytotoxicity assay

Cytotoxicity assays were performed by incubating confluent Caco-2 cells with different concentrations of DES, respective isolated compounds, AXT standard, or extracts, for 24 h, as described on Chapter 2, Part I, section 3.4.3.. Cell viability was evaluated at 490 nm after CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay. Experiments were performed in triplicate using at least three independent assays and cell viability was expressed as percentage of viable cells relative to the control. EC50 values were obtained from dose-response curves and expressed as $mg_{extract,\ AXT,\ DES,\ individual\ component,\ or\ physical\ mixture}/mL$.

3.7.1.4. Antiproliferative assay

Antiproliferative effect was evaluated on HT-29 cells at conditions of exponential growth, as previously described (Chapter 2, Part I, section 3.4.4.). Briefly, cells were incubated with different concentrations of DES, respective isolated compounds, AXT standard, or extracts, for 24 h, after which cell proliferation was determined as reported for cytotoxicity assay.

3.7.2. *Bacterial assays*

3.7.2.1. Bacterial test strains

Similar to what was described on Chapter 2, Part II, section 3.8.2.1., *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 strains were selected as representative of Gram-positive and Gram-negative species, respectively.

3.7.2.2. Sample preparation

DES, AXT standard solubilized in each DES, and DES extract stock dispersions were prepared in cation-adjusted Mueller Hinton broth (CAMHB, BD Difco, Sparks, NV, USA), immediately before antimicrobial susceptibility testing assays. Soxhlet extracts were previously solubilized in a mixture of water, DMSO, and NaOH (1 M, ACROS Organics, Geel, Belgium) (17:2:1). Pipetting up and down 20 times was performed to ensure a homogeneous stock solution/dispersion. When necessary, samples were heated at 35 °C for 5 min.

Solutions/dispersions were then 2-fold serially diluted in CAMHB to obtain a range of concentrations.

3.7.2.3. Antimicrobial susceptibility assay

Antimicrobial susceptibility was assessed according to what was previously described on Chapter 2, Part II, section 3.8.2.3.. Briefly, assays were performed by dispensing different concentrations of DES, AXT standard solubilized in each DES, and extracts, on microtiter plates, which were then inoculated with *S. aureus* or *E. coli*. Plates were incubated under aerobic conditions at 37 °C, for 16 to 20 h. Minimum inhibitory concentrations (MIC) were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited after incubation. When ambiguous MIC values were obtained, the cell viability reagent PrestoBlue™ (Invitrogen, San Diego, CA USA) was used according to the manufacturer's instructions. Three biological replicates were performed and results were expressed as μL_{sample}/mL and ng_{AXT}/mL.

3.8. Statistical analysis

The estimation of cytotoxicity, antiproliferative activity, and phytotoxicity, as well as the statistical significance of average differences determination, was performed using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance of average differences was assessed either by one-way analysis of variance followed by the Tukey test or calculated using an unpaired t-test. An alfa error of 5% was accepted in the hypothesis testing to decide for a significant effect. Data was reported as mean ± standard deviation values.

4. Results and discussion

4.1. DES preparation and physicochemical characterization

The decrease in the melting temperature of a DES when compared to its individual components, has been mainly attributed to hydrogen bond formation among the different components of the mixture[22]. Hydrogen bonding can occur in any system containing a proton donor group and a proton acceptor if the *s* orbital of the proton is able to overlap the *p* orbital of the acceptor[23]. In this work, PA, ME and MA were chosen as molecules that can act as hydrogen bond donors (HBD) through the hydroxyl group, while all five molecules (PA,

CA, ME, EU, and MA) can act as hydrogen bond acceptors (HBA), through their oxygen atoms. Additionally, due to their lipophilic character, it was expected that these systems would solubilize AXT. All mixtures were prepared using equimolar composition (1:1), except for ME:MA, which was prepared using a molar ratio of 8:1 (Table 1), since smaller ratios did not form stable DES, *i.e.*, some crystals were evident at room temperature. All DES were formed after a few minutes, resulting in clear and transparent liquids, maintaining this visual aspect when cooled to room temperature.

Table 1. DES prepared, respective molar ratios, and water content.

Component A	Component B	Molar ratio	Visual aspect	Water content (%)
PA	CA	1:1	Clear and transparent liquid at room temperature	0.21 ± 0.02
ME	PA	1:1		0.17 ± 0.01
ME	CA	1:1		0.18 ± 0.01
ME	EU	1:1		0.17 ± 0.01
ME	MA	8:1		0.15 ± 0.01

To confirm the formation of each system, different characterization techniques were applied, including DSC, FTIR-ATR, and NMR. Furthermore, important properties of each solvent, such as water content, density, viscosity, and polarity were also studied.

4.1.1. *DSC analysis*

DES and their individual components were analysed by DSC in order to evaluate if there were similar phase transitions among them. Although the melting point of CA is 174 – 179 °C,[24] the systems PA:CA and ME:CA were only studied until 80 °C, to avoid degradation. The thermograms obtained are presented on Figure 1.

Results showed that DES did not share any of the characteristic melting point peaks of their isolated components. Furthermore, the systems ME:CA, ME:EU, and ME:MA showed some endothermic transitions at lower temperatures than their components. These transitions were not evident for the systems PA:CA and ME:PA, since the melting point of PA is extremely low and, to the best of the author's knowledge, remains undetermined by the literature.

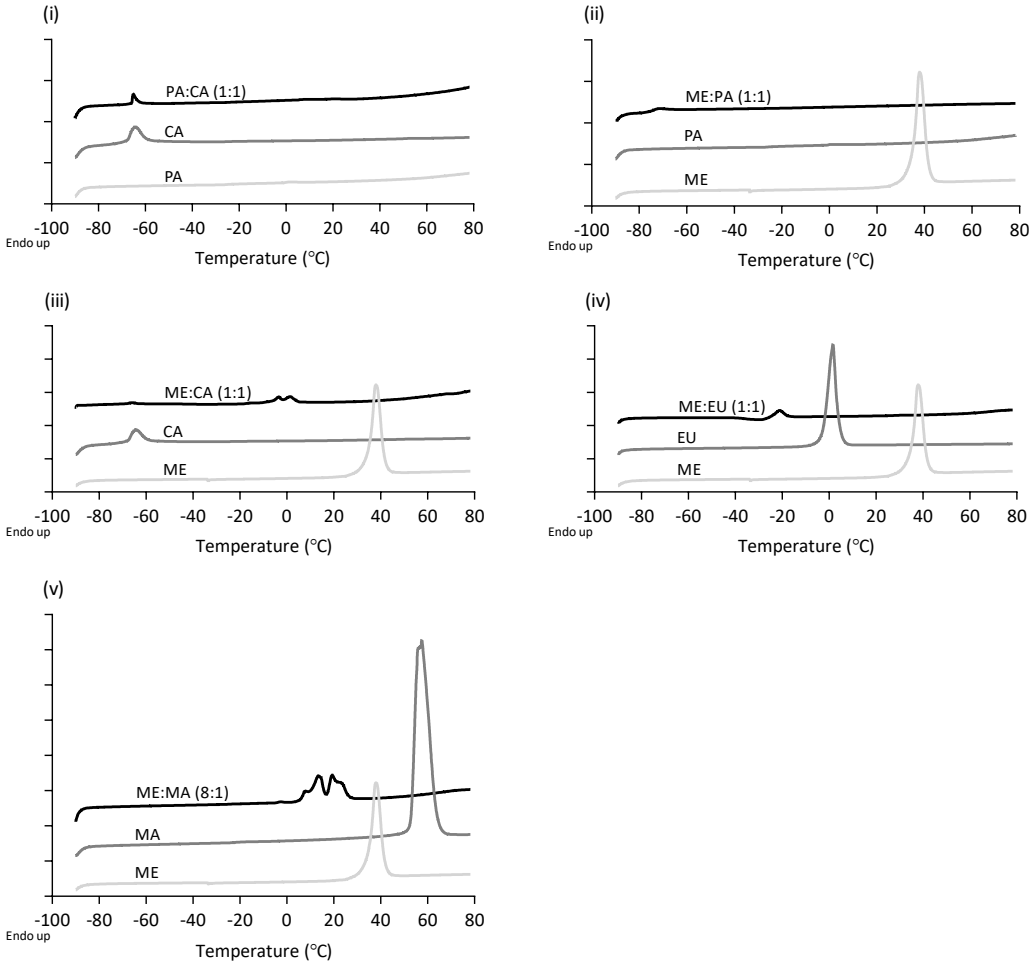

Figure 1. Thermograms obtained for DES and their individual components. (i) PA, CA and PA:CA (1:1); (ii) ME, PA and ME:PA (1:1); (iii) ME, CA and ME:CA (1:1); (iv) ME, EU and ME:EU (1:1); and (v) ME, MA and ME:MA (8:1).

4.1.2. *FTIR-ATR spectroscopy*

FTIR has been widely used to study the interactions among molecules. In particular, this technique has been used as indicative of hydrogen bond formation among the different components of a DES[25].

From the obtained FTIR spectra displayed on Figure 2, PA (Figures 2i and ii), as a terpene alcohol, exhibited a strong broad peak at 3313 cm^{-1}, which indicates O-H stretching vibration. Similarly, ME (Figures 2ii-v) exhibited O-H stretching at 3257 cm^{-1} [26,27]. CA spectrum (Figures 2i and iii) showed a strong absorption band at 1738 cm^{-1} resulting from C=O stretching vibration, which is characteristic of ketones[23,27]. EU (Figure 2iv) exhibited a characteristic spectrum of an ether, with absorption peaks at 1080 and 1215 cm^{-1},

corresponding to the symmetrical and asymmetrical stretching vibrations of the C-O-C system, respectively[28]. MA spectrum (Figure 2v) showed a characteristic band of carboxylic acids at 1697 cm^{-1}, which corresponds to C=O stretching vibration[29]. Absorption peaks at 2914 and 2846 cm^{-1} correspond to $–CH_2$ symmetrical and asymmetrical stretching vibration, respectively, being superimposed upon O-H stretch (between 3305 and 2690 cm^{-1})[23,29].

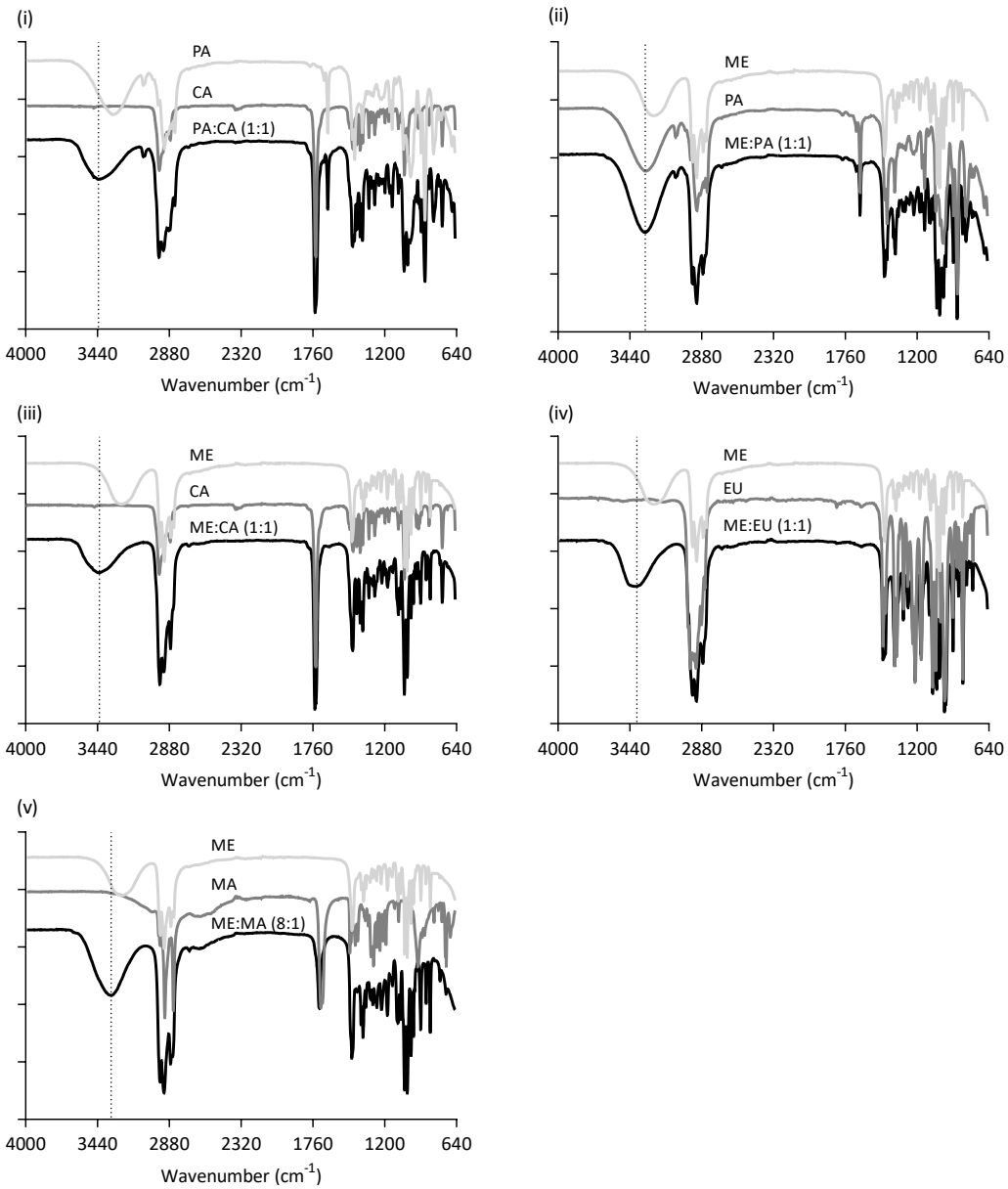

Figure 2. FTIR-ATR spectra of DES and their individual components. (i) PA, CA and PA:CA (1:1); (ii) ME, PA and ME:PA (1:1); (iii) ME, CA and ME:CA (1:1); (iv) ME, EU and ME:EU (1:1); and (v) ME, MA and ME:MA (8:1).

As mentioned above, the selected molecules can establish hydrogen bonds between the hydroxyl groups and the oxygen atoms present in each structure. By looking at the FTIR spectra of the different DES, it is possible to conclude that all mixtures presented similar absorption profiles to the individual components with the exception of the O-H stretching vibration bands. In fact, these peaks were shifted to lower frequencies (higher wavenumbers) when compared to the isolated components of the mixture, which suggests that hydrogen bonding occurred through the hydroxyl groups. Since hydrogen bonding modifies the force constant of both donor and acceptor groups, the frequencies of the stretching/bending vibrations were expected to be altered as well[23].

4.1.3. *NMR spectroscopy*

Several studies have shown that the chemical shifts of the H-bonded protons, for both intra- and intermolecular bonds, are deeply dependent on the temperature[17,30,31]. Typically, an increase in temperature results in an upfield shift (lower ppm) of the H-bonded proton chemical shifts, due to a decrease of the intermolecular hydrogen bonding[17,30]. Within this context, ^{1}H NMR spectra were acquired at different temperatures, ranging from 25 to 50 °C, to confirm this effect.

Figure 3i shows the spectra obtained for ME:MA as an example. When at 25 °C, the chemical shift of the OH proton was 5.35 ppm. However, increasing temperature up to 50 °C caused an upfield shift, ultimately resulting in chemical shift of 4.61 ppm. This same behaviour could be found for all DES studied (data not shown).

The magnitude of the shift can be evaluated through the calculation of the temperature coefficient (T_C), which can be obtained by plotting the chemical shifts as a function of temperature (Figure 3ii) and retrieving the slope from the resulting linear correlation. According to Li *et al.* and Hamuro *et al.*, in nonpolar solvents, a T_C below – 0.005 ppm/°C is usually related with intermolecular hydrogen bonding[32,33]. As shown on Figure 3ii, the T_C obtained for each system ranged from – 0.0297 ppm/°C to – 0.0165 ppm/°C, thus suggesting the existence of intermolecular H-bonding.

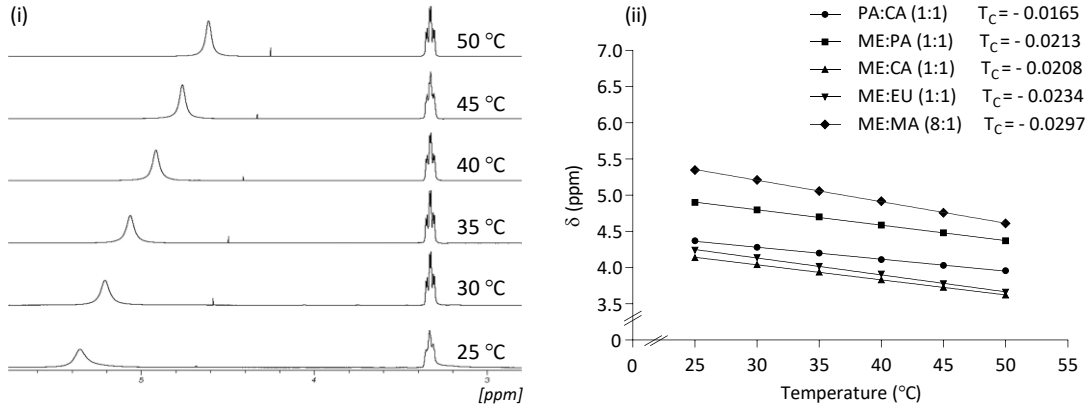

Figure 3. (i) Magnification of ^{1}H NMR spectra of ME:MA (8:1) as a function of temperature; (ii) plot of the chemical shifts (δ) of the OH protons as a function of temperature for each DES.

4.1.4. *Water content, density, viscosity and polarity of DES*

The water content is known to have an extensive impact on DES physical properties, including density, viscosity, and polarity[34]. Consequently, it is essential to accurately determine and eventually control this parameter. Table 1 shows that the weight percentage of water contained in each system was rather low, varying between 0.15% and 0.21%.

Both density and viscosity are two of the most important parameters to consider when choosing an extraction solvent. Accordingly, DES densities were studied as a function of temperature and are shown on Figure 4i. The values obtained ranged from 0.897 to 0.963 g/mL at 20 °C and from 0.867 to 0.932 g/mL at 60 °C, with densities increasing as follows: ME:MA < ME:EU < ME:CA < ME:PA < PA:CA. As it would be expected, densities decreased with increasing temperature. Additionally, DES densities showed to be inferior to the density of water and considerably lower than the values reported for most hydrophilic DES based, for example, on choline chloride[35]. The lower densities of terpene/fatty acid-based DES can possibly be explained by the lower densities of the compounds used in their preparation.

DES viscosities were also studied as a function of temperature (Figure 4ii) and increased as follows: ME:EU < PA:CA < ME:CA < ME:PA < ME:MA. Results showed that viscosity values tended to decrease with increasing temperature, being this decrease more pronounced for ME:MA, ME:PA, and ME:CA systems. In fact, it is known that temperature can regulate the cohesive forces in a DES, and when increased, it can cause a decrease in the internal resistance of molecules, thus decreasing the viscosity of the system[36]. DES are often characterized by their high viscosity, which has been mainly attributed to the presence of an

extensive hydrogen bond network among their components, but it may also be related with van der Waals and electrostatic interactions[37]. Figure 4ii shows that the DES prepared in this work presented fairly low viscosities when compared to other systems. Nevertheless, results are in accordance with the data reported by other authors for hydrophobic DES based on terpenes and monocarboxylic acids[38] or on menthol and organic acids[39].

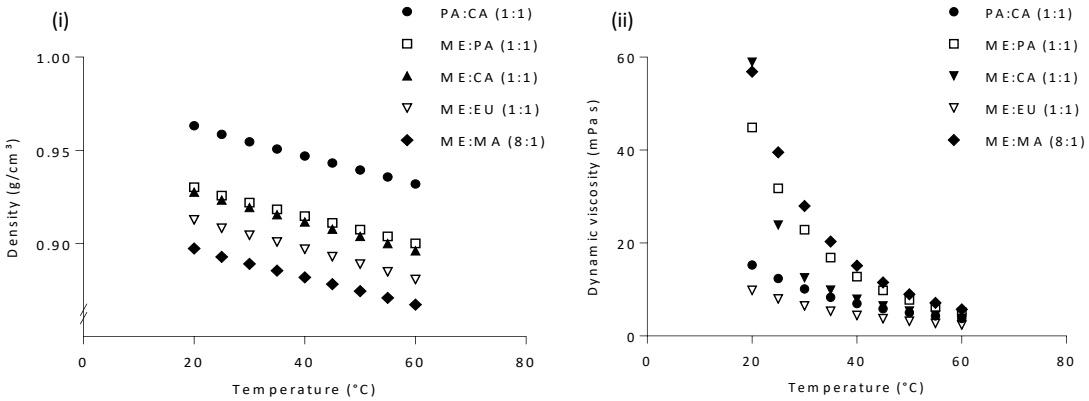

Figure 4. Variation of (i) density and (ii) viscosity of each DES as a function of temperature.

The use of solvatochromic dyes allows a direct probe of the relative polarity of a solvent affecting the absorption properties of the dye molecule. In particular, Nile red has been used to estimate the polarity of different solvents, including molecular or switchable solvents, ionic liquids (IL)[40] and DES[41,42]. When in the presence of higher polarity solvents, the λ_{max} of the dye shifts to higher wavelengths, resulting in a lower E_{NR}. By measuring these shifts and comparing them with the λ_{max} obtained for a solvent of reference, it is possible to estimate the relative polarity of a given solvent. E_{NR} values obtained for DES are displayed on Figure 5. The results obtained were compared with the polarity of acetone, which is typically used for AXT extraction[9], and ethanol. The relative polarity of DES increased as follows: ME:EU < ME:CA < ME:MA < PA:CA < ME:PA. Additionally, the experimental E_{NR} values obtained for ethanol (52.11 kcal/mol) and acetone (53.74 kcal/mol) were in agreement with the data previously reported[40]. DES based on PA presented the highest polarity, close to the value obtained for ethanol, but still very far from the polarity of water (E_{NR} of 48.20 kcal/mol)[40]. In contrast, DES based on EU or CA, which present some structural similarities, combined with ME, presented the lowest polarity, closer to the value obtained for acetone. The system ME:MA showed an intermediate polarity, possibly determined by the acid saturated

aliphatic chain. As it would be expected, the measured polarities were lower than those reported in the literature for DES based on organic acids, amino acids, sugars, or polyalcohols[43].

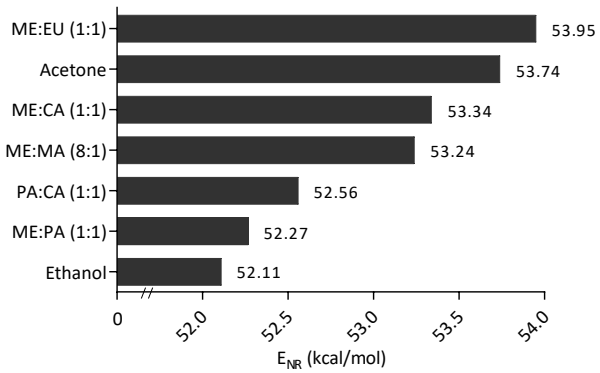

Figure 5. E_{NR} values obtained for each DES. Acetone and ethanol E_{NR} values were included as solvents of reference. Coefficient of variation ≤ 0.41%.

As above mentioned, an important factor to consider when measuring polarities is the water content[18,34]. However, in this work, no correlation between the water content and the polarity was found. This is in accordance with what was previously reported by Craveiro *et al.*, *i.e.*, when 0.1 to 15% of water (in mass) are present in a DES, the bathochromic shift is under 1.2% and the changes in E_{NR} values are minor[18].

4.2. DES *in vitro* phytotoxicity

DES have been frequently considered as environmentally safe or nontoxic by different authors. However, it is important to confirm their toxicological effect when considering commercial applications[44,45]. Within this context, the impact of terpene/fatty acid-based DES was evaluated on wheat (*T. aestivum*), a plant widely used in agriculture[46]. Growth parameters, namely germination and shoot height inhibition, were measured on wheat seeds treated with different concentrations of DES for 7 days. The respective EC50 were calculated and are summarized on Table 2.

Results show that DES inhibited both seed germination and growth, with EC50 ranging from 0.038 to 0.125 mg/mL, considering both markers, being ME:PA the most toxic and PA:CA the least toxic DES. It is also important to note that shoots grew in an uneven way on seeds

treated with higher concentrations of DES, although no signs of leaf necrosis were observed (data not shown).

Table 2. EC50 values (mg/mL) obtained on wheat seeds treated with terpene/fatty acid-based DES, after an incubation period of 7 days.

DES	Germination	Shoot height
PA:CA (1:1)	0.125 ± 0.047	0.072 ± 0.013
ME:PA (1:1)	0.051 ± 0.001	0.038 ± 0.018
ME:CA (1:1)	0.073 ± 0.023	0.058 ± 0.009
ME:EU (1:1)	0.063 ± 0.000	0.068 ± 0.014
ME:MA (8:1)	0.066 ± 0.019	0.062 ± 0.010

The phytotoxicity of IL and DES (mainly based on choline chloride) have already been explored by several authors on different model organisms[46–49], including wheat seeds[46]. In particular, Lapeña and co-workers followed a scale of toxicity proposed by Passino and Smith[50], to classify the ecotoxicity of the choline chloride-based DES studied[49]. In this classification scale, chemicals are organized into several categories of toxicity based on their effective concentrations (EC50 (µg/ml) less than 0.01 - supertoxic; 0.01 to 0.1 - extremely toxic; 0.1 to 1 - highly toxic; 1 to 10 - moderately toxic; 10 to 100 - slightly toxic; 100 - 1000 practically harmless; greater than 1000 - relatively harmless). Within this context, terpene-based DES can be regarded as practically harmless or slightly toxic to wheat.

MDA levels (Figure 6i), the content of photosynthetic pigments (CHL *a*, CHL *b*, and total CHL) (Figure 6ii), and the activity of antioxidant enzymes on plant leaves (Figure 7) have also been commonly used as markers of stress. Although MDA (the cytotoxic product of lipid peroxidation) levels were reduced (Figure 6i), possibly due to the known antioxidant capacity of terpenes[51], the CHL contents were still inhibited at higher DES concentrations (Figure 6ii), which correlates with the inhibition of seedlings growth.

With regard to the activity of antioxidant enzymes, namely SOD, GPX, CAT, and APX, Figure 7 shows that, while some DES caused the enzymatic activity to increase at specific concentrations, there were also cases where enzymes were slightly or fairly inhibited, which suggests that, as it would be expected, the defence mechanisms of wheat had different antioxidant and redox homeostasis response to oxidative stress, depending not only on the system tested, but also on the concentration applied.

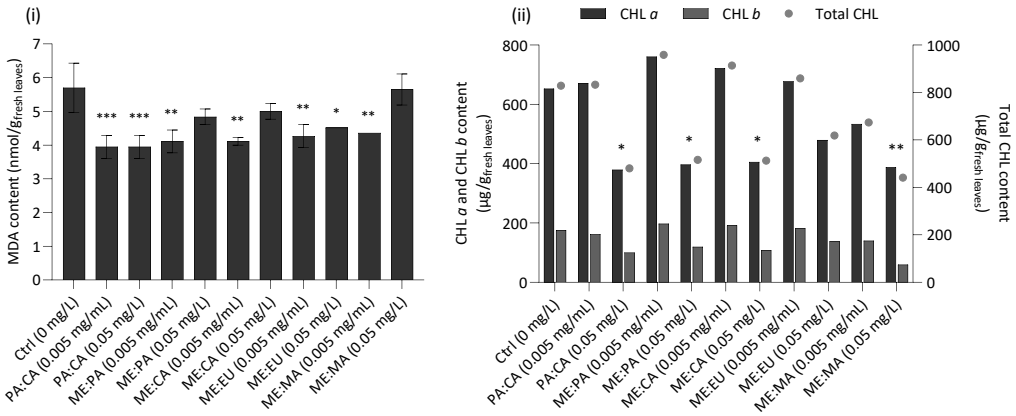

Figure 6. Effect of terpene/fatty acid-based DES on (i) MDA and (ii) photosynthetic pigments content. Statistically significant differences between the effect of DES and the control are represented by asterisks (*).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

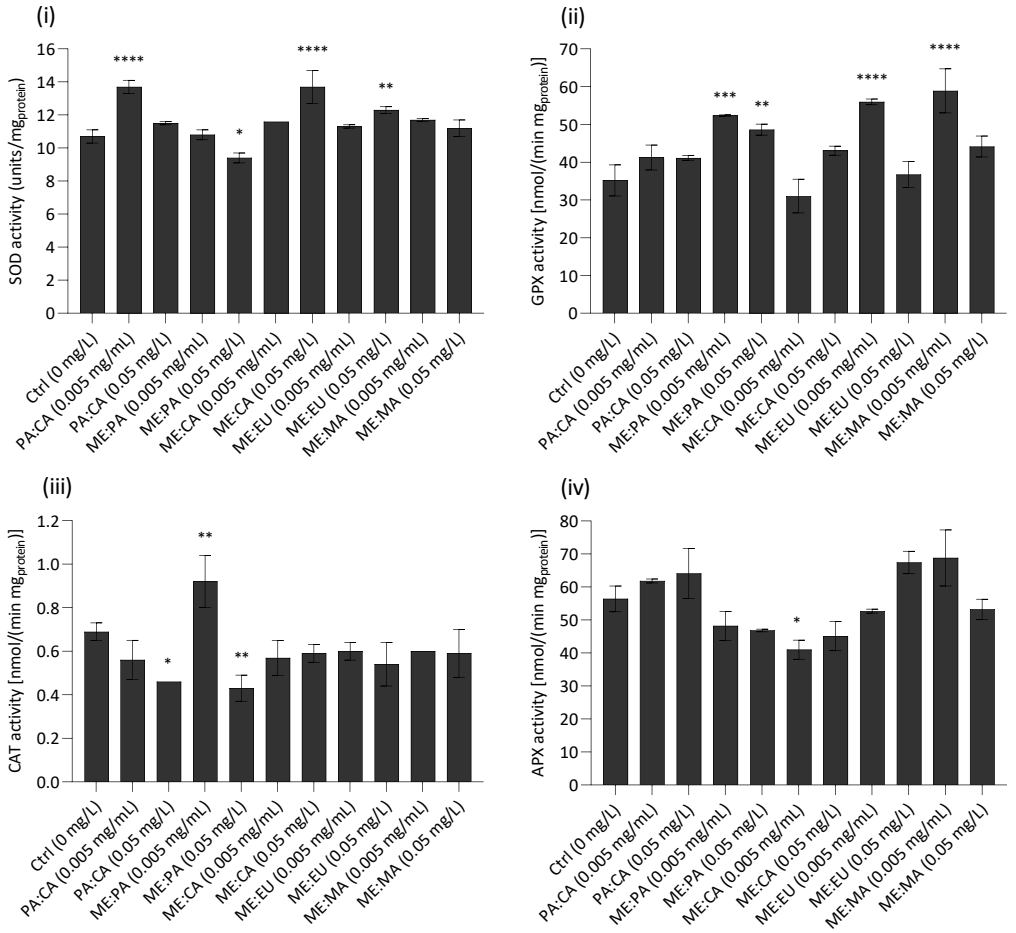

Figure 7. Effect of terpene/fatty acid-based DES on (i) SOD, (ii) GPX, (iii) CAT, and (iv) APX activities. Statistically significant differences between the effect of DES and the control are represented by asterisks (*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

In view of the results presented, it would be beneficial to consider using these DES as an integral part of the final product, thus avoiding their disposal, which could be potentially harmful, even if considered as slightly toxic, to the environment.

4.3. Astaxanthin extractions

Due to their hydrophobic nature, carotenoids are usually conventionally extracted using organic solvents. In particular, xanthophylls are frequently obtained using acetone, ethanol or ethyl acetate as solvents[9]. Accordingly, a conventional Soxhlet extraction with acetone was the chosen method to evaluate the AXT content of shells.

Results presented on Figure 8 show that a yield of 9.7 µg/g could be obtained by Soxhlet extraction. This value is about 4 to 16-fold higher than the one reported by Pires *et al.* for brown crabs from the Scottish and French coasts. However, in the work of Pires *et al.*, carotenoids were extracted with acetone at room temperature, which may not have been as efficient as an extraction at acetone's boiling point (56 °C). Furthermore, carotenoid and in particular AXT content, depends not only on the extraction conditions, but also on the crab species, on the harvesting season and site, and on the time and circumstances of storage[21].

Terpene/fatty acid-based DES were explored as an alternative to conventional organic solvents and applied for the extraction of AXT from crab shell residues. In order to achieve the highest AXT recovery, while minimizing energy costs (by reducing extraction time or temperature), the impact of different process parameters on the extraction performance of DES, namely operating temperature and extraction time, was studied for a fixed solid-liquid ratio (1:4). Figure 8 shows the extraction efficiencies for each DES. AXT yields varied greatly depending on the type of solvent used, as well as on the extraction parameters imposed, ranging from 1.8 (ME:CA, 30 °C, 2 h) to 9.3 µg/g (ME:MA, 60 °C, 24 h).

It is well known that the operating temperature has the ability of favouring extraction by enhancing the solubility of the solute, while decreasing the viscosity of the solvent and enhancing the diffusion rate. However, AXT is highly sensitive to temperature, being prone to oxidation due to its highly unsaturated structure. Therefore, it can undergo thermal degradation[52]. For this reason, in this work an operating temperature of 60 °C has not been exceeded.

Results from Figure 8 suggest that higher temperatures and extraction times tended to increase the AXT yield. However, this was not statistically significant in all cases. When compared with the other DES selected, ME:MA (8:1) was the most efficient solvent in extracting AXT from crab shells, being able to match the yield obtained by Soxhlet extraction with acetone, when operating at 60 °C. Since there were no statistically significant differences between 2, 6, or 24 h for ME:MA (8:1) at 60 °C, an extraction at 60 °C for 2 h could be accepted as the best extraction condition to achieve reasonable AXT yields.

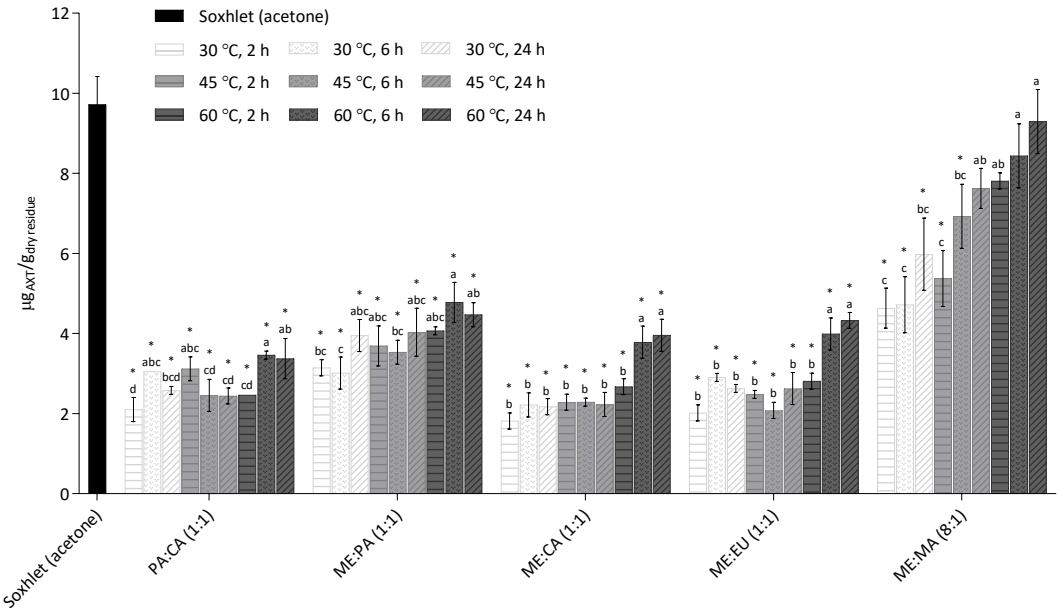

Figure 8. AXT mass yields obtained from crab shell residues, determined by UV-vis spectroscopy. For each DES, the statistically significant differences between the extraction conditions studied are represented by different lowercase letters. The significant differences between the extracts obtained with DES and the extracts obtained by Soxhlet with acetone are represented by asterisks (*). $P < 0.05$ was accepted as statistically significant in all cases.

DES have already been used by Lee and Row as additives to volatile organic solvents for AXT recovery from crab (*Portunus trituberculatus*) waste, using ultra-sound extraction (90 min, 65 W). The authors observed a 1.6-fold increase in AXT yield when using methyltriphenylphosphonium bromide:1,2-butanediol (1:4) DES as additive to acetone (0.25 mg/mL), compared to 1-ethyl-3-methylimidazolium bromide IL alone. According to Lee and Row neither DES nor IL alone were suitable for AXT extraction due to their high viscosity, which would lead to low mass transfer and low diffusivity rates. For that reason, the authors

have decided to use them as additives[53]. However, in the work described herein, the application of low viscosity terpene/fatty acid-based DES for the direct extraction of AXT from crab shell residues was demonstrated, using a straightforward solid-liquid extraction. As a proof of concept and based on the statistical analysis performed on crab shell extractions, the best extraction conditions (ME:MA (8:1), 60 °C, 2 h) were applied to three different feedstocks that are known to be rich sources of AXT: shrimp shells, mussels, and *H. pluvialis*. Results illustrated on Figure 9 show that, for all three biomasses, the system ME:MA (8:1) allowed a 3- to 657-fold increase in AXT yields, when compared to a 6 h Soxhlet extraction. Therefore, these results and alternative biomasses are worth further investigation.

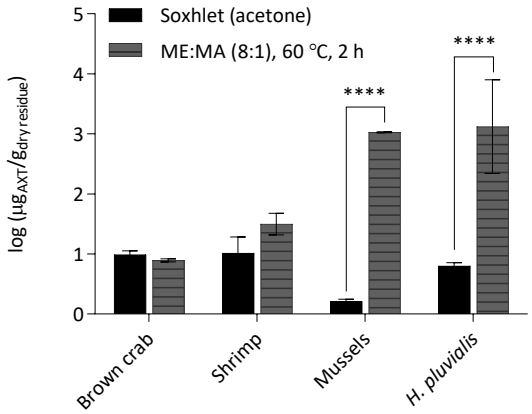

Figure 9. Logarithm of AXT mass yields obtained from crab and shrimp shell residues, mussels, and *H. pluvialis*, determined by UV-vis spectroscopy or HPLC. Statistically significant differences between the extracts obtained with DES and the extracts obtained by Soxhlet with acetone are represented by asterisks (*) (**** $P < 0.0001$).

Zhang *et al.* described the extraction of AXT from shrimp shells using an ultrasonic-assisted extraction (30 min, 70 W) with choline chloride:1,2-butanediol (1:5) DES with 10% of water. At these extraction conditions, the DES used was able to increase the AXT extracted by 1.4-fold, when compared to a conventional extraction with ethanol[54]. However, in the study reported herein it was shown that it was possible to increase the extraction of AXT from shrimp shells by 3-fold, when comparing a ME:MA (8:1) extraction with a Soxhlet extraction with acetone.

4.4. *In vitro* bioactivity evaluation

4.4.1. *Cytotoxicity and antiproliferative activity*

Some of the AXT-rich extracts obtained after DES and Soxhlet extractions were selected to be evaluated in terms of cytotoxicity and antiproliferative effect. Results summarized on Table 3 show that all extracts obtained with terpene/fatty acid-based DES had a cytotoxic effect on Caco-2 cells with EC50 values ranging from 0.52 to 3.28 mg/mL. However, neither AXT standard nor Soxhlet extracts presented cytotoxicity on Caco-2 cells at the concentrations tested. Additionally, when tested on HT-29 cells, none of crab shell extracts nor AXT standard showed to be effective in reducing cell viability at the concentrations tested. Nevertheless, it is important to highlight that AXT-rich extracts obtained using DES were tested in HT-29 cells at a maximum concentration of 0.4 mg/mL, which was not cytotoxic for Caco-2 cells.

The bioactive potential of AXT and AXT-rich extracts has been widely explored[5]. Wayakanon *et al.* reported that the cytotoxicity on Caco-2 cells of a supercritical CO_2 AXT-rich extract obtained from *Xanthophyllomyces dendrorhous* was superior to 5 mg/mL[55]**.** Hernandez *et al.* reported a similar effect displayed by carotenoid-rich extracts obtained from *Spirulina platensis* on a leukemic (K-562) cell line[56]. Additionally, two other studies reported the effects of AXT at lower concentration ranges (0 – 0.02 mg/mL) on both Caco-2 and HT-29 cell lines. After incubation times of 24 h and 72 h, no effects on viability, oxidative stress or DNA damage were observed[57,58]. When compared to the literature, the results obtained in this study allow for the conclusion that (i) the effects of the extracts obtained with DES on HT-29 cells may have been hampered by the concentrations used, which, as mentioned, were limited by the cytotoxicity of samples on Caco-2 cells; (ii) the effects of extracts obtained with Soxhlet on Caco-2 cells and AXT standard on both Caco-2 and HT-29 cell lines may have been restricted by the samples solubility in DMSO and by DMSO own toxicity on the cell models used.

Table 3. EC50 values (mg/mL) obtained for extracts, AXT standard, DES, their individual components, and physical mixtures in Caco-2 and HT-29 cells, after an incubation period of 24 h.

Samples	Cytotoxicity (Caco-2 cells)	Antiproliferative effect (HT-29 cells)
Extracts		
Crab shells		
PA:CA (1:1), 30 °C, 6 h	0.52 ± 0.07	> 0.40 [a]
ME:PA (1:1), 45 °C, 2 h	0.75 ± 0.05	> 0.40 [a]
ME:CA (1:1), 60 °C, 6 h	0.73 ± 0.06	> 0.40 [a]
ME:EU (1:1), 60 °C, 6 h	0.73 ± 0.10	> 0.40 [a]
ME:MA (8:1), 60 °C, 2 h	1.09 ± 0.32	> 0.40 [a]
Soxhlet extraction	> 0.25 [b]	ND
Shrimp shells		
ME:MA (8:1), 60 °C, 2 h	1.48 ± 0.85	ND
Soxhlet extraction	> 0.08 [b]	ND
Mussels		
ME:MA (8:1), 60 °C, 2 h	1.81 ± 0.14	ND
Soxhlet extraction	> 0.25 [b]	ND
H. pluvialis		
ME:MA (8:1), 60 °C, 2 h	3.28 ± 1.36	ND
Soxhlet extraction	> 0.25 [b]	ND
AXT standard	> 0.01 [b]	> 0.01 [b]
DES		
PA:CA (1:1)	0.89 ± 0.20	0.75 ± 0.36
ME:PA (1:1)	0.91 ± 0.08	0.57 ± 0.02
ME:CA (1:1)	1.26 ± 0.02	1.54 ± 0.24
ME:EU (1:1)	1.58 ± 0.08	1.21 ± 0.07
ME:MA (8:1)	3.67 ± 0.34	0.84 ± 0.18
Individual components		
Perillyl alcohol	0.74 ± 0.24	0.36 ± 0.03
Camphor	> 5.00 [c]	> 5.00 [c]
Menthol	1.68 ± 0.50	2.67 ± 1.28
Eucalyptol	> 5.00 [c]	3.09 ± 0.24
Myristic acid	> 1.50 [c]	> 1.50 [c]
Physical mixtures		
PA:CA (1:1)	2.45 ± 0.29	3.82 ± 0.37
ME:PA (1:1)	1.34 ± 0.11	1.61 ± 0.38
ME:CA (1:1)	3.63 ± 0.21	3.08 [d]
ME:EU (1:1)	3.31 ± 0.34	2.95 ± 1.31
ME:MA (8:1)	0.72 [d]	6.42 ± 1.58

[a] EC50 limited by the concentrations that revealed cytotoxicity on Caco-2 cells
[b] EC50 limited by the solubility in DMSO and by DMSO's own cytotoxicity
[c] EC50 limited by the solubility in culture medium
[d] GraphPad Prism 9 software was not able to calculate a standard deviation
ND: not determined

Since AXT-rich extracts obtained with DES were not separated from the solvents at any point, it was crucial to evaluate both cytotoxic and antiproliferative profiles of DES, considering that the bioactivity of extracts could be strongly influenced by the solvents' own bioactivity.

Table 3 shows that all systems tested presented toxicity on Caco-2 cells but were also able to inhibit the proliferation of HT-29 cells. When compared to the other systems, PA-based DES showed to be the most cytotoxic on Caco-2 cells, as well as the most powerful inhibitors of HT-29 cells proliferation. EC50 values have also shown that ME:PA, ME:EU, and ME:MA were the only systems capable of inhibiting HT-29 cell proliferation without compromising the cell viability on Caco-2 cells, *i.e.*, the concentration that allowed the inhibition of 50% of HT-29 cell population did not present cytotoxic effects on Caco-2 cells. Additionally, ME:MA showed the greatest difference between the EC50 values obtained on Caco-2 and HT-29 cells (3.67 and 0.84 mg/mL, respectively). Results summarized on Table 3 also show that extracts obtained with DES presented higher cytotoxicity on Caco-2 cells than the respective solvents, except for *H. pluvialis* AXT-rich extract, which showed similar toxicity to ME:MA.

In the last years, some studies regarding the cytotoxicity of DES, mostly choline chloride-based, have started to emerge. Overall, when comparing the results presented herein with other studies regarding DES cytotoxicity, terpene/fatty acid-based DES showed a 10-fold decrease in EC50 values, which may suggest that the bioactivity of these systems is stronger than DES based on quaternary ammonium salts[59,60]. Nevertheless, Radošević et al. reported similar EC50 values for a choline chloride:oxalic acid (1:1) DES on cervical (0.33 ± 0.03 mg/mL) and breast (0.56 ± 0.05 mg/mL) adenocarcinoma cell lines[61].

The bioactivity of terpenes and fatty acids has been widely described on the literature and in particular their anticancer and anti-inflammatory properties[62–64]. Dose-response curves were obtained for the cytotoxicity (on Caco-2 cells) of each individual component of the DES prepared, and compared with the cytotoxicity displayed by each system (Appendix C, Figure C1). Results show that all DES had a more prominent cytotoxic effect than the isolated compounds, except for ME:MA. Moreover, CA, EU and MA did not show any cytotoxic effect for the concentrations tested, while PA showed a strong cytotoxic effect, presenting a similar curve to those obtained for PA:CA and ME:PA. The results obtained for the antiproliferative activity of DES and their isolated compounds on HT-29 cells showed that the systems ME:CA, ME:EU, and ME:MA presented a completely different behaviour when compared to the isolated compounds, being more effective in inhibiting the proliferation of cancer cells than ME, CA, EU, or MA (Appendix C, Figure C2). Similar to the results obtained on Caco-2 cells, PA:CA and ME:PA showed identical effect to PA. When comparing the effect of PA:CA and

ME:PA with the isolated compounds on both Caco-2 and HT-29 cell lines, it is possible to conclude that the bioactivity shown by these DES might have been dictated by the presence of PA, since the effect shown by the DES was very similar to the one displayed by the PA itself.

The equivalent physical mixtures of each solvent were prepared by mixing the free forms of DES individual components in molar ratios that mimicked the original DES composition. The corresponding EC50 showed that the behaviour displayed by each DES was very different from the one displayed by the physical mixtures (Table 3). Overall, DES showed higher cytotoxicity than the physical mixtures, except for ME:MA. Regarding the antiproliferative effect, all DES demonstrated a stronger capability to inhibit HT-29 cell proliferation than the physical mixture of their components. In this way, ME:MA (8:1) can be regarded as the most promising DES, since it showed lower cytotoxicity and higher antiproliferative effect when compared to its physical mixture. Overall, these results help to confirm that DES are systems with a completely different behaviour/bioactivity than a simple mixture of two compounds at the same concentration, possibly due to an increase of the DES solubility on culture medium. In fact, similar behaviours have already been described in the literature, in which the solubility of the individual components of a DES was improved while in DES form[17,64].

4.4.2. *Antimicrobial activity*

The antimicrobial potential of AXT-rich extracts obtained with DES or Soxhlet extraction, AXT standard, and DES was evaluated on *S. aureus* and *E. coli* bacteria. MIC values represented on Figure 10 show that the behaviour of extracts, AXT standard, and DES was similar for both microorganisms.

All DES were able to inhibit the growth of *S. aureus* and *E. coli*, with MIC ranging from 31.25 µL/mL to 62.50 µL/mL. In fact, the antimicrobial effect of terpenes and fatty acids is well-known on both Gram-positive and Gram-negative bacteria[65–67]. For this reason, these compounds are already used in the cosmetic industry, not only as ingredients[68], but also as natural preservatives[69]. The antimicrobial activity of different DES, mainly based on choline chloride, betaine, organic acids, and sugars, towards Gram-positive or Gram-negative bacteria, has also been reported by several authors[48,61,70,71], being possibly related to bacterial cell wall distortion/disruption[48].

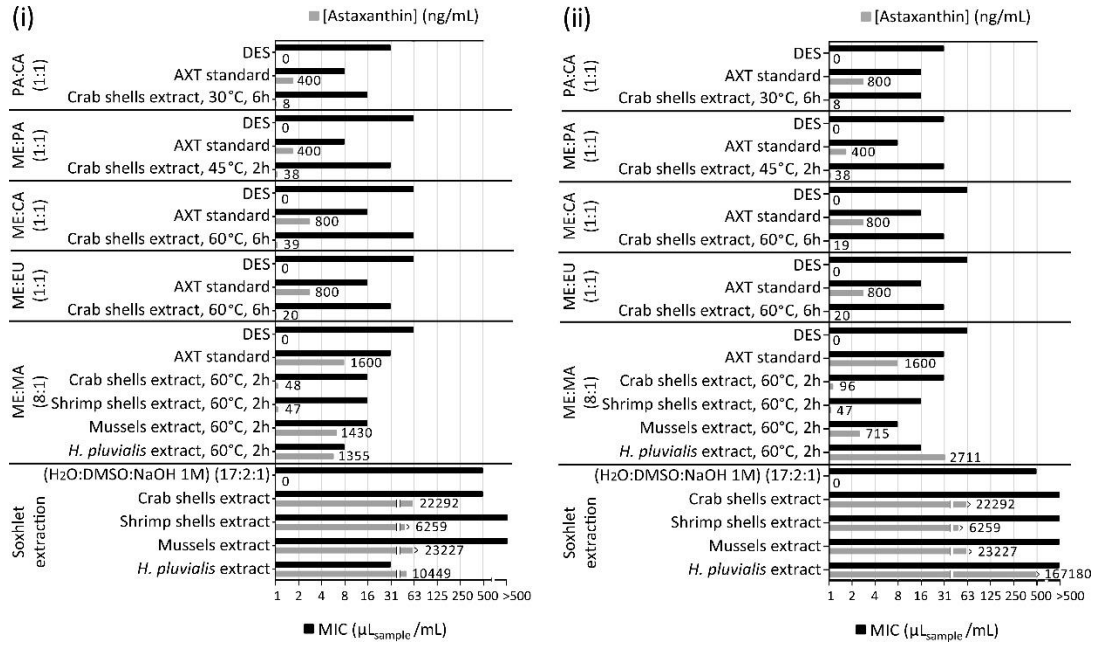

Figure 10. MIC obtained for DES, AXT standard, and AXT-rich extracts tested on the bacterial targets (i) *S. aureus* ATCC 6538 and (ii) *E. coli* ATCC 8739.

AXT is well-known for its antimicrobial and preservative potential[6,7]. In addition, the use of AXT-rich extracts as antibacterial agents has already been reported. In particular, Weeratunge and Perera described the antimicrobial potential of extracts obtained from shrimp shell waste with acetone, ethyl acetate and acetone:ethyl acetate (1:1) against *S. aureus*, *Salmonella typhimurium*, *Bacillus cereus*, and *E. coli*[72].

When solubilized in DES, AXT standard was able to enhance the antimicrobial activity of the solvents, with MIC ranging from 7.81 µL/mL to 31.25 µL/mL (Figure 10). With only one exception (ME:CA (1:1), crab shells extract, 60 °C, 6 h in *S. aureus*), the same behaviour could be found for all crab, shrimp, mussels, and *H. pluvialis* extracts obtained with DES, presenting MIC that ranged from 7.81 µL/mL to 31.25 µL/mL. However, when looking at the results obtained for samples resulting from Soxhlet extraction, all extracts presented MIC equal or higher than 500 µL/mL, which was the maximum concentration tested. The only exception was *H. pluvialis* extract, which presented some inhibitory potential against *S. aureus*, with a MIC of 31.25 µL/mL. The bioactivity of an extract can be strongly influenced by synergistic, additive, and antagonistic effects of its different components[73]. Considering the

results obtained for Soxhlet extracts, it can be hypothesized that there was a lack of selectivity of acetone towards the extraction of antimicrobial ingredients other than AXT.

In most cases, crab shell extracts obtained with DES showed higher MIC than AXT standard, except for the extract obtained with ME:MA (8:1). However, when looking at the AXT contained in each extract (grey bars illustrated on Figure 10) it is possible to conclude that extracts were able to inhibit *S. aureus* and *E. coli* growth with much lower concentrations of AXT. This suggests that the antimicrobial effect measured for DES extracts was not exclusively due to the AXT content, as some of the extracts with a lower AXT concentration presented similar or even higher antimicrobial activity. A synergistic and/or additive antimicrobial effect with other components that were concomitantly extracted from the biomass can be presumed. Additionally, the behaviour presented by DES and AXT-rich extracts obtained with DES suggests that it would be of interest not to separate the extracts from the solvents, in order to obtain a combined bioactivity.

5. Conclusions

This study highlighted the potential of terpene/fatty acid-based DES for the recovery of AXT-rich extracts, not only from crab shells, but also from other biomasses, including shrimp shells, mussels, and *H. pluvialis*.

Results from DSC, FTIR, and NMR experiments helped confirming the successful formation of the systems prepared. Furthermore, the different combinations of HBD and HBA have shown that it was possible to determine a wide range of physical properties, including density, viscosity, and polarity.

When compared to the other DES studied, ME:MA (8:1) could produce extracts from crab shell residues with an AXT yield up to 3-fold higher. Furthermore, when performing an extraction with this same system at 60 °C for 2 h, the AXT yield obtained was similar to the one obtained by a 6 h Soxhlet extraction, using acetone as solvent.

For the other biomasses under study, namely shrimp shells, mussels, and *H. pluvialis*, it was observed that AXT yields using ME:MA (8:1) at 60 °C for 2 h were up to 657-fold higher, than those obtained by Soxhlet extraction. This suggests that ME:MA (8:1) can be a viable alternative to boiling acetone for the efficient extraction of AXT from different biomass wastes.

Terpene/fatty acid-based DES showed to be slightly toxic or practically harmless to wheat, while having potential to inhibit colorectal cancer cells, as well as *S. aureus* and *E. coli* growth. In addition, AXT-rich extracts obtained with DES revealed to have antimicrobial potential towards both Gram-positive and Gram-negative bacteria. Consequently, due to the bioactivity displayed by DES, it would be of interest not to separate the extracts from the solvents in order to obtain a final product with a combined bioactivity.

6. Acknowledgments

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CHAPTER 3

Part II – Subcritical water extraction of protein hydrolysates

Contents

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The author was involved in the conceptualization and design of all experiments and performed all experimental work, except for the evaluation of the cytotoxicity of extracts (performed at iBET by M. Matos) and the determination of the free amino acid profile (acquired at University of Burgos by S. Beltrán and E. T. Andres). Data processing and interpretation, and results discussion were also performed by the author, as well as the preparation of the original manuscript.

1. Abstract

In recent years, a growing number of studies have demonstrated that protein hydrolysates have interesting functional properties and a strong antioxidant capacity, amongst other important bioactivities. In this work, the potential of subcritical water (scW) to efficiently extract bioactive protein hydrolysates contained in brown crab shell residues was evaluated. Aiming at maximizing the extraction of the target molecules, the impact of operating temperature (150, 200, and 250 °C), solid-liquid ratio (1:5, 1:10, and 1:15), and heating rate (3 and 6 °C/min) on scW extraction performance was assessed. Extracts were then evaluated in terms of total protein content, Maillard reaction products formation, free amino acid profile, and antioxidant potential through oxygen radical absorbance capacity assay. Results have shown that, although an increase in operating temperature allowed a significant increase in global extraction yields, there was a slight decrease (approximately 1.4-fold) in extraction selectivity towards proteins. Nevertheless, higher temperatures enabled the production of extracts with a higher antioxidant potential (up to 780 $\mu mol_{trolox\ equivalents}/g_{extract}$), possibly due to an increase of smaller peptides or Maillard reaction products.

2. Introduction

Protein hydrolysates is the designation generally given to the products that result from the hydrolysis of the protein's peptide bonds (C-N bond between the carboxyl and amine groups), leading to the production of peptides with different sizes and free amino acids. As a consequence of this modification in the structure of proteins, their physicochemical and functional properties also undergo modifications[1]. Notably, seafood-derived protein hydrolysates have been pointed to have remarkable functional properties, including good solubility in water, foaming, gelling, emulsifying, and water holding abilities, as well as important bioactive properties such as antioxidant, anticancer, antimicrobial, antihypertensive, immunomodulatory, anti-thrombotic activities, among others[2]. Furthermore, given their lower molecular weight, protein hydrolysates are usually more rapidly digested and absorbed than native proteins[3]. In particular, the proteins that form the skeletal organic matrix of marine calcifiers have been pointed has an unexplored and abundant source of novel proteins that could be of interest to different applications[4].

Therefore, the commercial value of protein hydrolysates has been growing, in particular when applied in infant or sports nutrition, dietary supplements, nutraceuticals, food products, and in the biotechnology industry, representing a market valued at over US$ 541 million (data from 2019) that should reach nearly US$ 800 million by 2026[5].

The production of protein hydrolysates can be performed either by using chemical or biological methods. While strong acid or alkaline hydrolysis are less expensive procedures, they usually require several pre-treatment steps, and the use of high temperatures for long times, leading to difficulties in controlling the process as well as to products with lower nutritional quality and functionality. Therefore, in spite of its higher costs and extraction times, the enzymatic hydrolysis has been frequently used as an alternative, and even as the preferred method, given its milder process conditions and higher specificity[1,6]. As the drawbacks associated to enzyme-based methodologies, mainly related to cost and operating times, have been limiting its implementation at industrial scale, there has been a growing need for alternative cost-effective processing technologies, so that high quality protein hydrolysates can be produced to meet the quality and functionality specifications demanded by the market[6].

Given its versatility, in recent years, environmentally friendly modern extraction technologies using water as solvent have attracted significant attention[7]. While supercritical water has been mostly used to treat municipal organic waste or in the decomposition of harmful substances like polychlorinated biphenyls or sodium sulphate, subcritical water (scW) has been explored for milder hydrolysis reactions, including for example the conversion of cellulose- or protein-rich biomass into valuable products[8]. Under subcritical conditions (attained by simply tuning conditions of temperature and pressure), the dielectric constant of water decreases, which allows it to interact with non-polar substances, while the ionic product increases, thus enabling it to act as an acid or alkaline catalyst[9]. In particular, peptide bonds can be rapidly hydrolysed in a hydrothermal system. Therefore, the potential of hydrothermal technologies, including scW extraction, to recover peptides and amino acids from several protein-rich feedstocks, such as marine wastes[9–11], hair[12], feathers[13], rice bran and soybean meal[14], has already been explored, representing a research topic that has been gaining interest in recent years[8].

Within this context, in the work reported herein, the application of scW was explored for the recovery of bioactive protein hydrolysates from brown crab shell residues. Accordingly, different operating conditions were applied aiming at maximizing the extraction of target compounds, namely temperature, solid-liquid ratio, and heating rate. The resulting extracts were then characterized in terms of total protein content, degree of browning, free amino acid profile, and evaluated with regards to antioxidant potential through oxygen radical absorbance capacity (ORAC) assay.

3. Materials and methods

3.1. Biomass

Brown crab (*Cancer pagurus*) shells were kindly provided by Tejo Ribeirinho, Portugal, in November 2017, and stored at -20 °C upon arrival. The residues were freeze-dried and milled as described on Chapter 2, Part I, section 3.1., and the particle size of the ground material was determined, after which samples were stored, as described on Chapter 3, Part I, section 3.1..

3.2. Subcritical water extractions

ScW extractions were performed in a 1200 mL high pressure reactor (series 4540, Parr Instrument Company, Moline, IL, USA). Briefly, depending on the solid-liquid ratio, a given mass of freeze-dried residue and 500 mL of distilled water were placed on the extraction vessel. Extractions were performed by varying conditions of temperature (150, 200, and 250 °C), solid-liquid ratio (1:5, 1:10, and 1:15 $g_{dry\ residue}/mL_{water}$), and heating rate (3 and 6 °C/min) for a fixed nitrogen (industrial grade, Air Liquide, Algés, Portugal) pressure (approximately 100 bar). Total extraction time varied from 40 to 80 min according to the operating temperature and heating rate (corresponding to the time necessary to reach the extraction temperature plus 15 min at the extraction temperature) (Table 1), and extract samples were taken at each 50 °C increase in temperature. After extraction, the system was depressurized, and the extract separated from the solid residue by vacuum filtration (designated as final extract). Extract aliquots were freeze-dried at -55 °C for approximately 24 h to determine the global extraction yield (expressed as $g_{extract}/100\ g_{dry\ residue}$), while the solids resulting from

scW extractions were dried in an oven at 80 °C for 24 h to determine the hydrolysis extent (expressed as a percentage, Equation 1). Extracts were stored at -20 °C until further analyses.

Table 1. Extraction conditions applied to brown crab shell residues. The operating pressure was maintained at approximately 100 bar in all experiments.

Run	Solid-liquid ratio (g/mL)	Temperature (°C)	Heating rate (°C/min)	Extraction time (min)
scW 1	1:10	150	6	40
scW 2		200		45
scW 3		250		50
scW 4	1:15	200		45
scW 5	1:5			
scW 6	1:10		3	80

$$Hydrolysis\ extent\ (\%) = \frac{Initial\ mass\ of\ feed - Final\ mass\ of\ feed}{Initial\ mass\ of\ feed} \times 100$$ **Equation 1**

3.3. Extract characterization

3.3.1. Lowry method for total protein quantification

Total protein content was determined by UV-vis spectroscopy, using the Folin & Ciocalteu's Phenol Reagent, as previously described on Chapter 2, Part II, section 3.7.1.. Experiments were performed in duplicate and results were expressed as $g_{protein}/100\ g_{extract}$, $g_{protein}/100\ g_{dry\ residue}$, or as $g_{protein}/L_{extract}$.

3.3.2. *Maillard reaction products formation*

As an indication of the extent of Maillard reactions, the absorbance of final scW extracts was evaluated at 294 and 420 nm (representing the intermediate and late stage in a nonenzymatic browning reaction, respectively), as previously described by Ajandouz *et al.*[15] and Hemmler *et al.*[16]. Samples were diluted in distilled water in order to obtain an absorbance reading lower than 1.2.

3.3.3. *Free amino acid profile determination*

Free amino acid profile of scW extracts was determined following the EZfaast™ amino acid analysis kit (Phenomenex, Torrance, CA, USA) specifications[6,17]. Samples were submitted to gas chromatography analysis immediately after derivatization using a gas chromatograph (Hewlett-Packard HP 6890 Series GC System) equipped with a flame ionization detector (GC-

FID), a Zebron ZB-AAA10 m × 0.25 mm capillary column (Phenomenex), and an Agilent Technologies 7683B series automatic injector. The oven temperature program started at 110 °C up to 320 °C, at a rate of 32 °C/min. Helium was used as carrier gas at 1 mL/min. The injector temperature was kept at 250 °C and the detector temperature at 320 °C. Amino acid standards (Sigma Aldrich) were used for method calibration and norvaline was used as internal standard. Arginine cannot be detected by this method of analysis and asparagine and glutamine were quantified as aspartic and glutamic acids, respectively. Results were expressed as $mg_{amino\ acid}/g_{extract}$, $mg_{amino\ acid}/g_{dry\ residue}$, or as relative mass percentage.

3.4. *In vitro* bioactivity evaluation

3.4.1. *Human cell-based assays*

3.4.1.1. Cell culture

Human intestinal Caco-2 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of heat-inactivated foetal bovine serum (FBS), 1% of non-essential amino acids (NEAA), and 1% penicillin-streptomycin (Gibco, Invitrogen, Paisley, UK). Cells were maintained as monolayers as detailed on Chapter 2, Part I, section 3.4.1..

3.4.1.2. Sample preparation

Freeze-dried extracts were prepared in DMEM supplemented with 0.5% of FBS and 1% of NEAA, immediately before the assays. To obtain a range of concentrations, samples were further diluted in DMEM supplemented with 0.5% of FBS and 1% of NEAA.

3.4.1.3. Cytotoxicity assay

Cytotoxicity assays were performed for 24 h using confluent Caco-2 cells, as described in detail on Chapter 2, Part I, section 3.4.3.. Cell viability was evaluated through CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay at 490 nm. Three independent assays were performed in triplicate and the half maximal effective concentrations (EC50) were calculated from dose-response curves. Results were expressed as $mg_{extract}$/mL.

3.4.2. *ORAC assay*

ORAC assay was performed as descried on Chapter 2, Part II, section 3.8.1., using fluorescein sodium salt as probe and 2,2'-azobis(2-methylpropionamidine) dihydrochloride as peroxyl

radical generator. Samples were analysed in duplicate and results were expressed as $\mu mol_{trolox\ equivalents}/g_{extract}$ (whenever possible) or $\mu mol_{trolox\ equivalents}/L_{extract}$.

3.5. Statistical analysis

GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to estimate the statistical significance of average differences by one-way analysis of variance followed by the Tukey test. An alfa error of 5% was accepted in the hypothesis testing to decide for a significant effect. Data was reported as mean ± standard deviation values.

4. Results and discussion

4.1. Protein hydrolysate extractions

As previously mentioned, scW has been successfully applied for the recovery of high value peptides and amino acids from both vegetable and animal biomasses, including shellfish[9–11,18]. Accordingly, in this work, scW was explored for protein hydrolysates extraction from brown crab shell residues.

Being one of the most important parameters in scW extraction[19], as a first approach, the impact of operating temperature (150, 200, and 250 °C) was studied at a constant solid-liquid ratio (1:10) and pressure (100 bar). The extraction time (40, 45, and 50 min) varied according to the target operating temperature. As it would be expected, the global extraction yields significantly increased with increasing temperature (Table 2). However, increasing temperature has also decreased the selectivity of scW extraction towards the recovery of protein hydrolysates (Table 2).

Table 2. Global extraction yields, extracts' protein content, and extent of hydrolysis of scW final extracts. (scW 1) 150 °C, 1:10 g/mL, 6 °C/min; (scW 2) 200 °C, 1:10 g/mL, 6 °C/min; (scW 3) 250 °C, 1:10 g/mL, 6 °C/min; (scW 4) 200 °C, 1:15 g/mL, 6 °C/min; (scW 5) 200 °C, 1:15 g/mL, 6 °C/min; (scW 6) 200 °C, 1:10 g/mL, 3 °C/min. Different lowercase letters represent a statistically significant difference among global extraction yields or extracts' protein content. $P < 0.05$ was accepted as statistically significant in all cases.

Run	Global extraction yield ($g_{extract}$/100 $g_{dry\ residue}$)	Protein content ($g_{protein}$/100 $g_{extract}$)	Hydrolysis extent (%)
scW 1	11.1 ± 0.11 ^{d}	48.6 ± 0.9 ^{a}	13.5
scW 2	14.2 ± 0.48 ^{c}	44.0 ± 1.5 ^{b}	18.6
scW 3	15.5 ± 0.11 ^{ab}	33.7 ± 1.0 ^{d}	19.7
scW 4	10.6 ± 0.42 ^{d}	44.6 ± 1.3 ^{b}	20.5
scW 5	15.8 ± 0.11 ^{a}	38.9 ± 1.1 ^{c}	18.1
scW 6	15.0 ± 0.11 ^{b}	43.2 ± 0.9 ^{b}	16.4

Results presented on Figure 1 suggest that the protein hydrolysates extracted from crab shells could significantly increase with increasing temperature, being this increment more pronounced between 150 and 200 °C. For extracts scW 1 and scW 2 (Figures 1i and ii), holding the final operating temperature for 15 min at the end of extraction has also shown to have to have a significant positive impact on yields. However, this yield was reduced for extract scW 3 (Figure 1iii), when maintaining 250 °C for 15 min. Conversely, as summarized on Table 2, the extent of hydrolysis increased with increasing temperature (1.4-fold from 150 to 200 °C, and 1.1-fold from 200 to 250 °C), which is in accordance with the increase of free amino acids content in these extracts (Table 3 and Appendix D, Tables D1 – D2). In fact, the dielectric constant of water is known to decrease with increasing temperature while the ionic product increases, thus facilitating the hydrolysis of complex matrixes[20,21]. Therefore, the decrease that was noticed in the total protein yield of extract scW 3 might be related to the method used for protein quantification and to an intensified hydrolysis. Although it is known that most di- and polypeptides can be detected by the Lowry method, free amino acids do not produce a coloured product with the Lowry reagent and cannot, therefore, be quantified by this method[22,23]. This conclusion can be further supported by the pronounced increase of free amino acids yield in scW 3 extract (Appendix D, Table D1).

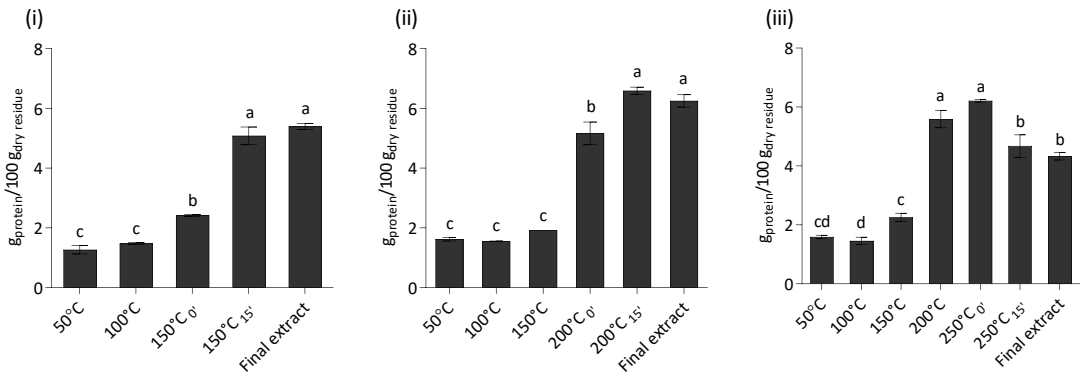

Figure 1. Total protein yields obtained from scW extraction of brown crab shell residues, determined by the modified Lowry method[24]. Extractions were performed at a final operating temperature of (i) 150 °C (scW 1), (ii) 200 °C (scW 2), and (iii) 250 °C (scW 3), at a solid-liquid ratio of 1:10 and a heating rate of 6°C/min. Extract samples were taken at each 50 °C increase in temperature. For each extraction, different lowercase letters represent a statistically significant difference between temperatures. $P < 0.05$ was accepted as statistically significant in all cases.

Table 3. Free amino acids content ($mg_{amino\ acid}/g_{extract}$) of scW final extracts quantified by GC-FID. (scW 1) 150 °C, 1:10 g/mL, 6 °C/min; (scW 2) 200 °C, 1:10 g/mL, 6 °C/min; (scW 3) 250 °C, 1:10 g/mL, 6 °C/min; (scW 4) 200 °C, 1:15 g/mL, 6 °C/min; (scW 5) 200 °C, 1:15 g/mL, 6 °C/min; (scW 6) 200 °C, 1:10 g/mL, 3 °C/min.

Amino acid	**scW 1**	**scW 2**	**scW 3**	**scW 4**	**scW 5**	**scW 6**
Alanine (Ala)	10.3	10.7	23.5	10.1	10.9	10.6
Aspartic acid (Asp)	1.8	3.6	2.3	4.6	2.7	3.6
Cysteine (Cys)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glutamic acid (Glu)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycine (Gly)	33.6	24.5	25.9	21.5	22.5	24.6
Histidine (His)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isoleucine (Ile)	3.5	3.1	4.4	3.2	2.7	3.2
Leucine (Leu)	2.9	3.6	7.9	3.9	3.0	3.8
Lysine (Lys)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methionine (Met)	1.0	1.3	2.4	1.5	1.2	1.5
Phenylalanine (Phe)	0.6	3.0	6.4	3.1	2.6	3.2
Proline (Pro)	5.0	5.7	8.9	5.1	6.0	5.9
Serine (Ser)	1.6	2.4	2.1	3.0	2.0	2.7
Threonine (Thr)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tryptophan (Trp)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyrosine (Tyr)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine (Val)	5.2	5.6	14.8	6.0	5.0	5.6
Total	65.4	63.5	98.4	62.1	58.5	64.7

n.d.: not detected
Coefficient of variation ≤ 2.9%

It is also interesting to note that extracts were enriched in more hydrophobic amino acids, such as Ala, Leu, Met, Phe, or Val[25] as temperature increased to 250 °C and the dielectric constant of scW decreased[26] (Table 3 and Appendix D, Table D2).

The solid-liquid ratio is another important parameter that affects scW extraction efficiency and recovery[19]. Figure 2 shows the results obtained at different solid-liquid ratios, at a final operating temperature of 200 °C. The extract scW 4, obtained at 1:15 g/mL (Figure 2i), revealed to be the most interesting regarding total protein content at 200 °C. As previously discussed by other authors, reducing the solid-liquid ratio often favours the extraction of the target molecules, and in particular proteins and amino acids[14], by increasing the concentration gradient as well as the diffusion rate of the solutes into the solvent[27]. Therefore, the residue was probably more prone to aggressive hydrolysis, which can be further supported by the higher hydrolysis extent obtained for scW 4 extract (Table 2). Another possible explanation is the fact that particle aggregation can occur at higher solid-liquid ratios, therefore impairing mass transfer. However, once 200 °C were reached, there was a significant decrease in protein yield for extract scW 4, which might have been caused

by decomposition of free amino acids into organic acids and ultimately volatile compounds and water[6], promoted by a lower solid-liquid ratio and a more severe hydrolysis. Possibly, as a consequence of this degradation, the scW extraction performed at the lowest solid-liquid ratio (scW 4) resulted in a significant decrease on the global extraction yield (Table 2), as well as in a decrease of free amino acids yield, when compared to extractions performed at intermediate (scW 2) or higher (scW 5) solid-liquid ratios (Appendix D, Table D1). Furthermore, even though scW 4 protein yield was higher at 200 °C than scW 2 or scW 5 extracts, it is worth considering that the concentration of protein hydrolysates in the extracts was lower (Figure 2, light grey dots), which might involve higher costs, given the need for extract concentration. It is also important to highlight that the protein yield of the final extract resulting from scW 4 greatly decreased when compared to the sample obtained after holding the temperature at 200 °C for 15 min, or to scW 2 and scW 5 extracts, possibly due to a continuous hydrolysis during the reactor cooling, as a result of its lower solid-liquid ratio. Therefore, to obtain an extract with higher quality, it would be essential to carefully monitor the reactor's cooling velocity (which required around 1 h, on average), aiming at stopping hydrolysis, and consequent amino acid degradation, at the earliest opportunity.

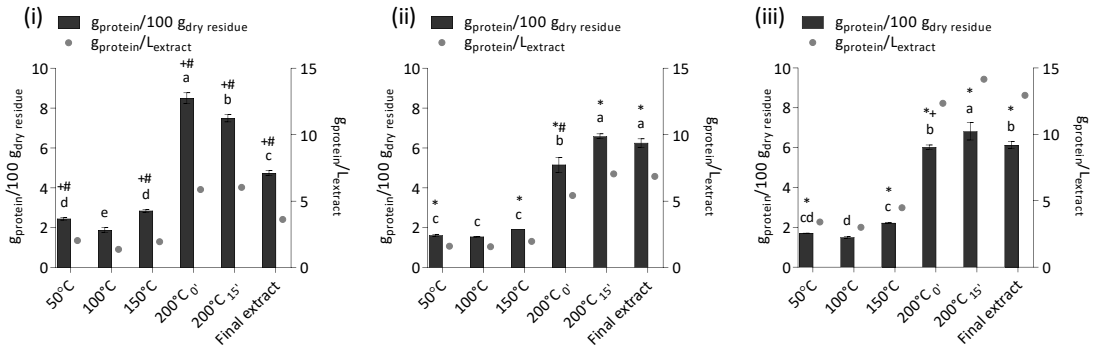

Figure 2. Total protein yields obtained from scW extraction of brown crab shell residues, determined by the modified Lowry method[24]. Extractions were performed at a solid-liquid ratio of (i) 1:15 (scW 4), (ii) 1:10 (scW 2), and (iii) 1:5 (scW 5), at a final operating temperature of 200 °C and a heating rate of 6°C/min. Extract samples were taken at each 50 °C increase in temperature. For each extraction, different lowercase letters represent a statistically significant difference between temperatures. For each temperature, different symbols represent a statistically significant difference between solid-liquid ratios: (*) represents a statistically significant difference with 1:15 ratio, (+) with 1:10 ratio, and (#) with 1:5 ratio. $P < 0.05$ was accepted as statistically significant in all cases.

Figure 3 summarizes the results obtained when comparing two different heating rates (3 and 6 °C/min). Although there was a tendency for a significant increase in protein yield as

temperature increased (from 100 to 200 °C) at 3 °C/min, as opposed to 6 °C/min, the final extracts resulting from both heating rates did not show statistically significant differences. It is also important to note that, as a consequence of a slower heating rate, the extraction time roughly doubled for extraction scW 6, which might have contributed for the significant increase of total protein until 200 °C were reached, as well as to a slight increase in the global extraction yield (Table 2). Nevertheless, the different heating rate conditions applied did not seem to have an impact on the free amino acid profile, which showed to be similar for both extracts (Table 3).

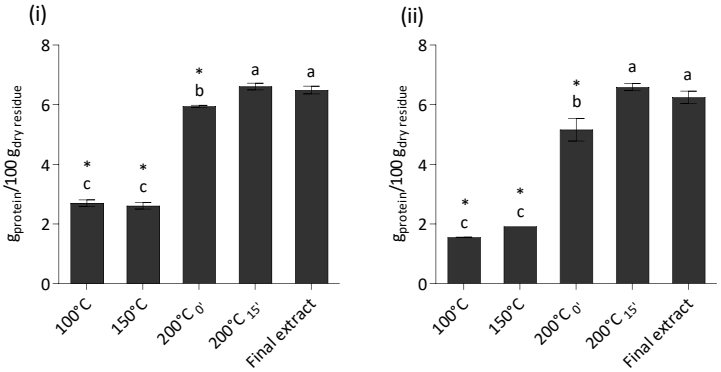

Figure 3. Total protein yields obtained from scW extraction of brown crab shell residues, determined by the modified Lowry method[24]. Extractions were performed at a heating rate of (i) 3 °C/min (scW 6), and (ii) 6 °C/min (scW 2), at a final operating temperature of 200 °C and solid-liquid ratio of 1:10. Extract samples were taken at each 50 °C increase in temperature. For each extraction, different lowercase letters represent a statistically significant difference between temperatures. For each temperature, the statistically significant differences between heating rates were represented by an asterisk (*). $P < 0.05$ was accepted as statistically significant in all cases.

According to the work of Pires *et al.*, in which brown crab (*C. pagurus*) shells were chemically characterized, the protein content of the shell biomass, determined after a conventional hydrolysis using hydrochloric acid and sodium hydroxide (aiming at demineralising and deproteinising the shells, respectively), could vary between 13 and 18%, depending on the crab gender and capture site[28]. With this in mind, it is possible to conclude that the scW processes developed in this work could extract up to 65% (at 200 °C, 1:15 g/mL, and 6° C/min) of all the protein contained in the shell residues.

4.2. Maillard reaction products determination

During heating, sugars and amino acids often undergo a series of subsequent and parallel complex reactions (Maillard reactions) leading to the formation of nitrogen-containing brown polymers[29,30]. Some of the products of such reactions (Maillard reaction products), including acrylamide, heterocyclic amines, 5-hydroxymethylfurfural, or advanced glycation end products, have been pointed as potentially toxic or carcinogenic[31,32]. However, there are other Maillard reaction products, such as melanoidins, which have been reported to display numerous bioactivities, including antioxidant, antibacterial, antihypertensive, anti-inflammatory, bifidogenic properties, among others, some of which have already been confirmed using *in vivo* animal and human clinical trials[31,33].

A simple approach to evaluate the progress of the different stages of these reactions is to measure the degree of browning. In the intermediate stage, Maillard reactions lead to chromophores that absorb at 294 nm, while reaction products of the final stage absorb light at a wavelength of 420 nm[16].

Figure 4 shows the degree of browning of scW extracts. As it would be expected, results presented on Figure 4 show that the polymerization reactions and the degree of browning in scW extracts (obtained at a fixed solid-liquid ratio) were favoured by temperature[6], *i.e.*, absorbances significantly increased with increasing temperature at both 294 and 420 nm (Figures 4i and ii, respectively), for extracts scW 1, scW 2, and scW 3. It is also possible to conclude that the heating rate did not have an influence on the extracts' browning degree, as the sample obtained at 3 °C/min (scW 6), presented similar results to the sample obtained at 6 °C/min (scW 2), thus correlating with the global extraction yield, and with the protein and amino acid contents (Tables 1 and 2). On the other hand, extracts scW 4 (obtained at a solid-liquid ratio of 1:15) and scW 5 (obtained at a solid-liquid ratio of 1:5) presented very different results when compared to scW 2 extract (obtained at a solid-liquid ratio of 1:10), possibly reflecting the concentration of Maillard reaction products that varied with the solid-liquid ratio applied.

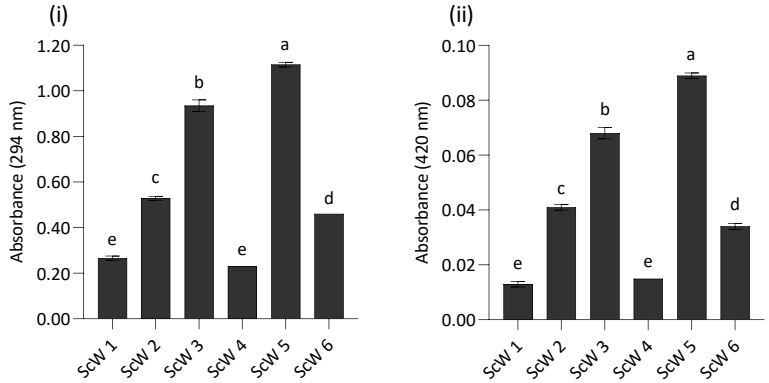

Figure 4. Formation of UV absorbing products in scW final extracts measured at (i) 294 nm and (ii) 420 nm. (scW 1) 150 °C, 1:10 g/mL, 6 °C/min; (scW 2) 200 °C, 1:10 g/mL, 6 °C/min; (scW 3) 250 °C, 1:10 g/mL, 6 °C/min; (scW 4) 200 °C, 1:15 g/mL, 6 °C/min; (scW 5) 200 °C, 1:15 g/mL, 6 °C/min; (scW 6) 200 °C, 1:10 g/mL, 3 °C/min. For each wavelength, different lowercase letters represent a statistically significant difference between extracts. $P < 0.05$ was accepted as statistically significant in all cases.

4.3. *In vitro* bioactivity evaluation

4.3.1. *Antioxidant activity*

The potential of scW extracts to scavenge peroxyl radicals was determined by ORAC assay, which has been widely used in the evaluation of the antioxidant capacity of different nutraceutical, pharmaceutical, and food products[34,35].

Figure 5i shows that all extracts exhibited peroxyl radical scavenging potential, having this potential significantly increased with increasing temperature (from 150 to 250 °C), which can also be confirmed by the results presented on Figure 5ii for extract scW 3 at different temperature points. Importantly, all extracts revealed to be low-cytotoxic when tested on a human intestinal cell line (EC50 > 13 mg/mL), except for extract scW 3, which revealed to be slightly more toxic, with an EC50 value of 4.8 mg/mL (Appendix E, Table E1).

As previously mentioned, melanoidins, *i.e.*, brown-coloured compounds that are formed through the Maillard reaction of sugars and amino acids at high temperatures, and other Maillard reaction products are known to be antioxidants, which may act through radical scavenging[29,30,36]. In fact, results illustrated on Figures 4 and 5i (light grey bars) show a significant positive correlation between the degree of browning and the antioxidant potential of each extract ($R^2 \geq 0.96$, $P \leq 0.0006$), *i.e.*, the antioxidant activity was modulated by the concentration of Maillard reaction products formed during scW extraction.

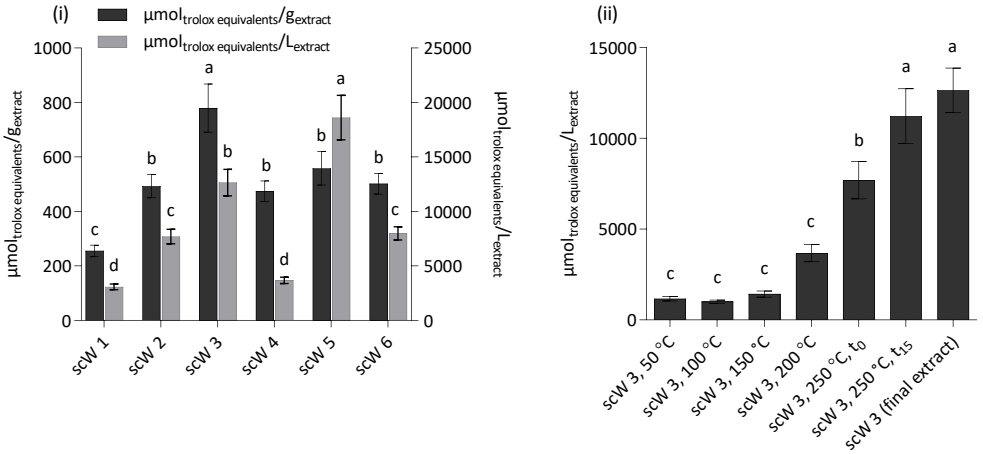

Figure 5. Variation of the antioxidant activity (evaluated through ORAC assay) of (i) scW final extracts obtained under different temperature, solid-liquid ratio, and heating rate conditions; (ii) extract scW 3 with temperature. (scW 1) 150 °C, 1:10 g/mL, 6 °C/min; (scW 2) 200 °C, 1:10 g/mL, 6 °C/min; (scW 3) 250 °C, 1:10 g/mL, 6 °C/min; (scW 4) 200 °C, 1:15 g/mL, 6 °C/min; (scW 5) 200 °C, 1:15 g/mL, 6 °C/min; (scW 6) 200 °C, 1:10 g/mL, 3 °C/min. Different lowercase letters represent a statistically significant difference between extracts (in (i)) or temperatures (in (ii)). $P < 0.05$ was accepted as statistically significant in all cases.

The ability of proteins to interact with free radicals has also been reported in different systems. Although amino acids are unlikely to possess a strong free radical scavenging activity when the protein is in its native state, there are methods that can be applied to increase a protein's overall antioxidant activity, such as its partial denaturation by increasing temperature or its hydrolysis to obtain smaller bioactive peptides or amino acids[36]. The antioxidant potential of protein hydrolysates is therefore dependent on the amino acid composition and on the disruption of the tertiary structure of proteins' native structures[37], and is possibly related to complex interactions between their ability to inactivate reactive oxygen species, scavenge free radicals, chelate prooxidative transition metals, reduce hydroperoxides, enzymatically eliminate specific oxidants, or alter the physical properties of food systems[36,38]. In fact, it is possible that scW extracts that resulted from more severe temperature conditions were richer in partially denatured or hydrolysed proteins, and consequently presented a higher peroxyl radical scavenging potential.

It is also important to note that, as it would be expected, given their identical protein and free amino acid contents (Tables 1 and 2, respectively), extracts scW 4, scW 5, and scW 6, obtained at different solid-liquid ratios or heating rates, presented a similar peroxyl radical

scavenging potential when compared to extract scW 2 (Figure 5i, dark grey bars), thus suggesting that the antioxidant effect of each extract was mainly governed by temperature.

5. Conclusions

The work reported herein highlights the potential of scW as an alternative extraction solvent, providing a single-step and swift process for the isolation of bioactive protein hydrolysates from brown crab shell biomass. Among all extraction parameters tested, temperature revealed to be the most crucial parameter to improve the extraction yield. The highest global extraction yield was obtained at 250 °C (scW 3 extract), as well as the highest free amino acid content, although the extraction selectivity towards protein hydrolysates slightly decreased with increasing temperature. The solid-liquid ratio applied has also revealed to be an important factor to consider, since a decrease in free amino acid and global extraction yields was apparent at a lower solid-liquid ratio, possibly due to the decomposition of free amino acids into organic acids and ultimately volatile compounds and water. All protein-rich extracts revealed to be good peroxyl radical scavengers, significantly increasing their antioxidant potential with increasing temperature, being the highest obtained at 250 °C (scW 3). This bioactive effect might have been due to an enrichment of the extracts in partially denatured or hydrolysed proteins and in Maillard reaction products, which are known antioxidants.

6. Acknowledgments

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CHAPTER 3

Part III – Deep eutectic system recovery of chitin

Contents

Adapted from:

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The DES phytotoxicity results were obtained during a visit to the Laboratory for Cell Culture Technology and Biotransformations, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia, within the scope of COST Action GREENERING (CA18224), under the supervision of Prof. I. R. Redovniković.

The author was involved in the conceptualization and design of all experiments and performed all experimental work, except for the characterization of chitin by FTIR (acquired at iBET by A. Ferreira), XRD (acquired at FCT NOVA by N. Costa), and SEM (acquired at IST-UL by I. Nogueira). Data processing and interpretation, and results discussion were also performed by the author, as well as the preparation of the original manuscript.

1. Abstract

The versatility of chitin and its derivatives has allowed their utilization in a wide range of applications, from wastewater treatment to pharmaceutical or biomedical industries. In the last part of this chapter, the potential of choline chloride (ChCl)/organic acid-based deep eutectic systems (DES) as alternative low-phytotoxic solvents was evaluated for the efficient recovery of chitin contained in brown crab shell processing wastes. Aiming at determining the phytotoxicity of DES, the systems were tested on wheat seeds, while measuring different growth parameters and stress biomarkers. DES were then explored for the recovery of chitin contained in the shell residues, by varying conditions of operating temperature (50, 80, and 130 °C) and processing time (2.5, 3, and 4 h), with and without the addition of water. The obtained chitin was characterized through different analytical techniques, including thermogravimetric analysis, Fourier transform infrared spectroscopy, X-ray diffraction, and scanning electron microscopy, and compared to a standard as well as to chitin obtained by a conventional acid/alkaline hydrolysis. Results showed that by applying a ChCl/lactic acid-based DES (which was the system that showed the least phytotoxic effects on wheat) at 130 °C, it was possible to obtain pure chitin (up to 98%) with characteristics similar to those presented by commercial chitin or chitin recovered by conventional hydrolysis. The proposed process was less time-consuming (more than 8-fold faster) and resulted in higher chitin recovery (95% compared to the 84% obtained with the conventional method), thus suggesting that ChCl/organic acid-based DES can truly represent a low-phytotoxic alternative extraction media for the recovery of chitin from crab shell biomass.

2. Introduction

Chitin, is a linear polysaccharide that plays a supportive and protective role in different living organisms[1,2]. In particular, chitin is one of the main constituents of crustaceans exoskeleton, a complex structure that provides the necessary mechanical strength to protect the soft body of crustaceans, and is comprised of three layers: an inner layer formed by chitin and proteins, a middle layer composed of chitin and minerals, and an upper layer consisting of calcium carbonate and proteins.[3]

Due to their stabilizing and emulsifying properties, chitin and its derivatives have been used in the food industry to improve food safety, quality, and shelf-life[2,4]. Additionally, owing to

properties such as low toxicity, biocompatibility and biodegradability, chitin and chitosan have also been used as excipients and as biological active agents in the cosmetic industry; or as biomaterials in the pharmaceutical and biomedical industries, including in bone and cartilage regeneration, wound healing and dressings, contact lenses, drug delivery systems, among others[4–6].

The demand for this naturally occurring polymer and its derivatives has recently increased, triggering a swift market growth, at a compound annual growth rate of 15.4% (from 2016 to 2021)[7].

In many countries, shellfish waste is already being used as a feedstock to produce chitin, chitosan, and glucosamine sulphate for a wide range of applications, especially in the biomedical field. However, due to the extensive covalent and hydrogen bonding between the different components of shells, an effective chitin isolation can be a challenge.[8]

The most commonly used method for obtaining pure and colourless chitin from crustacean shell biomass involves the disposal of large quantities of highly concentrated toxic chemicals into the aquatic ecosystem, including strong acids and bases, and does not allow the recovery of co-products, such as proteins or minerals[9]. Traditionally, this methodology involves a first demineralisation step with a strong acid at temperatures up to 100 °C and up to 2 days of reaction time, followed by deproteination with a strong base at temperatures up to 100 °C and up to 3 days of reaction time, and subsequent decolouration through oxidative bleaching[8,10]. Although there have been a lot of recent developments in the search for a more environmentally benign process for chitin extraction, namely biological methods, including enzymatic deproteination and fermentation using microorganisms, none of the methodologies proposed so far is competitive at commercial scales[6,8,10]. Therefore, there is a pressing need for the development of new technologies that allow a safer and cheaper chitin recovery.

Deep eutectic systems (DES) have emerged in the last years as viable alternatives to conventional solvents, due to their remarkable solubilizing power towards very different molecules, including lipid- or poorly water-soluble compounds[11,12]. In particular, their application in the fractionation of shrimp and lobster shells and in the recovery of chitin has already shown promising results[3,13–16].

In this work, low-phytotoxic DES based on choline chloride (ChCl) and different organic acids, namely malonic, DL-malic, and DL-lactic acids, were applied for the recovery of chitin from brown crab shell residues, a feedstock that, to the best of the author's knowledge, remains unexplored in the existing literature. Accordingly, before being applied in extraction experiments, the phytotoxicity of the DES prepared was assessed on wheat (*Triticum aestivum*) seeds, by evaluating their impact on seedlings growth (inhibition of germination and shoots height), lipid peroxidation, photosynthetic pigments content, and antioxidant enzymes activity. After determining their phytotoxicity, and aiming at maximizing the removal of minerals and proteins from the shells to yield pure chitin, ChCl/organic acid-based DES were tested at different operating conditions, namely temperature and processing time, with and without the addition of water. The structure and properties of the obtained chitin samples were determined by different analytical techniques and were then compared to a standard, as well as to chitin recovered from crab biomass through a conventional acid/alkaline hydrolysis.

3. Materials and methods

3.1. Biomass

Brown crab (*Cancer pagurus*) shells were kindly provided by Tejo Ribeirinho, Portugal, and processed and stored according to what was previously described on Chapter 2, Part I, section 3.1. (freeze-drying and milling), and Chapter 3, Part I, section 3.1. (determination of the particle size of the ground material and conditions of storage).

3.2. Biomass composition analysis

Total ash content in freeze-dried brown crab shells was determined as described in Norma Portuguesa NP2032 [17]. Briefly, crucibles containing a given amount of dried residue were placed in a muffle furnace at 550 °C for 6 h. Total mineral content was calculated through mass differences and results were expressed as $g_{minerals}/100\ g_{dry\ residue}$.

Total chitin content was determined as previously reported by Bradić *et al.*[3]. Briefly, 50 mL of 1 M hydrochloric acid (≥ 37%, Sigma-Aldrich, Austria) were added to 0.4 g of freeze-dried residue and heated to 105°C. After 1 h incubation, the mixture was filtered under vacuum and the residue was washed with distilled water until attaining a neutral pH. The remaining

solid was further extracted with 100 mL of a 5% sodium hydroxide (Acros Organics, Sweden) solution and heated to 105°C. After 1 h incubation, the mixture was filtrated under vacuum and the residue was washed with distilled water until achieving a neutral pH. The remaining solid was further washed with 30 mL of acetone (Fisher Chemical, Loughborough, UK). The resulting solid samples were dried in an oven at 110°C until achieving a constant weight, after which were incinerated in a muffle furnace at 600°C for 6 h. Total chitin content was calculated through the mass loss during the incineration process, and results were expressed as $g_{chitin}/100\ g_{dry\ residue}$.

Total protein content (which also included other minor compounds, such as fatty acids, pigments, etc.) was determined by mass balance, considering the results obtained for mineral and chitin, as previously described by Pires *et al.*[18]. Results were expressed as $g_{protein}/100\ g_{dry\ residue}$.

All experiments were performed in duplicates.

3.3. DES preparation

ChCl (ref. C7527) and DL-malic acid (MiA, ref. 240176) from Sigma-Aldrich (China), malonic acid (MoA, ref. A11526) from Alfa Aesar (Kandel, Germany), and DL-lactic acid (LA, ref. 125065000) from Acros Organics (USA) were used for DES preparation. Systems were prepared by heating the mixture of the two components to 80 °C in the case of ChCl:MoA and ChCl:LA, and 90 °C in the case of ChCl:MiA, under constant stirring, until a clear liquid was formed.

3.4. DES *in vitro* phytotoxicity determination

3.4.1. *Sample preparation*

DES stock solutions were prepared in distilled water immediately before the phytotoxicity assay. Samples were then two-fold serially diluted in distilled water to obtain a range of concentrations (0 – 20 mg_{DES}/mL).

3.4.2. *Phytotoxicity assay*

DES phytotoxicity was evaluated according to what was described in detail on Chapter 2, Part II, section 3.5.2.. Briefly, petri dishes were prepared with filter paper, cotton wool, and wheat (*T. aestivum*) seeds, which were then moistened with different concentrations of DES.

Seedlings were grown for 7 days and then harvested. The impact of DES on germination and early growth of wheat was determined and the half maximal effective concentrations (EC50) were calculated from dose-response curves. All experiments were performed in duplicates and results were expressed as mg_{DES}/mL.

3.4.3. *Lipid peroxidation (LPO) measurement*

LPO levels were evaluated on wheat leaves by measuring the malondialdehyde (MDA) content, after extraction of wheat leaves with 0.1% trichloroacetic acid and subsequent reaction with thiobarbituric acid, as previously described (Chapter 2, Part II, section 3.5.3.). Absorbances were read at 532 nm (subtracting the unspecific turbidity at 600 nm), as a mean of two independent measurements. Results were expressed as $nmol_{MDA}/g_{fresh\ leaves}$.

3.4.4. *Chlorophyll (CHL) content determination*

CHL was determined on wheat leaves as described on Chapter 2, Part II, section 3.5.4., by measuring the absorbances at 663 nm and 645 nm of the supernatants resulting from an extraction of the leaves with 80% acetone. Results were expressed as $\mu g_{CHL}/g_{fresh\ leaves}$.

3.4.5. *Antioxidant enzyme activity determination*

Antioxidant enzyme analysis was performed as previously described (Chapter 2, Part II, section 3.5.5.). Supernatants resulting from an extraction of wheat leaves with a potassium phosphate buffer solution were subjected to different specific reactions and superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX) activities were determined immediately after by UV-vis. Experiments were performed at least in duplicates and results were expressed as $units_{SOD}/mg_{protein}$ for SOD, $nmol_{oxidized\ guaiacol}/(mg_{protein}\cdot min)$ for GPX, $nmol_{decomposed\ H2O2}/(mg_{protein}\cdot min)$ for CAT, and $nmol_{oxidized\ ascorbate}/(mg_{protein}\cdot min)$ for APX.

3.5. Solid-liquid extractions

3.5.1. *Conventional recovery of chitin*

Conventional hydrolysis of chitin was performed according to Al Sagheer *et al.* following three sequential steps: demineralisation with strong acid, deproteination with strong base, and decolouration with ethanol and acetone[19]. Briefly, demineralisation was carried out with

0.25 M hydrochloric acid at a solid-liquid ratio of 1:40 g/mL for 90 min at room temperature. The acid was removed by decantation and this procedure was repeated 3 times. The resulting solids were washed with distilled water until neutral pH was attained and dried at 70 °C for 20 h before being used in deproteination experiments. Deproteination was performed using 1 M sodium hydroxide at a solid-liquid ratio of 1:20 g/mL for 30 min at 70 °C. The treatment was repeated 3 times, being the last treatment left overnight. The sodium hydroxide solution was removed by decantation and the resulting solids were washed to neutrality. To remove any impurities, such as pigments, the solids were washed with ethanol (Carlo Erba, Val de Reuil, France) at 70 °C and a solid-liquid ratio of 1:10 g/mL. After removing the ethanol, the remaining solids were boiled in acetone. The purified chitin was dried at 70 °C for 24 h, and then stored at room temperature, in a low-moisture environment, until further analyses. Mineral, protein, and chitin contents were determined through mass differences throughout the different extraction steps.

3.5.2. *Recovery of chitin using DES*

The recovery of chitin using DES was performed as previously described by Saravana *et al.*[15], with slight modifications. As a first approach, 12.5 g of DES were added to 0.5 g of freeze-dried residue. Extractions were carried out in silicon baths for 4 h at 50, 80, and 130 °C, under constant stirring (ca. 60 rpm). The resulting extracts were filtrated under vacuum and the solids were washed with distilled water until a neutral pH was attained. The solids were recovered and dried overnight at 70 °C.

A second set of extractions were conducted for 2 h, while maintaining the solid-liquid ratio, temperatures, and stirring rate as mentioned above. Distilled water was then added to the resulting extracts at a solid-liquid ratio of 1:25 g_{feed}/mL_{H2O}, and samples were stirred until reaching room temperature (approximately 30 min). The supernatant was filtrated under vacuum and the solids were washed with distilled water, allowing 5 min of contact time, with occasional manual stirring. This procedure was repeated twice. Samples were then filtrated under vacuum and the solids were repeatedly washed with distilled water until reaching a neutral pH. The solids were recovered, dried overnight at 70 °C, and the most promising samples were subjected to decolouration with hydrogen peroxide (30%, Carlo Erba, Val de Reuil, France), at a solid-liquid ratio of 1:10 g_{solids}/mL_{H2O2} and 80 °C for 30 min,

with manual agitation every 10 min. The resulting solids were washed with distilled water and dried in an oven for 24 h at 70 °C. Figure 1 schematically illustrates the process.

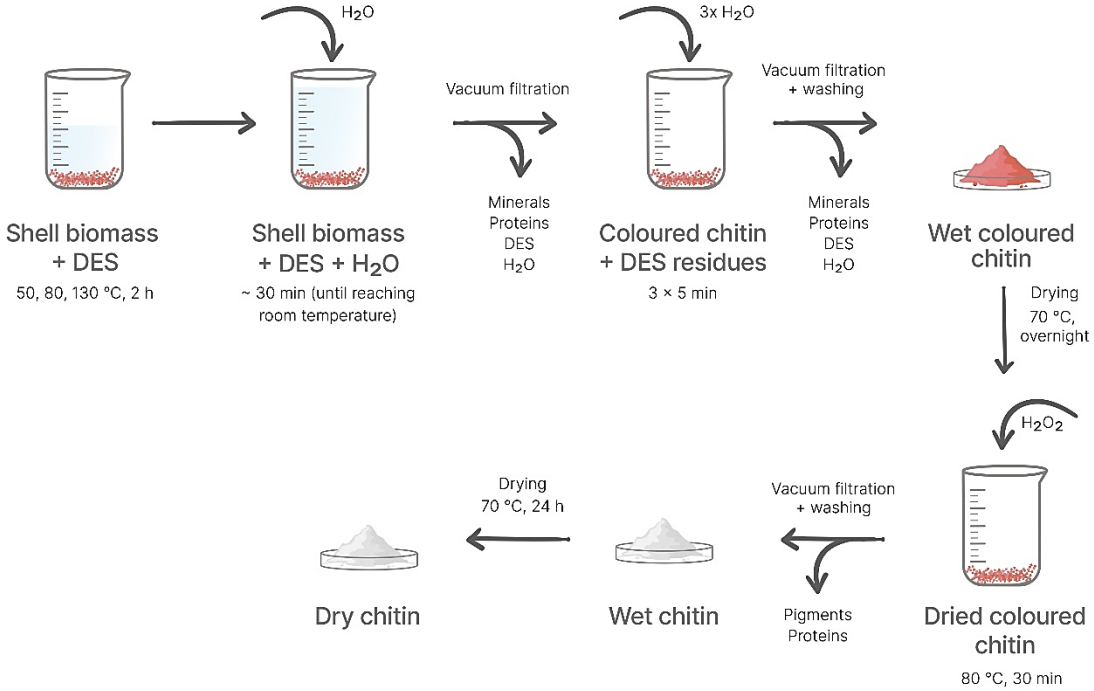

Figure 1. Schematic illustration of the second set of experiments for chitin recovery with DES.

Dried samples were stored at room temperature, in a low moisture environment, until further analysis. The hydrolysis extent was calculated as previously described (Chapter 3, Part II, section 3.2.). All experiments were performed at least in duplicate.

3.6. Chitin characterization

The ash content of the dried solids was measured using a muffle furnace at 550 °C for 5 h, while the protein content was determined through mass balances. Demineralisation and deproteination efficiencies were calculated according to Equations 1 and 2, respectively.

$$Demineralisation\ efficiency\ (\%) = \frac{Inicial\ mass\ of\ minerals - Final\ mass\ of\ minerals}{Inicial\ mass\ of\ minerals} \times 100 \quad \textbf{Equation 1}$$

$$Deproteination\ efficiency\ (\%) = \frac{Inicial\ mass\ of\ proteins - Final\ mass\ of\ proteins}{Inicial\ mass\ of\ proteins} \times 100 \quad \textbf{Equation 2}$$

The purity of the obtained chitin was calculated according to Equation 3.

$$Chitin\ purity\ (\%) = \frac{Final\ mass\ of\ chitin}{Final\ mass\ of\ feed} \times 100$$ **Equation 3**

Thermogravimetric analysis (TGA) was performed using a Q50 thermogravimetric analyser (TA Instruments, New Castle, DE) from 35 to 600 °C, at 10 °C/min, under a nitrogen atmosphere.

Fourier transform infrared-attenuated total reflection (FTIR-ATR) analyses were carried out using a Thermo Scientific FTIR spectrometer (Class 1 Laser Product Nicolet 6100, San Jose, CA). The equipment included ATR accessories with a diamond crystal of 42°. Spectra were recorded at room temperature between 4000 and 650 cm^{-1}. A background spectrum was recorded before acquisition and used as reference. The final spectrum corresponds to the average of 32 individual scans, obtained with a resolution of 4 cm^{-1}. The degree of acetylation of chitin samples was determined as previously described by Kasaai, using the absorbance obtained at 1560 cm^{-1} as the intensity of a probe band and the absorbance obtained at 1160 cm^{-1} as the intensity of a reference band[20].

Powder X-ray diffraction (XRD) spectra were recorded on a Miniflex II XRD (Rigaku, Tokyo, Japan) operated at 30 kV and 15 mA, with Cu/Kα as radiation source, in the 2θ range of 5 to 90°. Spectra were recorded at room temperature, at a scanning rate of 5°/min. The crystallinity indexes (*CrI*) were calculated as suggested by Segal *et al.*[21], according to Equation 4.

$$CrI\ (\%) = \frac{I_{110} - I_{am}}{I_{110}} \times 100$$ **Equation 4**

where I_{110} is the maximum intensity of the diffraction peak at 2θ ≈ 19° and I_{am} is the intensity of amorphous diffraction at 2θ ≈ 16 °.

The surface morphology of crab shells and chitin samples was examined under a scanning electron microscope (SEM, S2400, Hitachi High Technologies, Tokyo, Japan), operated at an acceleration voltage of 20 kV. Prior to imaging, samples were prepared on metal stubs, using an electrically conductive double-sided adhesive tape and then coated with a gold/palladium thin film using a sputter coater (Quorum Technologies, QT150T ES, Lewes, UK).

All characterization experiments were also performed on chitin from shrimp shells (chitin STD, coarse flakes, 98.0% acetylated, Sigma-Aldrich, Iceland) for comparison purposes.

3.7. Statistical analysis

The estimation of phytotoxicity, as well as the statistical significance of average differences determination was performed using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance of average differences was assessed by one-way analysis of variance followed by the Tukey test. An alfa error of 5% was accepted in the hypothesis testing to decide for a significant effect. Data was reported as mean ± standard deviation values.

4. Results and discussion

4.1. DES preparation and *in vitro* phytotoxicity

DES based on ChCl and different organic acids have already shown potential for the isolation of different polysaccharides, including chitin[3,13–16,22]. Therefore, in the work reported herein, ChCl was selected as hydrogen bond acceptor (HBA), while MoA, MiA, and LA were selected as hydrogen bond donors (HBD) for DES formation and subsequent chitin recovery from shells.

Although DES have been recurrently considered as environmentally safe or nontoxic by different authors, it is important to confirm their toxicological effect before envisioning a commercial application[12,23]. Within this context, in order to evaluate the impact of ChCl/organic acid-based DES on wheat (*T. aestivum*), seeds were treated with a range of concentrations of each system for 7 days. Growth parameters, namely germination and shoot height inhibition, were measured and the corresponding EC50 calculated.

Results presented on Table 1 suggest that the early growth of seedling shoots was more sensitive to the toxic effects of DES than seed germination, which is in accordance to the work of Radošević *et al.* on the toxic impact of DES based on mixtures of ChCl with glucose, glycerol, and oxalic acid on wheat seeds[24]. ChCl:LA (1:1) showed the lowest toxicity, while ChCl:MoA (1:2) was the system that affected the most both germination and shoot height, with EC50 ranging from 0.9 to 11.8 mg/mL, considering both markers (Table 1). It is also interesting to note that, although no signs of leaf necrosis were observed, shoots grew in an uneven way in seeds treated with the different systems, when compared to the control, which was exacerbated as the concentrations of DES increased (data not shown).

Table 1. EC50 values (mg/mL) obtained on wheat seeds treated with ChCl/organic acid-based DES, after an incubation period of 7 days.

DES	Germination	Shoot height
ChCl:MoA (1:2)	5.0 ± 0.1	0.9 ± 0.1
ChCl:MiA (1:2)	7.6 ± 1.9	1.3 ± 0.5
ChCl:LA (1:1)	11.8 ± 0.3	1.6 ± 0.6

The phytotoxicity of ChCl-based DES have already been explored by several authors on different model organisms, namely garlic (*Allium sativum*) cloves[25], microalgae (*Raphidocelis subcapitata*)[26], and wheat (*T. aestivum*) seeds[24]. In particular, the work Radošević *et al.* showed that, although the DES studied caused some degree of inhibition of seeds germination and shoot and root growth, the toxicity of the systems could still be regarded as low, since the germination EC50 was superior to 5 mg/mL[24]. Therefore, taking into consideration the work of Radošević *et al.* and the toxicity classification proposed by Passino and Smith, in which chemicals are classified into several categories of toxicity based on their effective concentrations, it is possible to conclude that the DES studied were "relatively harmless" to wheat (EC50 > 1 mg/mL, the least toxic category according to this classification system)[26,27].

Oxidative stress in known to be related to germination and growth inhibition. In this way, the determination of LPO has been widely used as marker of reactive oxygen species (ROS) mediated damage, in particular by measuring the accumulation of MDA, the cytotoxic product of the peroxidation of unsaturated fatty acids contained in phospholipids[28]. The accumulation of MDA on the leaves harvested from seedlings after treatment with two different concentrations of DES (5 and 10 mg/mL) is presented on Figure 2i and shows that ChCl:MoA (1:2) was the only system that significantly increased the MDA content ($P < 0.0001$) at 10 mg/mL. These results are probably justified by the higher toxicity displayed by this system on germination and early growth of seedling shoots (EC50 of 5.0 mg/mL and 0.9 mg/mL, respectively). Similar findings were reported for a ChCl:oxalic acid DES: the higher the concentration of the DES, the higher the MDA content, which shows an inability of the antioxidant enzymes to remove completely the accumulated ROS during DES treatment[24].

The content of photosynthetic pigments (CHL *a*, CHL *b*, and total CHL) in leaves have also been considered by several authors as an abiotic stress marker and as an indicator of plant health[29]. Generally, the CHL content did not show to be significantly affected by DES

treatment (Figure 2ii), except when using ChCl:MoA (1:2) at 10 mg/mL ($P < 0.05$). Similarly to what was reported in previous works for ChCl/organic acid-based DES[24,26], these results correlate not only with the higher toxicity displayed by ChCl:MoA (1:2), showing an increased inhibition of the plants' growth, but also with an increase in MDA content when treating wheat with this system at 10 mg/mL. This indicates that the accumulation of MDA may have contributed to the photosynthetic system damage, thus reducing photosynthesis, a key phenomenon that significantly contributes to the plant's growth and development under stress[30].

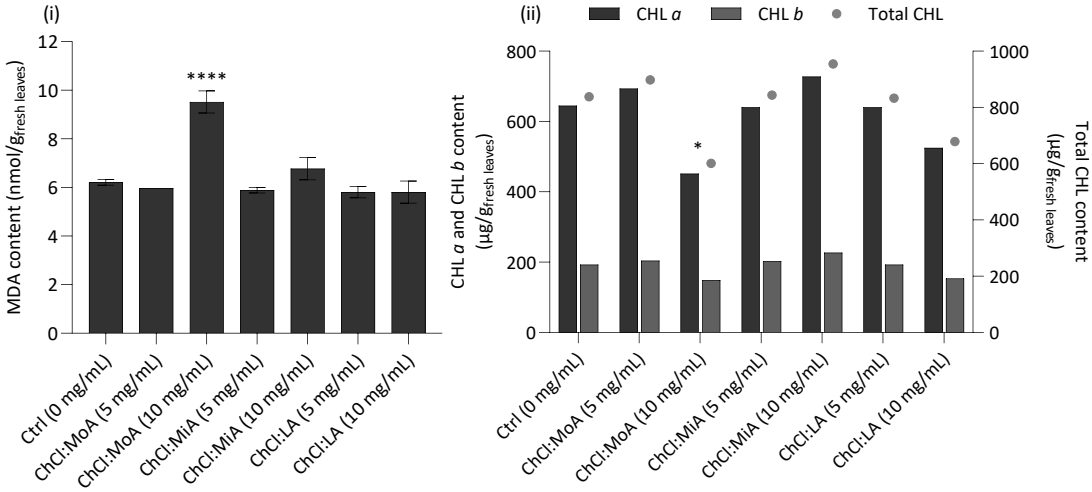

Figure 2. Effect of ChCl/organic acid-based DES on (i) MDA and (ii) photosynthetic pigments content. Statistically significant differences between the effect of DES and the control are represented by asterisks (*).*$P < 0.05$, ****$P < 0.0001$.

Antioxidant enzymes such as SOD, GPX, CAT, and APX, are part of a complex antioxidative defence system, which is of the utmost importance for plant survival and adaptation[28]. These and other enzymes are responsible for maintaining cell homeostasis and for providing a concerted response to oxidative stress[28,31]. Consequently, considering that plants are known to fine tune their response to different stress factors, being the severity of stress symptoms highly dependent on their capacity to tolerate a specific stress inducer, their up- or downregulation is considered to be a good indicator of the oxidative stress level in the plant[32,33]. Within this context, SOD, GPX, CAT, and APX were selected as biomarkers to determine the oxidative stress caused by ChCl/organic acid-based DES on the enzymatic antioxidant defence system of wheat (Figure 3).

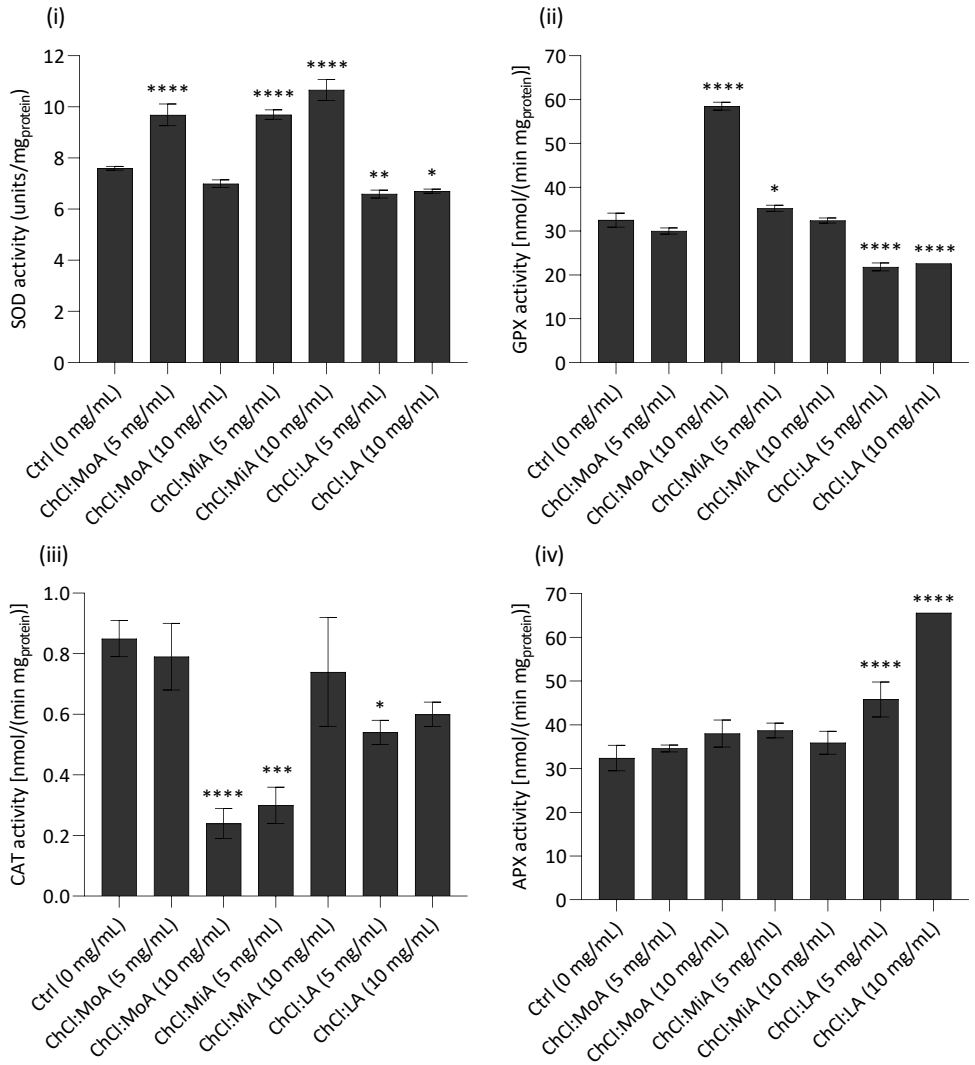

Figure 3. Effect of ChCl/organic acid-based DES on (i) SOD, (ii) GPX, (iii) CAT, and (iv) APX activities. Statistically significant differences between the effect of DES and the control are represented by asterisks (*).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Figure 3 shows that, while some DES caused the activity of antioxidant enzymes to increase at specific concentrations, there were also cases where enzymes were slightly or fairly inhibited. This implies that the DES' toxicities were mostly affected by their specific characteristics and could eventually have been triggered by their HBD, which might have induced different free radicals. The most toxic DES regarding germination and growth of seedlings, namely ChCl:MoA and ChCl:MiA, showed a tendency to upregulate SOD and GPX, while inhibiting CAT and maintaining APX activity. Conversely, the least toxic DES ChCl:LA

caused a downregulation of SOD, CAT, and GPX, while inducing APX activity. These results suggest that the defence mechanisms of wheat had a different antioxidant and redox homeostasis response to toxic levels of oxidative stress, depending not only on the system tested, but also on the DES concentration applied and on the free radicals induced.

4.2. Chitin recovery

Table 2 shows the proximate composition of brown crab shell residues. As it would be expected, since the residues were mainly composed of shells, the most prominent component of the matrix were minerals (65%), followed by proteins and other minor compounds (24%), and chitin (11%).

Although the relative percentage of each of these components is extremely dependent on the crab species and on seasonal changes, the results described in this work for minerals, proteins, and chitin were quite similar to those presented by other authors for both male and female *C. pagurus*, caught in Scottish and French waters[18].

Table 2. Composition of freeze-dried brown crab shell residues.

Compound	**g/100 g** $_{\text{dry residue}}$
Minerals	64.8
Other compounds [a]	23.8
Chitin	11.4

Coefficient of variation ≤ 8.7%

[a] Mainly proteins and other minor compounds such as fatty acids or pigments

As previously mentioned, minerals, proteins, and chitin form a complex network, which can pose a challenge when the aim is to separate the different components of the crustacean's exoskeleton. In this way, for a successful chitin isolation, the solvent applied must be able to demineralise and deproteinise the shells. Demineralisation usually requires acidic conditions, while high temperatures under acidic or alkaline conditions are crucial for deproteination, so that proteins can be denatured[3]. Therefore, the traditional procedure for the fractionation of crustacean shell biomass involves the removal of minerals and proteins, and subsequent decolouration to obtain a pure and colourless chitin[8,10]. Although this conventional process is extremely time-consuming and harmful to the environment[8], it is in fact a highly efficient methodology to obtain pure chitin, as shown on Table 3. However,

this process has resulted in considerable losses of chitin (approximately 16%) during the subsequent steps of extraction.

Table 3. Operating conditions, hydrolysis extent, and demineralisation and deproteination efficiencies for the different DES tested.

	Temperature (°C)	Processing time (h)	Hydrolysis extent (%)	Demineralisation efficiency (%)	Deproteination efficiency (%) [a]
Conventional method	Room T - 70	25	90.4	100.0	100.0
ChCl:MoA (1:2)	50	4	45.2	47.5	60.5
	80		52.1	57.0	63.9
	130		50.8	47.9	83.0
ChCl:MiA (1:2)	50		29.1	27.3	47.8
	80		31.3	28.3	54.4
	130		65.6	71.3	81.5
ChCl:LA (1:1)	50		32.9	31.4	52.8
	80		39.6	40.1	57.2
	130		65.1	68.0	88.2
ChCl:MoA (1:2) | H_2O	50	2 | 0.5	77.5	99.7	54.4
	80		78.7	100.0	58.4
	130		76.0	92.3	68.2
ChCl:MiA (1:2) | H_2O	50		75.7	99.6	47.0
	80		76.8	99.9	50.7
	130		81.1	99.7	69.5
ChCl:LA (1:1) | H_2O	50		72.4	99.3	33.9
	80		74.3	100.0	40.1
	130		85.1	99.6	86.7

[a] Includes not only proteins but also other impurities (*e.g.*, fatty acids, pigments)
Coefficient of variation ≤ 13.2%

The ChCl/organic acid-based DES prepared, as well as the operating conditions applied, were selected based on the previous works, where these systems have proven to be effective in the extraction of chitin from shellfish biomass[3,14–16].

In the first set of experiments, the DES prepared were studied for the isolation of chitin at different operating temperatures for 4 h. However, as summarized on Table 3, DES showed to be very ineffective in removing both minerals and proteins. Although, in general, efficiencies increased with temperature, probably facilitated by a decrease of DES viscosities, it was not possible to exceed 71% of demineralisation (ChCl:MiA (1:2) at 130 °C) or 88% of deproteination (ChCl:LA (1:1) at 130 °C) efficiencies.

Therefore, a second set of experiments was designed, in which extractions were performed for 2 h with the selected DES at different temperatures. After the first extraction step, water was added to the mixture, which was stirred for about 30 min, until reaching room temperature. The addition of water was expected to have a double function: on one hand, it should cause the disruption of the DES structure, thus leading to the precipitation of any solubilized chitin; and on the other hand, the presence of water should cause an acidic environment through the formation of charged species, which was expected to improve the reaction of organic acids with minerals and further promote the acidic hydrolysis of proteins[3]. However, it is important to note that other authors have found that adding high water contents at the beginning of the extraction could negatively influence the removal of proteins[16]. Therefore, in this work, water was only added to the mixtures after 2 h of extraction with DES.

As expected, results displayed on Table 3 show that the addition of water after the first extraction step was crucial to increase the demineralisation efficiency to close to 100% for most of the conditions tested. However, the deproteination was not as efficient as the demineralisation, ranging from 34 to 87%, being ChCl:LA (1:1) the most promising system when used at 130 °C. Nevertheless, it was possible to further enhance the deproteination efficiency during the decolouration step (Table 4), as hydrogen peroxide was able to remove the proteins that remained in the matrix, as previously reported by other authors[34,35].

Table 4. Operating conditions, hydrolysis extent, demineralisation and deproteination efficiencies, and chitin purity for the different DES tested at the most promising temperature conditions, after decolouration with hydrogen peroxide.

	Temperature (°C)	Processing time (h)	Hydrolysis extent (%)	Demineralisation efficiency (%)	Deproteination efficiency (%) [1]	Chitin purity (%)
ChCl:MoA (1:2) | H_2O | H_2O_2	80	2 | 0.5 | 0.5	84.2	100.0	81.9	72.5
ChCl:MiA (1:2) | H_2O | H_2O_2	130		84.2	99.7	82.5	72.4
ChCl:LA (1:1) | H_2O | H_2O_2	130		89.2	99.7	100.0	98.2

[1] Includes not only proteins but also other impurities (*e.g.*, fatty acids, pigments)
Coefficient of variation ≤ 7.1%

The fact that ChCl:LA DES outperformed ChCl:MoA and ChCl:MiA in the deproteination of shells (up to 1.2-fold) might be related to the acidity of the HBD (MoA, MiA, or LA), as it has been demonstrated by Zhou *et al.* through a linear positive correlation between the

deproteination efficiency and the pKa of the HBD.[36] Furthermore, it can also be hypothesized that this behaviour might be related to the viscosity of each system (which increased as follows: ChCl:LA < ChCl:MoA < ChCl:MiA)[3,37], and to the consequent problems arising from limitations in mass transfer and diffusivity for more viscous systems.

Therefore, similarly to what was described for other biomass matrices[3,15,36], by applying a methodology composed of a first step of demineralisation and partial deproteination with ChCl:LA (1:1) DES and water, followed by a step of decolouration and complete deproteination with hydrogen peroxide, it was possible to obtain high purity chitin (98%) with fewer losses than those caused by the conventional hydrolysis (5% compared to 16% chitin losses). Furthermore, it can be of great benefit to use ChCl:LA (1:1) as solvent, since, as previously discussed, this was the system that showed the least phytotoxicity on wheat seeds.

As shown on Table 2, shell residues still hold a significant amount of minerals in their composition (around 65%). Therefore, and, if desirable, this mineral fraction can be isolated from the DES and eventually commercialized, either by precipitation with ethanol or sodium hydroxide, while the DES can be recycled and reused for further extractions[3,16,38].

4.3. Chitin characterization

The thermal stability of chitin is a critical factor when determining its potential applications. TGA curves corresponding to crab shell residues, chitin STD, and chitin samples isolated by the conventional method or ChCl/organic acid-based DES (obtained at the processing conditions summarized on Table 4) are presented on Figure 4. A first slight mass loss, which was common to all samples, could be perceived between 35 and 100 °C, possibly due to the evaporation of chemisorbed water[14,15,39]. A second stage of degradation between 100 and 250 °C was followed, being mainly noticeable in crab shells, which was probably due to the breakdown of proteins and lipids. The absence of an evident mass loss at this temperature range in the chitin samples isolated with DES or by conventional hydrolysis helps to confirm that proteins were removed from the matrix[36,39,40]. As a result of chitin degradation, a third abrupt decomposition step could be noticeable between 250 and 400 °C[14,15,36,38–40].

The thermal stability of a given material can be inferred by looking at the initial decomposition temperature. As shown on Figure 4, the stability of the chitin samples

obtained after DES treatment was very similar to the chitin STD, whereas the stability of the chitin obtained by conventional hydrolysis was superior. This may be related to the molecular weights of the different fractions of chitin, suggesting that the chitin isolated with DES and the standard have similar molecular weights, which were probably lower than the molecular weight of the chitin obtained by conventional acid/alkaline hydrolysis. It is possible that the higher temperatures applied during DES treatment resulted in an increased hydrolysis of chitin, consequently leading to macromolecules with a lower molecular weight[38].

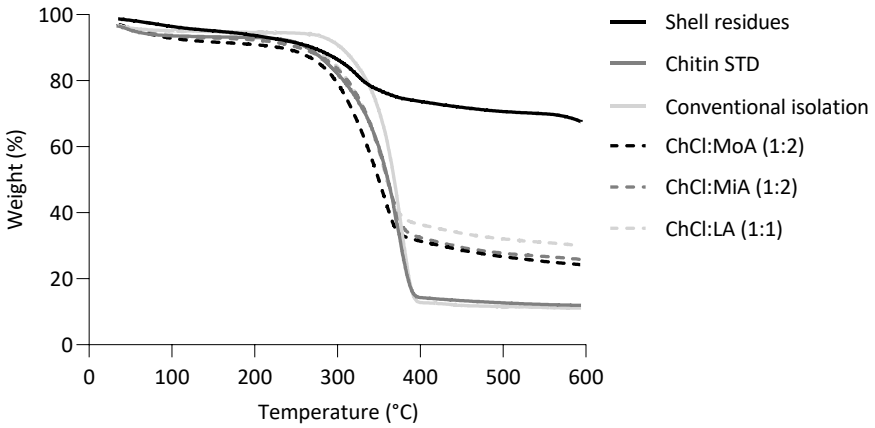

Figure 4. TGA curves of shell residues, chitin STD, and chitin recovered from crab shell biomass by the conventional method and ChCl/organic acid-based DES (obtained at the processing conditions summarized on Table 4).

The FTIR spectra of the crab shells, chitin STD, and chitin recovered from the shell biomass by acid/alkaline hydrolysis and DES (obtained at the processing conditions summarized on Table 4) are represented on Figure 5. It is interesting to note that, except for crab shells, all samples showed very similar patterns, presenting all typical absorption bands of chitin STD. The assignments of chitin absorption peaks were according to previous literature[14,15,36,38–40], namely the symmetric stretching vibration of O-H at 3444 cm^{-1} and N-H at 3260 and 3102 cm^{-1}; the amide I band split at 1652 and 1620 cm^{-1}, attributed to the existence of intermolecular (-CO··HN-) and intramolecular (-CO··$HOCH_2$-) hydrogen bonds; the amide II band at 1554 cm^{-1} attributed to in-plane N-H bending and C-N stretching; and the amide III band at 1308 cm^{-1} attributed to C-H bend.

Regarding the crab shells spectrum, it is possible to conclude from Figure 5 that the chitin amide I band was not clearly split, due to the overlapping of the protein amide peaks[39,40]. This suggests that both conventional hydrolysis and ChCl/organic acid-based DES were able to remove proteins from the shell matrix.

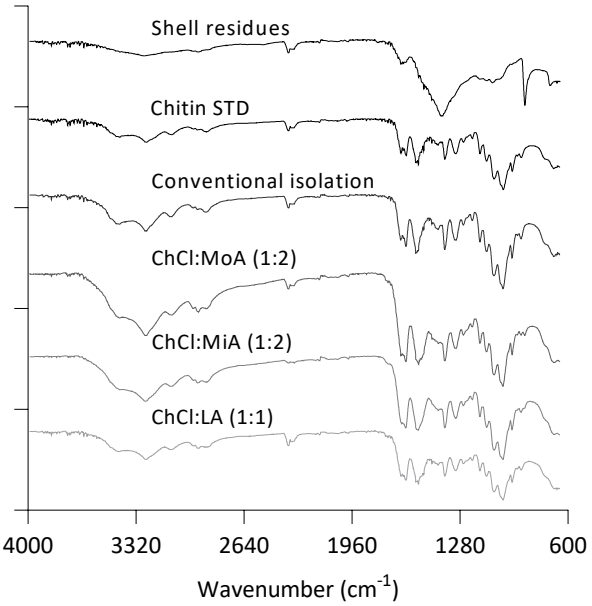

Figure 5. FTIR-ATR spectra of shell residues, chitin STD, and chitin recovered from crab shell biomass by the conventional method and ChCl/organic acid-based DES (obtained at the processing conditions summarized on Table 4).

It is also worth highlighting that the deacetylation degree of samples obtained after DES treatment was lower than 6.4%, with the acetylation degree increasing as follows: ChCl:MiA (93.6%) < ChCl:MoA (96.8%) < chitin STD = conventional acid/alkaline hydrolysis (98.0%) < ChCl:LA (98.5%).

To evaluate the crystal structure and the crystallinity of samples, XRD analysis was performed on crab shell residues, on chitin STD, and on the chitin samples isolated by the conventional method and ChCl/organic acid-based DES (obtained at the processing conditions summarized on Table 4). The profiles illustrated on Figure 6 show that the chitin samples isolated with DES are in good agreement with what was obtained for the chitin STD or for chitin isolated through the conventional method. Unlike shell residues, all chitin samples displayed two main diffraction peaks at $2\theta \approx 9.4°$ and $2\theta \approx 19.3°$, and three weaker diffraction peaks at $2\theta \approx 12.8°$, $2\theta \approx 23.2°$, and $2\theta \approx 26.4°$, which are characteristic of the

crystalline structure of α-chitin[14,15,36,39]. When comparing the diffraction patterns displayed by chitin with the pattern of shells, it is interesting to note that the diffraction peak at 2θ ≈ 29.8°, characteristic of calcium carbonate[38–40], decreased in chitin samples isolated either by the conventional method or by DES, while the peaks characteristic of α-chitin increased. This suggests that the chitin concentration increased as calcium carbonate was removed.

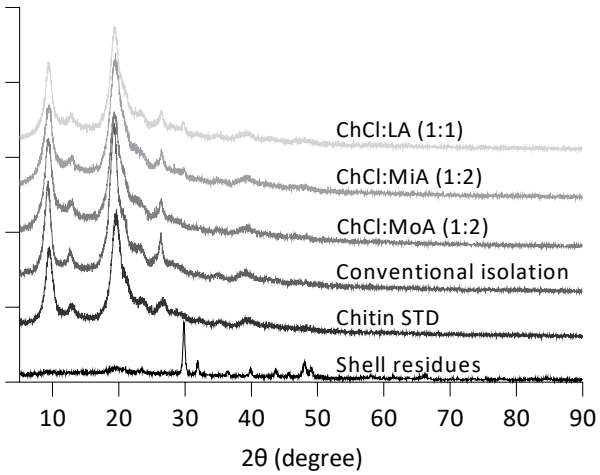

Figure 6. XRD profiles of shell residues, chitin STD, and chitin recovered from crab shell biomass by the conventional method and ChCl/organic acid-based DES (obtained at the processing conditions summarized on Table 4).

Crystallinity indexes of crab shell residues, chitin STD, and chitin samples isolated by acid/alkaline hydrolysis, ChCl:MoA, ChCl:MiA, and ChCl:LA DES, were as follows: 43.0%, 84.5%, 85.7%, 78.6%, 79.2%, and 82.9%. The increase of chitin crystallinity, when comparing to the shells, suggests that both minerals and proteins were successfully removed from the matrix[38–40]. Furthermore, ChCl:LA DES was able to produce chitin with a crystallinity index close to those of chitin STD or chitin obtained by conventional hydrolysis.

SEM images of the shell residues, chitin STD, and chitin isolated after conventional acid/alkaline hydrolysis and DES treatment (obtained at the processing conditions summarized on Table 4) are presented on Figure 7. It is possible to conclude from SEM observations that there was considerable modification of the surface of the recovered chitin samples when comparing to those of crab shells or the standard. A rough surface without pores was observed for the shells as well as for the standard (Figures 7i and ii, respectively), while smoother surfaces with pores were visible in chitin recovered with the conventional

method or with DES, due to mineral and protein removal (Figures 7iii-vi). It is interesting to note that, as reported by other authors, chitin presenting a porous surface structure may be interesting for applications such as adsorption of metal ions or dyes[14,36,38].

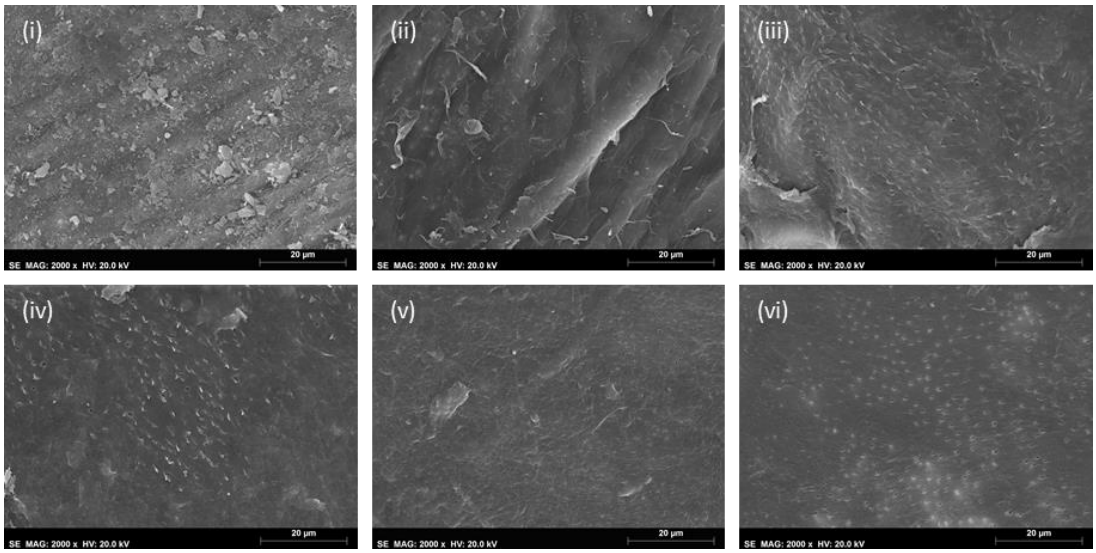

Figure 7. SEM images of (i) shell residues, (ii) chitin STD, and chitin recovered from crab shell biomass by (iii) conventional hydrolysis, (iv) ChCl:MoA (1:2), (v) ChCl:MiA (1:2), and (vi) ChCl:LA (1:1) treatment (obtained at the processing conditions summarized on Table 4).

Although the particle size of the standard revealed to be higher than the remaining materials, the particle size of the chitin samples obtained after the different treatments was quite similar to the size of the crab shell particles (Appendix F, Figure F1), confirming that the intrinsic structure of chitin was not degraded into smaller particles. These results are in accordance with the work developed by Zhou *et al.*, in which chitin was isolated from black soldier flies with ChCl:lactic acid and betaine:urea DES[36].

5. Conclusions

The study developed herein demonstrated an alternative approach for chitin recovery from brown crab biomass, using ChCl/organic acid-based DES, which has the potential to become competitive at commercial scales.

Overall, DES have shown to be low-toxic to wheat seeds, with EC50 ranging from 5 to 12 mg/mL considering germination, and from 0.9 to 1.6 mg/mL considering shoot height, being ChCl:MoA the most toxic and ChCl:LA the least toxic DES.

Due to the multifunctional properties of these systems, the shell residues could be demineralised, deproteinised, and decoloured to yield chitin in just 3 h (in a 3-step process using DES, water, and hydrogen peroxide), as opposed to the 25 h necessary to obtain pure chitin through a conventional hydrolysis (in a 4-step process using hydrochloric acid, sodium hydroxide, ethanol, and acetone, at temperatures up to 70 °C). ChCl:LA (1:1) revealed to be the most interesting system for chitin isolation at 130 °C, resulting in the highest demineralisation and deproteination efficiencies. Minerals were mainly removed by the acidic conditions provided by the DES and the added water, while proteins could be removed, in a first stage, by the high temperatures applied, and in a second stage by hydrogen peroxide, which had as main function the removal of pigments and other impurities that remained in the recovered chitin.

The characterization experiments performed on chitin obtained by ChCl:LA DES treatment have shown that it was possible to obtain chitin with similar features to a commercial product or to chitin obtained by a conventional hydrolysis, namely similar thermal stability, degree of acetylation, and crystallinity. Furthermore, the chitin obtained after DES treatment revealed to have a porous surface structure, which might enable its application in market sectors such as adsorption of metal ions or dyes, that would not be viable if chitin presented a non-porous structure.

It is also interesting to note that it has already been demonstrated by other authors that it is possible not only to recover the calcium carbonate extracted from the shells while using DES as solvents, but also to recycle and reuse the DES, which can greatly contribute to the environmental and economic viability of the process.

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CHAPTER 4

Process sustainability assessment

CHAPTER 4

Contents

Sections 3 and 4 contain parts from:

Gargalo, C. L., Rodrigues, L. A., Paiva, A., Carvalho, A. Life cycle assessment of a canned sardine residue upcycling process in Portugal. In preparation.

Gargalo, C. L., Rodrigues, L. A., Paiva, A., Carvalho, A. Life cycle assessment for early- stage design: upcycling of crab shell. In preparation.

The author was involved in the conceptualization and design of all experiments and performed all experimental work, including mass and energy balances, except for the characterization of extracts by GC-FID (acquired at INIAV by A. M. C. Partidário), the *in vitro* evaluation of cytotoxicity, antiproliferative, and anti-inflammatory effects (performed at iBET by M. Cardeira), the evaluation of antimicrobial activity (performed at iBET by I. C. Leonardo under the supervision of F. B. Gaspar), the characterization of chitin by FTIR (acquired at iBET by A. Ferreira), XRD (acquired at FCT NOVA by N. Costa), and SEM (acquired at IST-UL by I. Nogueira), and the LCA (performed at DTU by C. L. C. L. Gargalo under the supervision of A. I. Carvalho from IST-UL). Data processing and interpretation, and results discussion were performed by the author, having also been involved in the preparation of the original manuscript.

1. Abstract

Sustainability is a concept that has been gaining increased attention in the design and development of new products. In this chapter, two different sustainability assessment tools, namely process integration (PI) and life cycle assessment (LCA), were explored for the evaluation of the sustainability of the most promising processes, developed throughout this thesis and selected in the previous chapters, for the valorisation of waste streams resulting from sardine and crab processing companies. Furthermore, an experimental scale-up (up to 70-fold increase in scale) was performed before PI, to be able to identify some of the problems that could arise from a scale increase. Results have shown that, although the scale-up did not cause considerable changes on both composition and bioactive potential of the recovered products, some of them undergone a few modifications, mainly in terms of bioactivity, after PI. In particular, the antimicrobial potential of protein derivative-rich extracts obtained from sardine residues shifted, being more effective towards the Gram-negative than the Gram-positive target bacterium tested, as opposed to the effect displayed by the extract resulting from the isolated process, which was more effective towards the Gram-positive bacterium. Another example was the chitin obtained from crab shell residues through PI, which revealed to be contaminated with the deep eutectic system (DES) used for astaxanthin (AXT) extraction, thus affecting some of its characteristics, such as the thermal stability, which decreased considerably when compared to the chitin obtained by applying the isolated process. Regarding LCA, hotspot analysis has shown that the processes with the higher environmental footprint were the extraction of protein derivatives from sardine residues with betaine/propylene glycol-based DES and the extraction of AXT from crab shells with a menthol/myristic acid-based DES, being these impacts mainly related to the production of the chemicals used for DES preparation.

2. Introduction

In 1987, the World Commission on Environment and Development introduced the term sustainability defining it as the “development that meets the needs of the present without compromising the ability of future generations to meet their own needs”[1]. Nowadays, sustainability is regarded as a broad and complex concept, with no universally consensual definition. Nonetheless, the 1980s definition has evolved, even with myriad interpretations,

to be based on the integration and acknowledgement of three fundamental principles, namely economic growth, environmental protection, and social development, as well as on the 17 United Nations' Sustainable Development Goals, which should be considered throughout the decision-making process[2,3].

In particular, the sustainability evaluation of already existing or potential future manufacturing processes, or even biorefineries that convert biomass feedstocks into bioproducts, has been mainly performed through the exclusive analysis of a single criterion, mostly related to economic or environmental impacts[4,5], although this is far from ideal. Ubando *et al.* have discussed the five methodologies that have been used for sustainability assessment of biorefineries, namely life cycle assessment (LCA), techno-economic analysis (TEA), social-economic analysis, multi-criteria decision analysis, and process integration (PI) and optimization, concluding that PI and optimization, LCA, and TEA were by far the most explored tools[4].

PI started being developed in the 1970s, aiming at achieving a higher energy efficiency, as a response to the oil crisis, which had caused fuel to become a scarce and expensive resource[6]. Nowadays, PI provides a key holistic approach to the optimal design, planning and operation of a process, supply chains, and systems, allowing the minimisation of material consumption, energy demand, water use, waste generation, and emissions[6,7]. Therefore, PI provides a powerful tool for the design of cleaner and more sustainable processes, by optimizing resource deploy and waste discharge[8].

This methodology has already been successfully applied to different biorefinery processes towards the production of biodiesel and bioproducts from lignocellulosic biomass[9,10], algae[11,12], food waste[11], among others, aiming at improving their sustainability. Some of these studies have also been complemented or validated either through LCA[9] or TEA[12].

LCA is a tool that allows the evaluation of the environmental impact of a service, process or product[4]. In particular, it is considered to be of the utmost importance for a sustainable design of emerging processes/technologies, as it enables the identification of opportunities for improvement, even for research at very early stages of technology development[13]. This tool consists of 4 major dynamic procedures, *i.e.*, (i) the definition of a goal and a scope, where the purpose and the extent of the study is identified and the functional unit defined; (ii) the life cycle inventory analysis, which involves the data collection, either experimentally

or in databases or in the literature, and its analysis; (iii) the assessment of the life cycle environmental impact, which evaluates the significance of the environmental impacts; (iv) and the interpretation of the results[4].

LCA studies performed on biorefineries (*e.g.*, agricultural residues-, sugar crops-, starch-, lignocellulosic-, empty fruit bunch-, or algae-based[14]), biobased products, or food production processes have been mainly performed with data provided by the industry, while studies involving process and product improvement at the early development stages are still very limited in the literature. Nonetheless, and although such studies can be challenging due to the lack of inventory data, they can be extremely important, as they might allow design decisions at an early stage of product development, such as the selection of alternative materials, techniques, or production processes, thus providing a basis for process design at full commercial scales[15]. Furthermore, it is known that about 80% of all environmental effects associated to a product are determined in the design phase of development[16].

Even though the sustainability of processes and products can be assessed at an early stage of their development, ultimately, the main goal of all efforts invested on fundamental and applied research for biomass alternative extraction and fractionation methods, is the development of a new generation of sustainable processes and products at commercial scales. Within this context, it is important to carry out optimization and scale-up studies prior to sustainability assessments, not only to better understand the overall process, but also to facilitate its validation and implementation at full scale[17].

When scaling-up a process, there are some parameters, including thermodynamic and mass transfer criteria, that should be preserved (*e.g.*, temperature, pressure (if applicable), solvent velocity (if applicable), extraction bed geometry (*i.e.*, length-diameter ratio, L/D), while others need to be increased (*e.g.*, feed, solvent or solvent flow rate, extraction bed dimensions (while maintaining the L/D ratio)), so that the smaller scale process outcomes may be reproducible at a larger scale. Furthermore, the results that are to be reproduced, such as the extraction yield, the extraction velocity (*i.e.*, mass transfer rate), the physical and/or chemical properties of the extract (*e.g.*, composition, viscosity, density, thermal properties), the extract quality (*e.g.*, biological activity, flavour, aroma), among others, should also be defined, so that an accurate evaluation of the results can be performed[18].

Within this context, and as a proof of concept, in this chapter, the most promising processes developed throughout this research work were experimentally scaled up (up to 70-fold scale increase, although still at laboratory scale) and then integrated. These processes included the extraction of fatty acids with supercritical CO_2 (sc-CO_2) and protein derivatives with a betaine:propylene glycol deep eutectic system (DES) from sardine heads and offal; and the extraction of astaxanthin (AXT) with a menthol:myristic acid DES, protein hydrolysates with subcritical water (scW), and chitin with a choline chloride:lactic acid DES from brown crab shell residues. It is noteworthy that the factor by which scale was increased for each individual process was chosen based on the limitations of the equipment available. The results of scale-up and PI were then evaluated taking into consideration different characterization and bioactivity endpoints. Furthermore, the results obtained after integration of the processes proposed for the valorisation of both biomasses were evaluated through LCA, aiming at quantifying their environmental impact and at identifying the hotspots of those impacts to provide recommendations and suggestions that might contribute to improve the processes, before moving towards their development and potential implementation at commercial scales.

3. Materials and methods

3.1. Biomasses

Canned sardine (*Sardina pilchardus*) heads and offal were kindly provided by Fábrica de Conservas A Poveira, Portugal, in September 2020, and brown crab (*Cancer pagurus*) shells by Tejo Ribeirinho, Portugal, in November 2017. Biomasses were stored at -20 °C upon arrival. The biomasses were freeze-dried and then milled, as previously described on Chapter 2, Part I, section 3.1., and the particle size of crab shells ground material was determined as described on Chapter 3, Part I, section 3.1.. After processing, the residues were protected from light and stored at -20 °C, in a nitrogen atmosphere, in the case of sardines, and at room temperature, in a low moisture environment, in the case of crab.

3.2. DES preparation

Betaine:propylene glycol (B:PG) (1:3), menthol:myristic acid (ME:MA) (8:1), and choline chloride:lactic acid (ChCl:LA) (1:1) DES were prepared as previously described (Chapter 2,

Part II, section 3.2.; Chapter 3, Part I, section 3.2.; and Chapter 3, Part III, section 3.3., respectively) by heating the mixtures to 80, 55, and 80 °C, respectively, under constant stirring until a clear liquid was formed (Table 1).

Table 1. Summary of the DES prepared, respective molar ratios, preparation temperatures, and target molecules.

DES	Preparation temperature (°C)	Target molecule
B:PG (1:3)	80	Protein derivatives from sardine
ME:MA (8:1)	55	Astaxanthin from crab
ChCl:LA (1:1)	80	Chitin from crab

3.3. Solid-liquid/supercritical fluid extractions

3.3.1. *Sc-CO_2 extraction of fatty acids from sardines*

Sc-CO_2 extractions were carried out in a supercritical fluid extraction system with independent control of temperature and pressure, as previously described on Chapter 2, Part I, section 3.2.2., with slight modifications regarding the scale used. Briefly, 10 or 50 g of freeze-dried residue were extracted at 550 bar, 35 °C, and a CO_2 flow rate of 5 or 25 g_{CO2}/min, respectively, in order to maintain the solid-fluid ratio. Samples were recovered at different time points during an extraction time of 105 min. Experiments were performed in duplicate and samples (extracts and resulting sardine residues) were stored at -20 °C, in the absence of light, until further analyses. Results were expressed as $g_{extract}$/100 $g_{dry\ residue}$.

The CO_2 velocity and the residence time were calculated according to Equations 1 and 2[19], respectively.

$$v_{CO2} = \frac{CO_2\ flow\ rate}{Bed\ cross\ section\ area} \quad \textbf{Equation 1}$$

$$t_{residence} = \frac{Bed\ void\ fraction \cdot Solvent\ density \cdot Feed\ mass}{CO_2\ flow\ rate \cdot Feed\ density} \quad \textbf{Equation 2}$$

3.3.2. *DES extraction of protein derivatives from sardines*

Briefly, 9.6 or 400 g of B:PG (1:3) DES were added to 1.2 or 50 g of sardine freeze-dried residue (for PI, the feedstock used was the residue resulting from sc-CO_2 extraction). Extractions were performed at 80 °C for 18 h under constant magnetic stirring. Samples were filtrated under vacuum and the supernatant recovered. Extracts were stored at -20 °C in the absence of light, until further analyses. All experiments were performed in triplicate.

3.3.3. *DES extraction of AXT from crab shells*

Briefly, extractions were performed under constant magnetic stirring at 60 °C for 2 h, by adding 5 or 200 g of ME:MA (8:1) DES to 1.25 or 50 g of freeze-dried crab shell residue. Samples were filtrated under vacuum and the supernatant recovered. Extracts were analysed immediately after by UV-vis spectroscopy to determine the AXT content. After quantification, extracts were stored at -20 °C in the absence of light. Experiments were performed at least in triplicate.

3.3.4. *ScW extraction of protein hydrolysates from crab shells*

ScW extractions were performed in a 1200 mL high pressure reactor as described on Chapter 3, Part II, section 3.2., with slight modifications. Briefly, 500 mL of distilled water were added to 50 g of shell residue (the residue resulting from AXT extraction was used for PI). Extractions were performed at 250 °C, 100 bar, and a heating rate of 6 °C/min. After extraction, the extract was separated from the solid residue by vacuum filtration. Extracts were freeze-dried at -55 °C for approximately 24 h, while the solids were dried in an oven at 80 °C for 24 h. Extracts were stored at -20 °C until further analyses. Global extraction yield results were expressed as $g_{extract}$/100 $g_{dry\ residue}$ and hydrolysis extent as weight percentage.

3.3.5. *DES recovery of chitin*

The recovery of chitin using DES was performed as previously described on Chapter 3, Part III, section 3.5.2., with slight modifications. Briefly, 12.5 or 875 g of ChCl:LA (1:1) DES were added to 0.5 or 35 g of feedstock (either freeze-dried crab shell residue or the dried residue resulting from protein hydrolysates extraction with scW). Samples were maintained under constant stirring at 130 °C for 2 h. Distilled water was then added to the resulting mixtures at a solid-liquid ratio of 1:25 g_{feed}/mL_{H2O}, until reaching room temperature. The solids were recovered through vacuum filtration and washed with distilled water, until a neutral pH was attained. The filtrated solids were dried overnight at 70 °C. Decolouration of the dried solids was performed with hydrogen peroxide at 80 °C for 30 min, using a solid-liquid ratio of 1:10 g_{solids}/mL_{H2O2}. The resulting solids were washed with distilled water and dried for 24 h at 70 °C. Dried samples were stored at room temperature, in a low moisture environment, until further analysis. All experiments were performed in duplicate.

3.4. Extract characterization

3.4.1. *Fatty acid profile determination*

The fatty acid profile of the extracts obtained after sc-CO_2 extractions was determined by gas chromatography-flame ionization detection (GC-FID) analyses, as described on Chapter 2, Part I, section 3.3.. Briefly, extracts were transesterified before being injected into the GC column. Separation of fatty acid methyl esters (FAMES) was achieved using helium as carrier gas in a DB-23 capillary column at an oven temperature program as follows: 70 to 195 °C (5 °C/min), 30 min isotherm at 195 °C, 195 to 220 °C (5 °C/min), 65 min an isotherm at 220 °C. The injector and detector temperatures were maintained at 220 and 280 °C, respectively. FAMES were identified by comparison of relative retention times with those of a standard mixture. Results were expressed as $mg_{fatty\ acid}/g_{extract}$ or $mg_{fatty\ acid}/100\ g_{dry\ residue}$.

3.4.2. *Lowry method for total protein quantification*

Total protein content was determined as previously described (Chapter 2, Part II, section 3.7.1.) by UV-vis spectroscopy, using the Folin & Ciocalteu's Phenol Reagent. Experiments were performed in duplicate and results were expressed as $mg_{protein}/g_{dry\ residue}$.

3.4.3. *AXT quantification*

AXT was quantified by UV-vis as described on Chapter 3, Part I, section 3.6., by analysing extracts immediately after extraction at 484 nm. Results were expressed as $\mu g_{AXT}/g_{dry\ residue}$.

3.4.4. *Chitin characterization*

Chitin was characterized through determination of ash and protein contents, hydrolysis extent, purity, thermogravimetric analysis (TGA), Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectroscopy, powder X-ray diffraction (XRD), and scanning electron microscopy (SEM), as described on Chapter 3, Part III, sections 3.5.2 and 3.6.. Characterization experiments were also performed on a standard (chitin STD) for comparison purposes.

3.5. *In vitro* bioactivity evaluation

3.5.1. *Oxygen radical absorbance capacity (ORAC) assay*

ORAC assay was performed as previously descried (Chapter 2, Part II, section 3.8.1.), using 2,2'-azobis(2-methylpropionamidine) dihydrochloride as peroxyl radical generator and fluorescein sodium salt as probe. Samples were analysed in duplicate and results were expressed as $\mu mol_{trolox\ equivalents}/L$ or as $\mu mol_{trolox\ equivalents}/g_{extract}$.

3.5.2. *Human cell-based assays*

3.5.2.1. Cell culture

Human intestinal Caco-2 and HT-29 cell lines were cultured as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of heat-inactivated foetal bovine serum (FBS), 1% of non-essential amino acids (NEAA), and 1% penicillin-streptomycin (in the case of Caco-2 cells), as previously described on Chapter 2, Part I, section 3.4.1..

3.5.2.2. Sample preparation

Sample stock solutions/dispersions were homogenized in DMEM supplemented with 0.5% of FBS and 1% of NEAA, immediately before the assays. When required, solutions/dispersions were heated to 37 °C for a few minutes and thoroughly vortexed. To obtain a range of concentrations, samples were further diluted in DMEM supplemented with 0.5% of FBS and 1% of NEAA.

3.5.2.3. Cytotoxicity assay

Caco-2 cells were incubated with different concentrations of samples for 24 h, as described on Chapter 2, Part I, section 3.4.3.. Cell viability was evaluated using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, by measuring absorbances at 490 nm. Cell viability was expressed as percentage of viable cells relative to the control. Three independent assays were performed in triplicate and half maximal effective concentrations (EC50) were calculated from dose-response curves. Results were expressed as $mg_{extract}/mL$.

3.5.2.4. Antiproliferative assay

HT-29 cells were used at conditions of exponential growth to evaluate the antiproliferative effect of samples, as previously described (Chapter 2, Part I, section 3.4.4.). Briefly, cells were

incubated with different concentrations of samples for 24 h and cell proliferation was determined as described for cytotoxicity assay. Three independent experiments were performed in triplicate and EC50 values were calculated from dose-response curves.

3.5.2.5. Anti-inflammatory assay

Experiments were performed as fully detailed on Chapter 2, Part I, section 3.4.8. Briefly, confluent and differentiated Caco-2 cells were stimulated with a pro-inflammatory cocktail and co-incubated with extracts (2 mg/mL) for 48 h. Interleukin-8 (IL-8) secretion was quantified on cell supernatants by enzyme-linked immunosorbent assay. Three independent experiments were performed in triplicate and results were expressed as percentage of secretion in relation to the positive control.

3.5.3. *Bacterial assays*

3.5.3.1. Bacterial test strains

Staphylococcus aureus ATCC 6538 and *Escherichia coli* ATCC 8739 strains were selected as representative of Gram-positive and Gram-negative species, respectively.

3.5.3.2. Sample preparation

Solutions/dispersions were prepared in cation-adjusted Mueller Hinton broth (CAMHB, BD Difco, Sparks, NV, USA), immediately before antimicrobial susceptibility testing assays. Pipetting up and down 20 times was performed to ensure a homogeneous stock solution/dispersion. When necessary, samples were heated at 35 °C for 5 min. Solutions/dispersions were then 2-fold serially diluted in CAMHB to obtain a range of concentrations.

3.5.3.3. Antimicrobial susceptibility assay

Assays were performed as previously described (Chapter 2, Part II, section 3.8.2.3.). Briefly, different concentrations of samples were dispensed on microtiter plates, which were then inoculated with *S. aureus* or *E. coli*. Plates were incubated under aerobic conditions at 37 °C, for 16 to 20 h. Minimum inhibitory concentrations (MIC) were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited after incubation. An additional MIC value, denominated MIC*, was also determined and defined

as the lowest concentration of an antimicrobial agent at which bacterial growth was visually and differentially affected, when compared to the positive control. Three biological replicates were performed, and median results were expressed as μL_{sample}/mL.

3.6. Mass and energy balances

Mass balances were calculated following the general conservation equation (Equation 3), while heat energy balances were estimated based on the transferred heat equation for mixtures (Equation 4)**.**

$$m_{out} = m_{in} + m_{generated} - m_{consumed} - m_{accumulated} \quad \textbf{Equation 3}$$

$$Q = \sum_{i=1}^{n} m_i \int_{T_i}^{T_f} Cp_i \, dT \pm \Delta h_{vap} \quad \textbf{Equation 4}$$

Where, Q is the transferred heat (J), T_i and T_f are the initial and final temperatures, respectively (°C), m_i is the mass of component *i* (g), Cp_i is the specific heat of component *i* (J/g·°C), and Δh_{vap} is the vaporisation or condensation enthalpy (J/g), which may be disregarded if there are no phase transitions[20]. Although the heat of mixing (heat of solution) of liquid mixtures and solutions may be significant, there were no tabulated values for these mixtures (or equivalent) that would allow to include these variables on the calculations.

3.7. Life cycle assessment

LCA studies were conducted following the methodological recommendations given by ISO 14040 and 14044 standards[21,22]. Consequential modelling was primarily applied[23] in the inventory analysis, for all data regarding the background system, including the data pertained to water supply, chemicals, energy carriers, and wastewater treatment, as presented by Ekvall and Weidema[24] and Weidema *et al.*[24], using the consequential version of the ecoinvent database (ecoinvent, Zurich, Switzerland), Agri-footprint database (Agri-footprint | Blonk Consultants, Gouda, The Netherlands), and SimaPro version 9.1 (PRé Sustainability, Amersfoort, The Netherlands). The ReCiPe method, comprising 18 midpoint categories, was applied to compute the midpoint life cycle environmental impacts, following the Hierarchist approach[26].

The processes under study and the system boundaries for sardine heads and offal and brown crab shell residues are schematically shown on Figures 1 and 2, respectively, where the

different aspects of the integrated processes, as well as inputs and outputs, are represented. The "cradle-to-gate" approach was used, which included all the supporting activities such as the production of solvents, chemicals, and energy sources, as well as the different steps inbuilt in the proposed process for the valorisation of sardine heads and offal or brown crab shells.

The functional unit was chosen based on the amount of residue that entered the system and that was used as feedstock, *i.e.*, 149.3 g of fresh sardine heads and offal or 100.8 g of fresh crab shell residue. As a proof of concept, the lab scale integrated process was modelled as a batch production process.

The primary data sources in the inventory analysis were obtained from the original data (experimental tests), ecoinvent databases, literature review, and estimations. The detailed data sources, modelling, and assumptions made for both processes are reported on Appendix G, Tables G1 – G4.

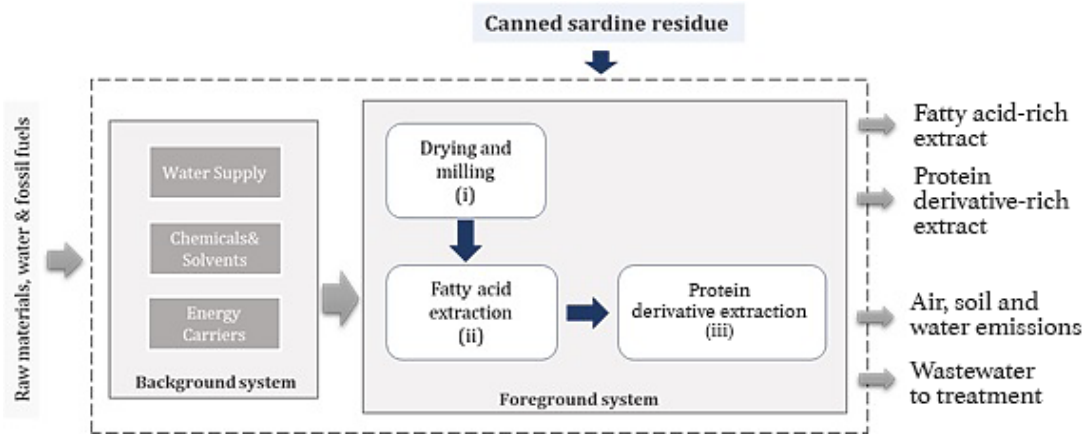

Figure 1. System boundaries and flow of the integrated processes proposed for sardine heads and offal valorisation (cradle-to-gate approach).

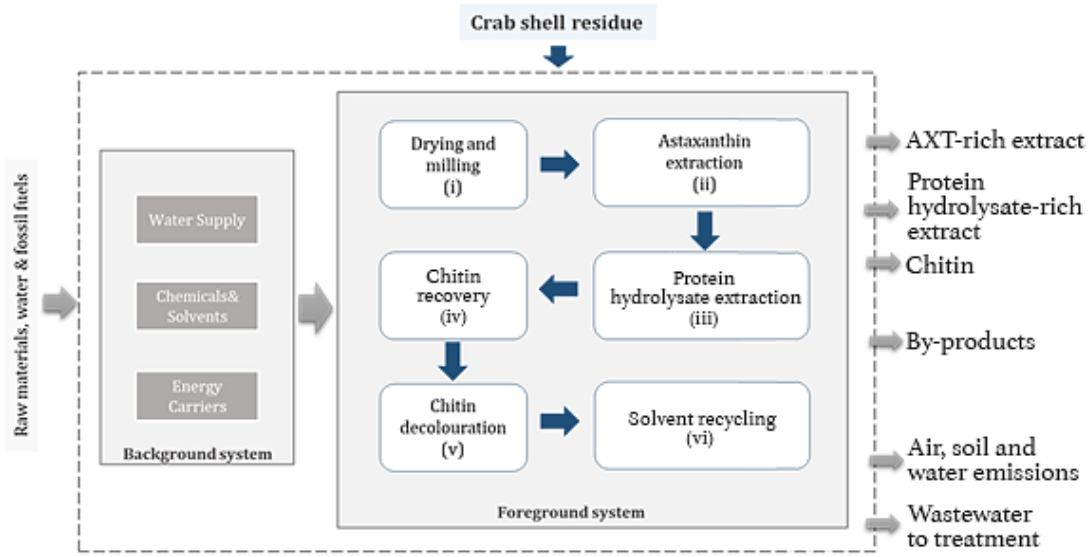

Figure 2. System boundaries and flow of the integrated processes proposed for brown crab shells valorisation (cradle-to-gate approach).

It is also important to mention that as waste streams, both sardine and crab residues come into the system with no associated environmental burdens, *i.e.*, it was considered that the environmental burdens fell solely on the sardine and crab fishery industry, which produced them.

3.8. Statistical analysis

GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to estimate the cytotoxicity and antiproliferative activity, as well as the statistical significance of average differences. Statistical significances were calculated either by one-way analysis of variance followed by the Tukey test, or by using an unpaired *t*-test. An alfa error of 5% was accepted in the hypothesis testing to decide for a significant effect. Data was reported as mean ± standard deviation values.

4. Results and discussion

4.1. Experimental scale-up and process integration

In order to validate the methodologies developed in Chapters 2 and 3 and to establish a proof of concept, an experimental scale-up of the most promising processes involved on the valorisation of both sardine and crab shell residues was performed. Although it was not

possible to conduct pilot scale experiments due to equipment limitations, an up to 70-fold scale increase was performed, by moving from the 0.5 – 10 $g_{feedstock}$ range to the 35 – 50 $g_{feedstock}$ range, using up to 1.9 $L_{solvent}$.

These experiments aimed at reproducing the results obtained at smaller scale in terms of extraction yield, and extracts' chemical composition and/or physical/bioactive properties. To achieve this goal, the most promising extraction parameters, such as temperature, pressure, solid-liquid ratio, and extraction time were preserved, while other parameters, including the amount of feed, solvent amount or solvent flow rate, and the extraction bed dimensions (whenever possible) were increased. The processes and the extraction conditions were selected based on what was previously discussed on the previous chapters, and decisions were made taking into account the scale limitations of each equipment as well as the best combination between extraction yields, extracts' composition, and extracts' bioactive or physical properties (Figure 3). Furthermore, after scale-up experiments, the same processes were integrated, aiming at minimising the consumption of feedstock.

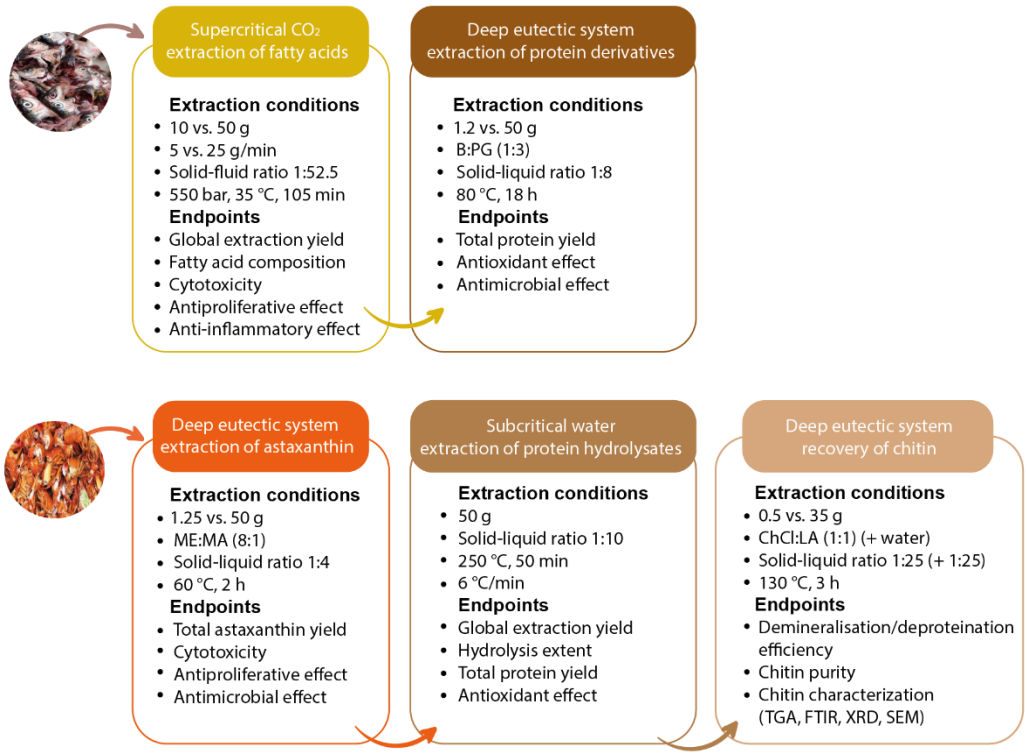

Figure 3. Schematic representation of the most promising processes developed for the valorisation of sardine heads and offal and brown crab shell residues.

4.1.1. *Valorisation of sardine heads and offal residues*

4.1.1.1. Processes description

The flow of operation of the proposed processes for sardine heads and offal residues valorisation is illustrated on the block diagram represented on Figure 4.

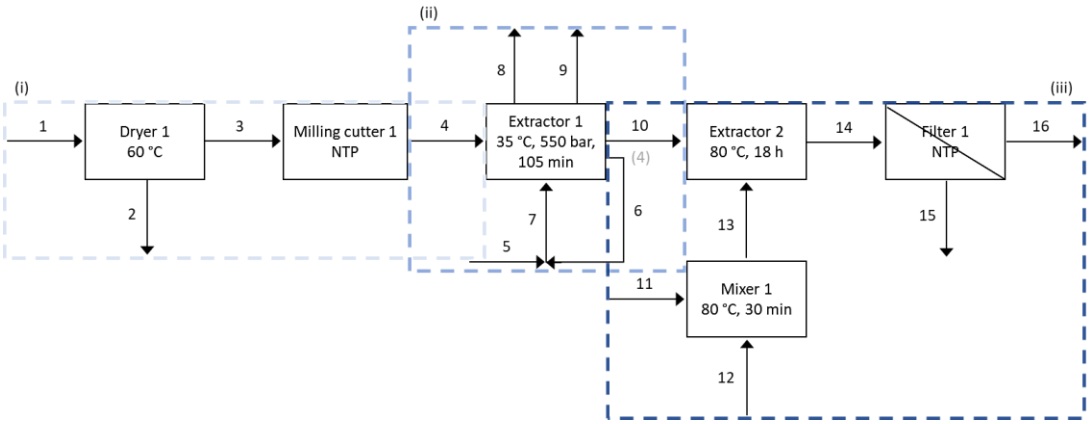

Figure 4. Block diagram of the processes involving canned sardine residues. (i) Drying and milling process, (ii) sc-CO_2 extraction of fatty acids, and (iii) DES extraction of protein derivatives. Stream (4), represented in light grey, refers only to the isolated process.

The first step of both isolated and integrated processes involved the drying and milling of the biomass. It is known that the efficiency of an extraction process may be impaired by the moisture content or the particle size[27], therefore, these parameters should be controlled. The dried and milled residue was then directed to the first extractor, in which fatty acids were recovered from sardine residues, using sc-CO_2 as solvent. After extraction, the CO_2 flow stream was expanded, resulting in a fatty acid-rich extract (stream 8) – one of the final products – and in a sardine residue (stream 10), a good source of proteins and minerals. In the isolated process, the latter may be considered as waste, or directed, at best, to agriculture or animal feed applications, while in the integrated process the remaining residue may be further used for protein derivatives extraction.

Protein derivatives were recovered from sardine residues in Extractor 2 using B:PG (1:3) DES, previously prepared in Mixer 1, either using the dried and milled residue (isolated process, stream (4)) or the residue resulting from the sc-CO_2 extraction of fatty acids (integrated process, stream 10). After DES extraction, the protein derivative-rich extract was separated from the solid residue by filtration, resulting in a protein derivative-rich extract (stream 16)

– the second final product, composed of protein-derived ingredients and the DES used as solvent – and in a mineral-rich residue (stream 15), which could still be applied in the agriculture sector.

4.1.1.2. Fatty acid extraction with sc-CO_2

Aiming at obtaining bioactive fatty acid-rich extracts, sc-CO_2 was applied to 10 or 50 g of freeze-dried canned sardine residues. Although the extraction vessel used had capacity for 500 mL, due to pump limitations, it was not possible to work at a larger scale. The operating conditions were set at 35 °C and 550 bar, for 105 min, at a solid-fluid ratio of 1:52.5, since, as previously discussed on Chapter 2, Part I, these settings allowed to obtain the highest yield amongst the extraction conditions tested, while maintaining interesting bioactive properties regarding antiproliferative and anti-inflammatory effects.

Figure 5i shows that higher cumulative extraction yields could be obtained at a faster rate when 50 g of residue were used as feed, reducing the extraction time in 45 min, given the higher superficial velocity caused by the higher CO_2 flow rate applied (Table 2 shows the process parameters for extractions performed at both scales). In fact, it is known that the solvent velocity can have a direct effect on the convective mass transfer coefficient, axial dispersion, and accumulation in the supercritical phase[18,28]. However, due to shorter CO_2 residence times, this reduction on extraction time was only achievable at the expense of higher operational costs, not only due to a pump energy increase, but also a higher amount of CO_2 consumed (approximately 2-fold), as shown on Figure 5ii.

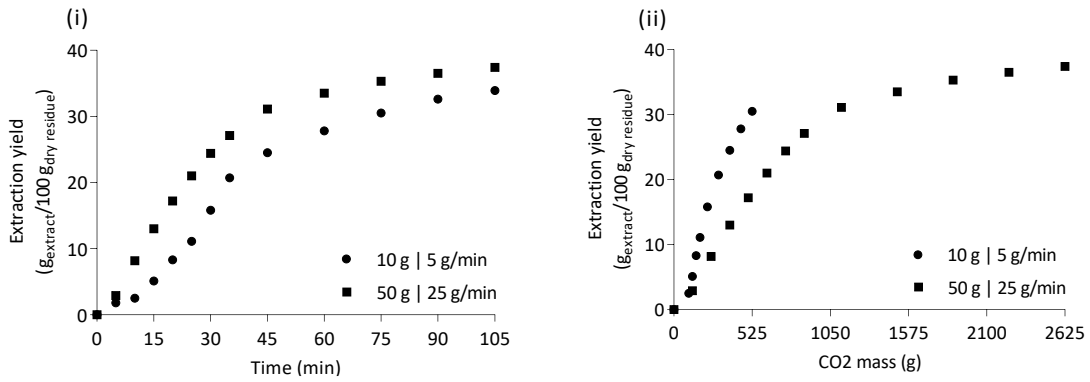

Figure 5. Cumulative extraction yields as a function of (i) time and (ii) CO_2 mass, obtained at 35 °C, 550 bar, and a solid-fluid ratio of 1:52.5.

Table 2. Bed characterization and process parameters for the sc-CO_2 extraction of sardine residues at both scales, at 35 °C and 550 bar.

Feed mass (g)	10	50
CO_2 flow rate (g/min)	5	25
CO_2 mass (g)	525	2625
Extractor volume (cm^3)	500	
Extractor height (cm)	24.5	
Extractor diameter (cm)	5.1	
Bed cross section area (cm^2)	20.4	
S/F [(g_{CO2}/min)/g_{feed}]	0.50	0.50
CO_2 velocity (cm/min)	0.24	1.20
Residence time (min)	1.30	0.65

As previously mentioned, to ensure that a process is reproducible and consistent at a higher scale, there are some guidelines that should be followed. However, deciding which criteria to maintain or vary requires in-depth knowledge of the factors that might limit the extraction process (either of thermodynamic or mass transfer nature). In the case of sc-CO_2 extraction, when solubility limits the process, the ratio between the mass of sc-CO_2 spent and the feed mass should be kept constant when moving from laboratory to industrial scale; whereas when diffusion is the major limitation, the ratio of sc-CO_2 flow rate to feed mass should be kept constant[18,28]. Figure 5i shows that in the first 35 min the extraction rate was controlled by solubility and external mass transfer resistance. However, as extraction progressed (between 35 and 60 min), the controlling factors were diffusion and the internal mass transfer resistance, thus resulting in a much lower extraction rate[29]. Since what conditioned extraction the most was essentially the slow diffusion process, even though the solvent was not saturated, the scale-up criterion adopted consisted in maintaining the ratio between the CO_2 flow rate and the mass of feed constant. The fact that extraction curves are not exactly similar is probably related to the variation of the extraction bed geometry, as smaller and larger scale extractions were performed using the same extraction equipment, thus implying a higher L/D ratio for the larger scale; to differences in biomass aggregation or compaction; or to distinct residence times (smaller residence times for the larger scale).

In order to confirm that the quality of the extract in terms of fatty acid composition remained unchanged after the upscaling, samples were analysed by GC-FID. Table 3 summarizes the major compounds found in both extracts and shows that their composition was very similar. Palmitic acid was the most prominent fatty acid, followed by eicosapentaenoic, myristic,

palmitoleic, oleic, and docosahexaenoic acids, which accounted for 73% of the extracts' total fatty acid composition. A more complete fatty acid profile can be found on Appendix H, Tables H1 – H2.

Table 3. Major fatty acids in sardine sc-CO_2 extracts obtained at 35 °C, 550 bar, and solid-fluid ratio of 1:52.5, quantified by GC-FID. Results were expressed as $mg_{fatty\ acid}/g_{extract}$.

Fatty acid (FA)	10 g | 5 g/min	50 g | 25 g/min
C14:0 Myristic acid	92.4	92.8
C16:0 Palmitic acid	207.9	210.3
C16:1 (9) Palmitoleic acid	85.0	89.9
C18:0 Stearic acid	41.0	38.8
C18:1 (9) Oleic acid	84.2	84.0
C18:1 (11) cis-Vaccenic acid	32.8	32.3
C18:4 (6, 9, 12, 15) Stearidonic acid	13.8	14.2
C20:1 (9) Gadoleic acid	20.2	19.2
C20:5 (5, 8, 11, 14, 17) Eicosapentaenoic acid	97.2	92.1
C22:1 (11) Cetoleic acid	15.8	14.4
C22:6 (4, 7, 10, 13, 16, 19) Docosahexaenoic acid	65.9	58.0
Other FA	105.3	109.8
Saturated FA	377.8	378.0
Monounsaturated FA	266.1	272.3
Polyunsaturated FA	217.4	205.4
TOTAL FA	861.3	855.8

In order to determine the potential cytotoxic effects of the obtained fatty acid-rich extracts, cell-based experiments were performed using confluent and non-differentiated Caco-2 cell monolayers. EC50 values summarized on Table 4 show that both extracts presented similar cytotoxicity, with values ranging from 7.0 to 8.4 mg/mL.

Table 4. EC50 values (mg/mL) obtained for sardine fatty acid-rich extracts obtained at 35 °C, 550 bar, and solid-fluid ratio of 1:52.5 evaluated on Caco-2 and HT-29 cells, after an incubation period of 24 h.

	Cytotoxicity (Caco-2 cells)	**Antiproliferative effect** (HT-29 cells)
10 g	7.0 ± 1.2	2.4 [a]
50 g	8.4 ± 0.3	3.5 ± 0.3

[a] GraphPad Prism 9 software was not able to calculate a standard deviation

Non-cytotoxic extracts' concentrations were then tested on HT-29 cells, with results showing that both extracts were able to decrease the viability of human colorectal adenocarcinoma cells at similar concentrations, with EC50 values ranging from 2.4 to 3.5 mg/mL (Table 4).

The last endpoint to be evaluated was the potential of fatty acid-rich extracts to reduce the secretion of IL-8, a pro-inflammatory chemokine which is commonly used as biomarker of inflammatory bowel disease[30], upon pro-inflammatory stimulus. As represented on Figure 6, both extracts were able to decrease by more than half IL-8 levels, when compared to the positive control.

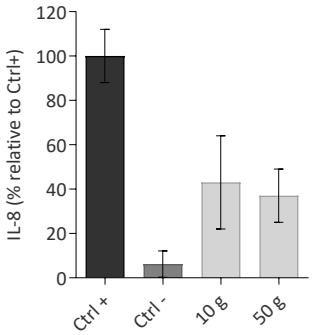

Figure 6. Modulation of IL-8 by sardine fatty acid-rich extracts obtained at 35 °C, 550 bar, and solid-fluid ratio of 1:52.5, after 48 h incubation of differentiated Caco-2 cells. No statistically significant differences between extracts were observed.

4.1.1.3. Protein derivatives extraction with DES

As previously discussed on Chapter 2, Part II, the DES that allowed the recovery of a higher protein yield was B:PG (1:3) at 80 °C for 18 h. Furthermore, although a solid-liquid ratio of 1:80 has shown better results in terms of protein yield, preliminary experiments performed on *S. aureus* and *E. coli* demonstrated that extracts prepared at a solid-liquid ratio of 1:8 could yield similar or even better results on *S. aureus* and *E. coli*, respectively (data not shown), possibly due to a higher concentration of protein derivatives in the extract. Therefore, aiming at obtaining protein derivative-rich extracts, in the experimental scale-up, the most promising conditions of temperature and extraction time were applied, while using a solid-liquid ratio of 1:8, thus allowing a reduction of the amount of solvent by 10-fold. Figure 7i compares the protein yields obtained for both scales tested (smaller scale and 42-fold scale-up), as well as for the isolated and integrated process (using the residue that remained after sc-CO_2 extraction of fatty acids) and shows that although the results obtained for the small and larger scale were very similar, the integrated process resulted in a significantly higher protein yield. Similar findings have already been reported by different authors for protein extracted from edible insects[31], squid viscera[32], mackerel skin[33], among

others, and have been mainly attributed to an increased accessibility of the solvent to extractable solutes, due to the lower content of interfering lipophilic molecules[34].

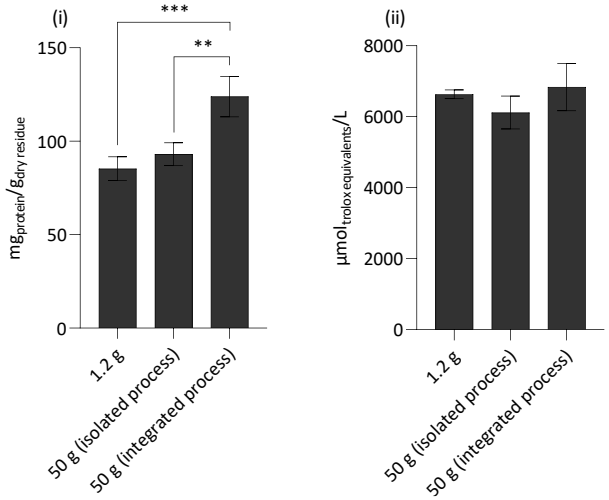

Figure 7. (i) Total protein yields and (ii) antioxidant capacity evaluated by ORAC assay of the protein derivative-rich extracts obtained from sardine residues with B:PG (1:3) at 80 °C for 18 h and a solid-liquid ratio of 1:8. The significant differences between extracts are represented by asterisks (*). ** $P < 0.01$; *** $P < 0.001$.

However, even though the extract obtained by the integrated process allowed to reach a higher protein yield, that increase was not reflected in the extract's antioxidant activity (Figure 7ii). It is possible that the extract resulting from the isolated process (at both small and large scale) could have contained a small amount of antioxidant lipids (which were not present in the integrated process' sample, as they had been previously removed by sc-CO_2 extraction), thus increasing the antioxidant activity of these extracts, even though they presented a lower protein yield when compared to the integrated process. Conversely, when tested on *S. aureus* and *E. coli*, the integrated process extract showed a different antimicrobial potential, when compared to the isolated process samples. As represented on Figure 8, while the isolated process resulting extracts showed a higher potential to inhibit the growth of *S. aureus*, the integrated process extract revealed to be more efficient in inhibiting *E. coli* growth. In fact, it is known that defatting a raw material prior to extraction or even a protein concentrate, may lead to changes in extracts bioactivity[34] and functional properties[35]. A possible explanation for these results is related to what was previously mentioned for the samples' antioxidant response, *i.e.*, the extraction of a small amount of antimicrobial lipids during the isolated process (at both small and large scales), might have

led to a different behaviour towards the studied bacteria. Another possible justification might be linked to the fact that proteins can be destabilized by sc-CO_2, leading to unfolding and denaturation[36,37], which might have had an impact on the way protein derivatives contained in the extract resulting from PI interacted or permeated into the microorganisms' membranes, thus leading to a different bioactive response.

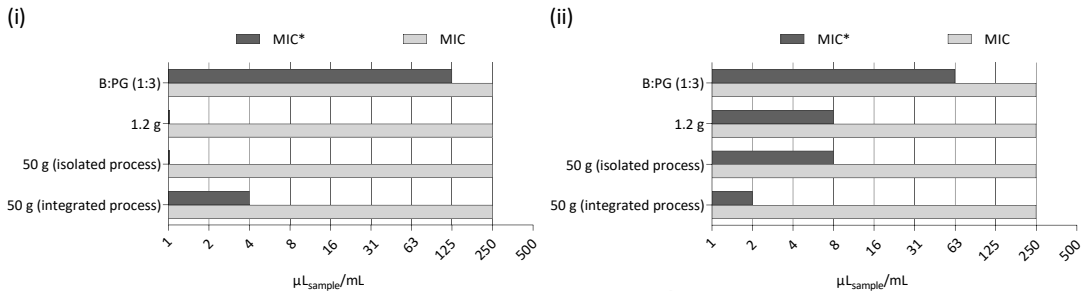

Figure 8. MIC* and MIC values of B:PG (1:3) and protein derivative-rich extracts obtained at 80 °C for 18 h and a solid-liquid ratio of 1:8 in the bacterial targets: (i) *S. aureus* ATCC 6538 and (ii) *E. coli* ATCC 8739.

Overall, the integrated process involving sardine residues allowed a 1.3-fold increase in the quantity of final products obtained, while using the same amount of biomass feedstock. Even though the protein derivative-rich extract revealed to have a different potential towards Gram-positive and Gram-negative bacteria than the extract obtained through the application of the isolated process, the obtained product still showed an interesting antimicrobial effect and could still be applied in high value markets, such as in nutraceuticals or cosmetic products.

4.1.2. *Valorisation of crab shell residues*

4.1.2.1. Processes description

Figure 9 represents the block diagram that illustrates the flow of operation of the proposed isolated and integrated processes for the valorisation of crab shell residues.

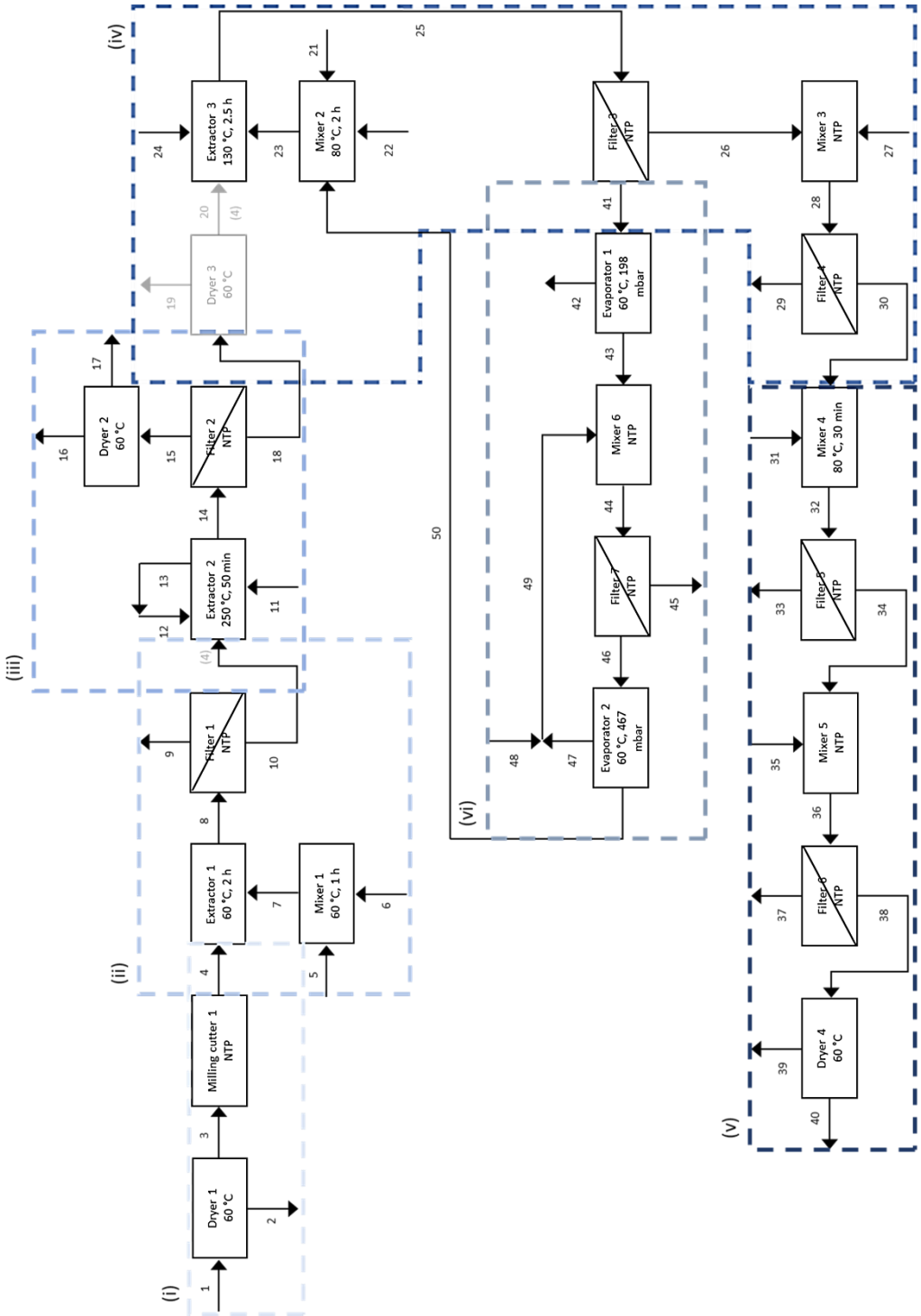

Figure 9. Block diagram of the processes involving brown crab shell residues. (i) Drying and milling, (ii), DES extraction of AXT, (iii) scW extraction of protein hydrolysates, (iv) recovery of chitin with DES, (v) decolouration of chitin, and (vi) ChCl:LA (1:1) recycling. Operation units or streams represented in light grey refer to only one of the processes (either isolated or integrated).

Similarly to the processes involving sardine residues, the first step of crab shell processes also included the drying and milling of the residues. The milled biomass was then used for AXT extraction in Extractor 1, using ME:MA (8:1) DES as solvent, which was previously prepared in Mixer 1. After extraction, the AXT-rich extract was separated from the solid residue by filtration, resulting in an AXT-rich extract (stream 9) – the first final product, comprising the extract and the DES used as solvent – and a crab shell residue (stream 10) – which was still a good source of proteins, chitin, and minerals. In the isolated process, stream 10 was considered to be a waste (due to ME:MA contamination), while in the integrated process, the remaining residue was used for protein hydrolysates recovery in the subsequent extraction step.

Protein hydrolysates were extracted from the shells using scW as solvent in Extractor 2, under nitrogen pressure (stream 12), either using the dried and milled residue (isolated process, stream (4)) or the residue resulting from the DES extraction of AXT (integrated process, stream 10). The resulting extract was then separated from the solid residue by filtration and dried to obtain a protein hydrolysate-rich extract – the second final product (stream 17) – and a water vapour stream (stream 16). The remaining crab residue (stream 18), which still harboured considerable amounts of chitin and minerals, could be considered as waste, or eventually applied in agriculture, if following the isolated process. When applying PI, the shell residue could be dried (Dryer 3) and further used for chitin and mineral recovery.

Chitin was recovered either using the dried and milled residue (isolated process, stream (4)) or the dried residue resulting from the scW extraction of protein hydrolysates (integrated process, stream 20) with a ChCl:LA (1:1) DES, partly prepared in Mixer 2, partly recirculated after DES regeneration (stream 50). After a 2 h extraction (Extractor 3), the heating was switched off and water was added to the mixture (stream 24), continuing the extraction for further 30 min. The protein/mineral-rich extract was separated from the solid (mainly chitin and a small percentage of proteins and pigments) by filtration (Filter 3) and the isolated chitin was directed to Mixer 3 where it was washed with water until attaining a neutral pH, resulting in a stream of wastewater (stream 29) contaminated with ChCl:LA, and in a stream of impure chitin (stream 30).

In order to eliminate residual pigments and proteins that were not completely removed in Extractor 3, the impure chitin was subjected to a treatment with hydrogen peroxide (Mixer 4). After extraction, the solid was separated from the liquid by filtration resulting in a stream of waste hydrogen peroxide (stream 33) contaminated with the removed pigments and proteins, and a wet chitin stream (stream 34). Any residue of hydrogen peroxide was removed from chitin by washing the solid with water (Mixer 5) resulting, after filtration, in a chitin stream – the third final product – which was then dried (Dryer 4), and in a wastewater stream, contaminated with hydrogen peroxide (stream 37).

The final step involved the regeneration of the ChCl:LA (1:1) used for chitin extraction and the eventual recovery of calcium carbonate from the DES. The process started with the evaporation of the water contained in the mixture (Evaporator 1), resulting in a water vapour stream (stream 42) and in a DES stream (stream 43). After water removal, the calcium carbonate solubilized in the DES could be precipitated with ethanol (Mixer 6) and recovered after filtration (stream 45), while the ethanol and DES could be separated by evaporation (Evaporator 2) and then recycled back into the system. It is important to highlight that this final step was not performed experimentally. However, as it is technically possible[38–40], it was considered to be relevant for the environmental impact evaluation through LCA.

4.1.2.2. AXT extraction with DES

In order to achieve the highest AXT recovery, an extraction with ME:MA (8:1) DES at 60 °C for 2 h and a solid-liquid ratio of 1:4, was applied to dried crab shell residues, since these extraction conditions were able to produce extracts with yields equivalent to the conventional method used, *i.e.*, a Soxhlet extraction with acetone, as previously discussed on Chapter 3, Part I. Furthermore, ME:MA (8:1) have also shown the lowest toxicity towards human intestinal cells, while preserving its antimicrobial potential towards both Gram-positive and Gram-negative bacteria.

Results represented on Figure 10 show that an increase of 40-fold in scale did not lead to statistically significant changes in AXT yield. Nevertheless, it is important to note that changes in heat-transfer mechanisms are recurrent when scaling-up, which can affect the extraction efficiency[18,41]. Furthermore, overheating can occur in certain areas of the

extraction vessel[10], which can lead to the degradation of compounds that are more sensitive to temperature increase, such as AXT, possibly justifying the slight decrease in AXT yield obtained after the scale increase (Figure 10)[42].

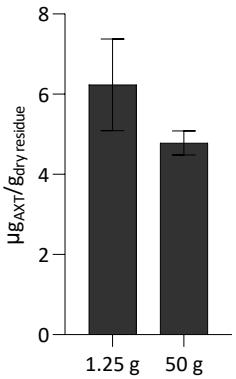

Figure 10. AXT yields determined by UV-vis spectroscopy in the extracts obtained from crab shell residues with ME:MA (8:1) at 60 °C for 2 h and a solid-liquid ratio of 1:4. No statistically significant differences between extracts were observed.

These extracts were then evaluated in terms of cytotoxicity (on Caco-2 cells) and antiproliferative effect (on HT-29 cells). The calculated EC50 values are summarized on Table 5 and show that, although the extracts presented a higher cytotoxicity when compared to ME:MA (8:1), their antiproliferative effect was very similar to the DES. Nevertheless, these results helped to confirm that the bioactivity of the extracts did not change with the scale-up, as both samples showed similar cytotoxicity and antiproliferative effect towards human intestinal cells.

Table 5. EC50 values (mg/mL) obtained for ME:MA (8:1) and AXT-rich extracts obtained at 60 °C for 2 h and a solid-liquid ratio of 1:4 evaluated on Caco-2 and HT-29 cells, after an incubation period of 24 h.

	Cytotoxicity (Caco-2 cells)	**Antiproliferative effect** (HT-29 cells)
ME:MA (8:1)	4.84 ± 0.74	0.58 ± 0.04
1.25 g	2.90 ± 0.52	0.65 ± 0.07
50 g	2.08 ± 0.91	0.54 ± 0.02

As a second endpoint, the antimicrobial potential of both DES and extracts were also studied on *S. aureus* and *E. coli*, a Gram-positive and a Gram-negative bacterium, respectively. Results illustrated on Figure 11 show that AXT-rich extracts maintained their antimicrobial

potential when the extraction process was performed at a higher scale, being this potential similar to ME:MA (8:1) when tested on *E. coli*, and higher when assessed towards *S. aureus*.

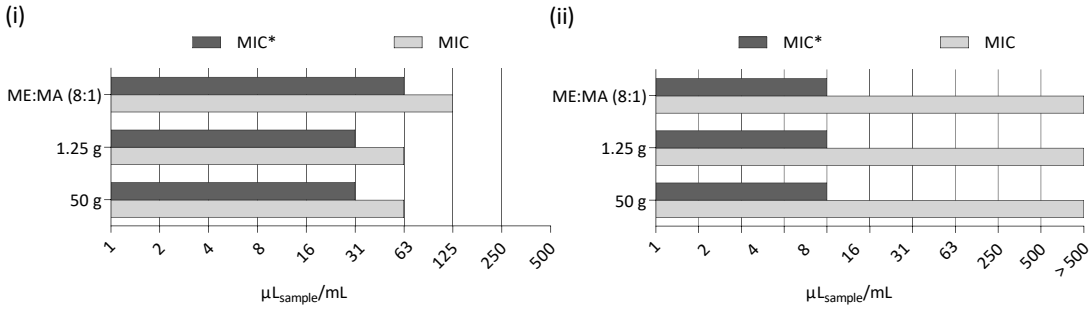

Figure 11. MIC* and MIC values of ME:MA (8:1) and AXT-rich extracts obtained at 60 °C for 18 h and a solid-liquid ratio of 1:4 in the bacterial targets: (i) *S. aureus* ATCC 6538 and (ii) *E. coli* ATCC 8739.

4.1.2.3. Protein hydrolysates extraction with scW

Due to equipment limitations, it was not possible to test more than one scale during scW experiments. Therefore, this section only presents a comparison between the isolated and integrated processes performed on 50 g of freeze-dried residue. As described in Chapter 3, Part II, 250 °C, a solid-liquid ratio of 1:10, and a heating rate of 6 °C/min were the conditions that allowed the highest global extraction yield, as well as the highest antioxidant activity. These conditions were, therefore, applied to freeze-dried crab shells and to the solid residue resulting from the extraction of AXT with DES, following an isolated and integrated process, respectively (Figure 12).

Figure 12 represents the different endpoints evaluated, namely global extraction yield (Figure 12i), hydrolysis extent (Figure 12ii), protein yield (Figure 12iii), and antioxidant activity (Figure 12iv), and shows that all outcomes were similar for both processes, thus suggesting that the extract resulting from the integrated process was not affected by the first extraction step.

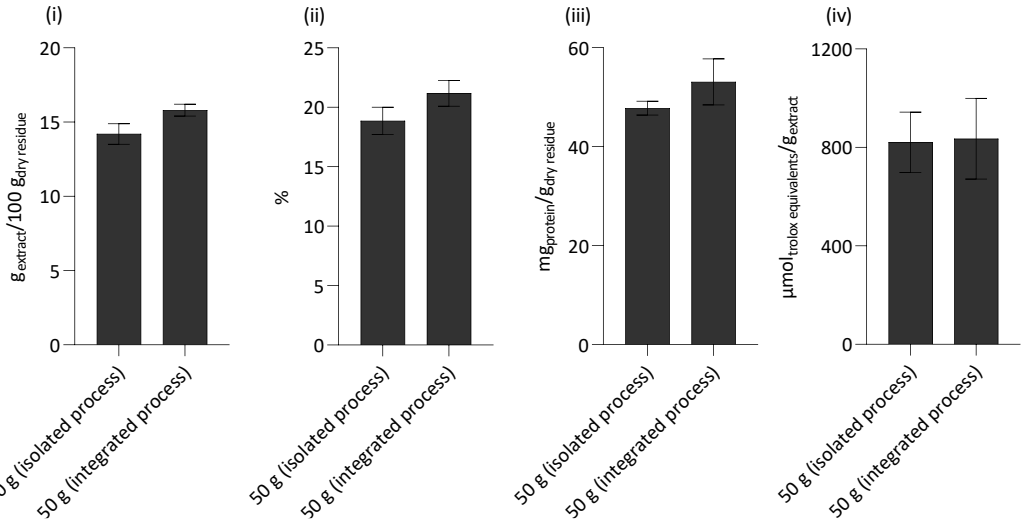

Figure 12. (i) Global extraction yields, (ii) hydrolysis extent, (iii) total protein yields, and (iv) antioxidant capacity evaluated by ORAC assay of the protein hydrolysate-rich extracts obtained from crab shell residues with subcritical water at 250 °C, a solid-liquid ratio of 1:10, and a heating rate of 6 °C/min. No statistically significant differences between extracts were observed.

4.1.2.4. Chitin recovery with DES

The last target molecule being isolated from crab shell residues was chitin. ChCl:LA (1:1) was identified in Chapter 3, Part III as being the most promising DES for both demineralisation and deproteination of shells, thus yielding chitin with high purity. In this way, isolation of chitin was performed with ChCl:LA (1:1) at 130 °C for 2 h and a solid-liquid ratio of 1:25. After extraction, chitin was washed with water and dried, and a treatment with hydrogen peroxide was performed aiming at removing residual impurities. Table 6 shows the results obtained not only for the small scale and the 70-fold scale-up, but also for the integrated process, using the residue that remained after scW extraction of protein hydrolysates as feedstock. Extractions performed on dried crab shells at both scales resulted in similar hydrolysis extents, as well as demineralisation/deproteination efficiencies and purity. However, results obtained after integrating AXT extraction, protein hydrolysates extraction, and chitin recovery showed that only 54% of proteins and other impurities could be removed from the shells, possibly resulting from a contamination with the hydrophobic DES used for AXT extraction. Therefore, the extent of hydrolysis and the purity of chitin were lower.

Table 6. Hydrolysis extent, demineralisation and deproteination efficiencies, and chitin purity obtained after extraction with ChCl:LA (1:1) at 130°C for 2 h, with water addition (*c.a.* 30 min), and decolouration with hydrogen peroxide (30 min).

	Hydrolysis extent (%)	Demineralisation efficiency (%)	Deproteination efficiency (%) [a]	Chitin purity (%)
0.5 g	89.2	99.7	100.0	98.2
35 g (isolated process)	88.0	100.0	97.5	95.2
35 g (integrated process)	82.1	100.0	54.0	63.6

[a] Includes not only proteins but also other impurities/contaminants (*e.g.*, fatty acids, pigments)
Coefficient of variation ≤ 5.4%

Aiming at identifying the contaminants that remained in the chitin obtained by the integrated process, different techniques were applied for its characterization, including, TGA, FTIR, XRD, and SEM, and were then compared to a standard, as well as to the sample obtained by the isolated process (Figure 13).

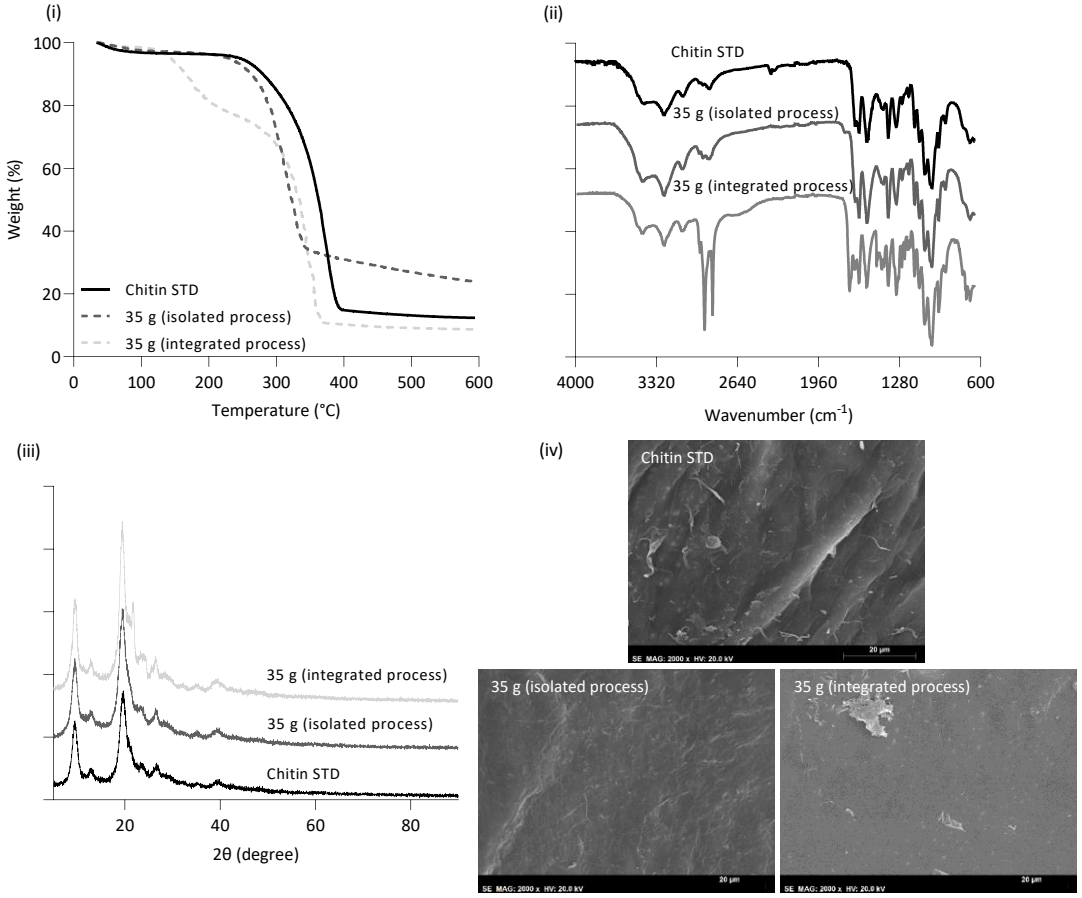

Figure 13. (i) TGA curves, (ii) FTIR-ATR spectra, (iii) XRD profiles, and (ii) SEM images of chitin STD and chitins obtained after extraction with ChCl:LA (1:1) at 130°C for 2 h, and decolouration with hydrogen peroxide.

TGA curves represented on Figure 13i show that the chitin STD and the chitin obtained by the isolated process presented similar profiles, although the chitin isolated with DES presented a slightly lower thermal stability, starting to degrade at 233 °C, as opposed to 258 °C in the case of chitin standard, which might be related to the molecular weights of the different samples.

When analysed by FTIR and XRD (Figures 13ii and iii, respectively), chitin STD and chitin obtained by the isolated process have also shown similar profiles, presenting all typical absorption bands and diffraction peaks of α-chitin[40,43–47], as previously discussed in detail in Chapter 3, Part III, section 4.3.. However, the profiles presented by chitin obtained by the integrated process after TGA, FTIR, and XRD analysis (Figures 13i – iii) displayed some differences when compared to chitin STD or to the sample obtained by the isolated process. In particular, the TGA curve showed a stage of degradation that started at a much lower temperature, around 132 °C, suggesting a contamination of chitin, which can be supported by the low efficiency of deproteination (whose aim was to remove not only proteins, but also other impurities). Furthermore, FTIR spectra and XRD diffractograms also presented absorption and diffraction peaks, respectively, that are not characteristic of chitin, namely at 2915 and 2848 cm^{-1} ($–CH_2$ symmetrical and asymmetrical stretching vibration, respectively), and 1699 cm^{-1} (C=O stretching vibration, typical of carboxylic acids) for FTIR, and $2\theta \approx 21.8°$ and $2\theta \approx 23.5°$ for XRD. The combination of the results obtained by these three analytical techniques suggest that the chitin isolated by PI eventually became contaminated with myristic acid, one of the components of the DES used for AXT extraction, which can be supported by the existent literature on this fatty acid[48–50], thus confirming what was previously hypothesized. Since menthol has a higher solubility in water than myristic acid (456 mg/L vs. 22 mg/L)[51,52], it is possible that it solubilised in the protein hydrolysate-rich extract during scW extraction, although this had no effect on the biological activity of the extract (Figure 12iv); while myristic acid probably precipitated and remained in the solid state, being isolated together with chitin. To improve the integrated process and increase the purity of the recovered chitin, it would be, therefore, advisable to wash the shell biomass with an organic solvent, such as ethanol, prior to the scW extraction, in order to remove any myristic acid residues.

Regarding SEM images (Figure 13iv), both chitins isolated with DES showed similar morphology with smooth and porous surface structures, due to the removal of minerals and proteins, whereas chitin STD displayed a rough non-porous surface. Nevertheless, as previously discussed in Chapter 3, Part III, section 4.3., a porous structure may be important if applications such as adsorption of metal ions or dyes are envisioned[40,43,46].

Altogether, the results obtained for the integrated process involving crab shell residues suggest that the quantity of final products produced could be increased by 3-fold, while starting from the same amount of feedstock. However, it is important to highlight that the purity of chitin was heavily affected by PI, which will certainly have a strong influence on its value as well as on its possible applications. Therefore, it would be interesting to explore, as future work, the economic impact of both processes in order to understand if it would be economically viable to implement the integrated process (with or without an additional washing step), even with chitin's potential being compromised by its lower purity.

4.2. Life cycle assessment

The LCA study performed aimed at quantifying the environmental impacts of the novel processes developed for the extraction of the different target molecules, towards the valorisation of sardine heads and offal and brown crab shell waste streams, as well as at understanding how the environmental impacts of the proposed processes could be improved before moving towards the development and potential implementation of these processes at commercial scales. Therefore, and even though the processes were at an early technology readiness level (TRL 4, *i.e.*, processes have been validated in laboratory environment)[13], this section describes and discusses the environmental burdens of each process, and identifies the hotspots to provide suggestions for further improvement, thus preventing propagation of potential damaging effects to commercial scales. In fact, in recent literature, the application of LCA at low TRL has been gaining increased attention, since it has the potential to enable the development of emerging technologies with improved environmental performances[13]. It is also important to note that the processes have been developed considering their transferability to pilot and industrial scales, *i.e.*, the selected set

of processing units is fully transferable to commercial stages. Therefore, the upscaling of such processes is a reliable hypothesis.

4.2.1. *Valorisation of sardine heads and offal residues*

The global inventory of the integrated processes targeting the valorisation of sardine residues is presented on Table 7. Furthermore, the results regarding the energy requirements are shown on Appendix I, Table I1, on which a breakdown per equipment and process stage is presented.

Table 7. Summary of the global inventory for the valorisation of sardine residues.

	Inputs from technosphere			**Outputs to technosphere**	
Feedstock, chemicals, and solvents	**Feedstock**		**Products and emissions**	**Final products**	
	Sardine residue (fresh weight)	149.3 g		Fatty acid-rich extract	15.3 g
	Chemicals and solvents			Protein derivative-rich extract	204.2 g
	CO_2	8.9 g		**Solid waste, organic**	
	Betaine	94.3 g		Sardine residue	108.4 g
	Propylene glycol	183.6 g		**Outputs to environment**	
				Water (to river)	99.3 g
Energy	Heating	475.1 kJ		CO_2 direct emissions	8.9 g
	Cooling water	665.2 kJ			

The absolute values for the life cycle impact assessment are reported on Appendix J, Table J1 and show that terrestrial ecotoxicity, land use, and global warming were the categories that contributed the most to the overall environmental burden of the processes involved on the valorisation of sardine residues. Aiming at understanding how those impacts came to be, as well as the relative contribution of the different production stages to the overall environmental performance, Figure 14 highlights the relative negative impacts and contributions associated to the various production steps onto each of the 18 environmental impact categories assessed by the ReCiPe method[26]. The protein derivatives extraction step, which was the least heating/cooling energy demanding process involved in the valorisation of sardine residues (Appendix I, Table I1), was the stage that carried the highest environmental burden (≥ 91%), noticed across all impact categories.

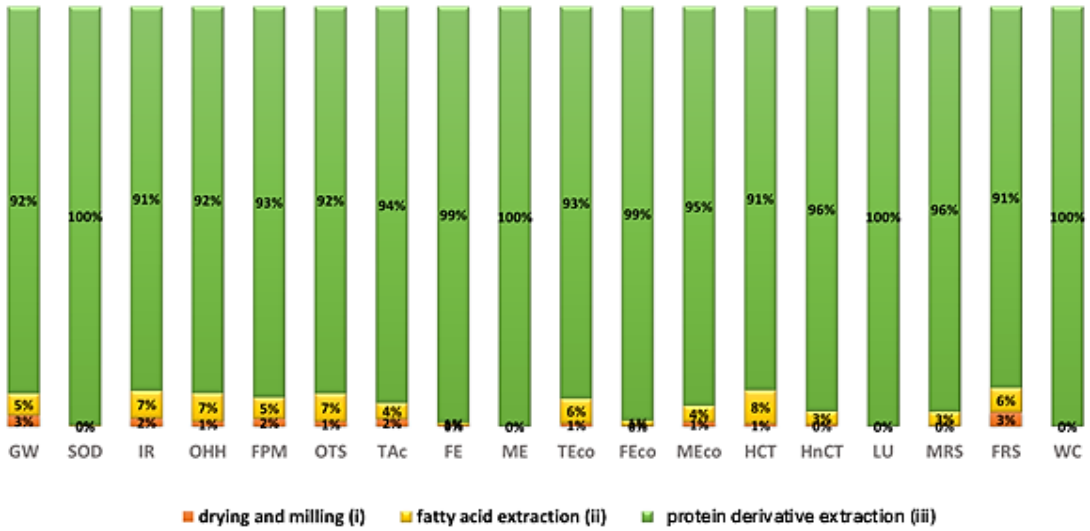

Figure 14. Relative contributions of each process step involved in the valorisation of sardine residues to the 18 environmental impact categories. Absolute values are reported on Appendix J, Table J1. (GW, global warming; SOD, stratospheric ozone depletion; IR, ionizing radiation; OHH, ozone formation, human health; FPM, fine particulate matter formation; OTS, ozone formation, terrestrial ecosystems; TAc, terrestrial acidification; FE, freshwater eutrophication; ME, marine eutrophication; TEco, terrestrial ecotoxicity; FEco, freshwater ecotoxicity; MEco, marine ecotoxicity; HCT, human carcinogenic toxicity; HnCT, human non-carcinogenic toxicity; LU, land use; MRS, mineral resource scarcity; FRS, fossil resource scarcity; WC, water consumption).

As shown on Figure 15, in which the most significant cradle-to-gate effects of each process step are represented (namely on global warming (Figure 15a), terrestrial ecotoxicity (Figure 15b), and land use (Figure 15c)), the use of DES based on betaine and propylene glycol was the main factor contributing to the high environmental impact of this process step. In particular, the impact category related to land use was the second most affected (after terrestrial toxicity, as shown on Appendix J, Table J1), since betaine is obtained directly from the processing of sugar beet molasses, a waste stream resulting from the processing of sugar beets to produce sugar[53], which contain around 3 to 6% of betaine in their composition[53,54]. Therefore, its environmental burden is closely related to sugar beets cultivation and sugar production. Conversely, it is noteworthy that betaine obtained from natural sources can have a 76 to 80% lower carbon footprint than the synthetic alternative pathways, outperforming in categories such as climate change; fossil, metal, and ozone depletion; marine and fresh water eutrophication; terrestrial acidification; among others, thus being a significantly less impactful and more sustainable product[55]. It is also important to mention that the database used to model the impact of betaine production[56] did not take into account that sugar beet molasses are a waste resulting from the production of sugar and,

for that reason, should have entered the system with no associated environmental burdens. Therefore, before moving towards commercial scales, it would be important to model the production of betaine considering that the environmental impacts associated to sugar beet molasses fall solely on the sugar industry, which produces them.

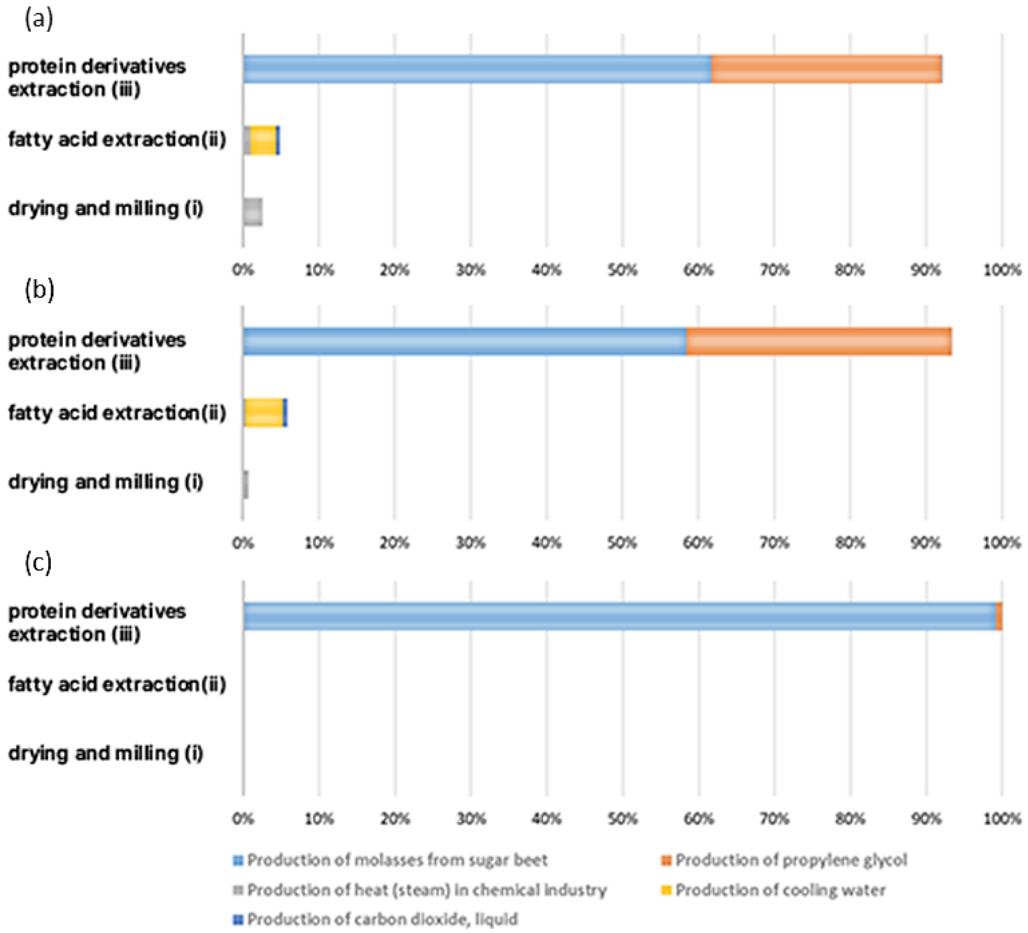

Figure 15. Most significant cradle-to-gate effects on (a) global warming, (b) terrestrial ecotoxicity, and (c) land use of using/performing the different activities involved in the valorisation of sardine residues (a cut-off of approx. 1% was used in (a), while no cut-off was used in (b) and (c))).

When looking at the two remaining processes, namely drying and milling, and the extraction of fatty acids, it is possible to conclude that their environmental impacts were mainly related to the production of heat and cooling water, which can be improved by a high degree of heat integration, thus allowing a higher energy saving and efficiency.

4.2.2. *Valorisation of crab shell residues*

As for sardine residues, the global inventory of the integrated processes targeting the valorisation of crab shell residues is summarized on Table 8, while the detailed energy requirements are reported on Appendix I, Table I1.

Table 8. Summary of the global inventory for the valorisation of crab shell residues.

	Inputs from technosphere			**Outputs to technosphere**	
Feedstock, chemicals, and solvents	**Feedstock**		**Products and emissions**	**Final products**	
	Crab shell residue (fresh weight)	100.8 g		AXT-rich extract	186.6 g
				Protein hydrolysates	20.7 g
	Chemicals and solvents			Chitin	7.2 g
	Menthol	169.1 g		**By-products**	
	Myristic acid	30.9 g		Protein	0.6 g
	Fresh water	3732.6 g		Minerals	32.4 g
	Choline chloride	112.5 g		Lipids	0.3 g
	Lactic acid	72.6 g		ME:MA (8:1)	0.6 g
	Ethanol	76.4 g		ChCl:LA (1:1)	169.5 g
	Hydrogen peroxide	57.1 g		Ethanol	76.4 g
				Water effluent to treatment	
Energy	Heating	6339.8 kJ		Wastewater	2184.7 g
	Cooling water	1457.5 kJ		ME:MA (8:1)	8.5E-03 g
				ChCl:LA (1:1)	15.6 g
				Astaxanthin	2.5E-04 g
				Protein	0.7 g
				Hydrogen peroxide	57.1 g
				Outputs to environment	
				Water (to river)	1599.7 g

As illustrated on Figure 16, which summarizes the relative contributions of each process step to the overall environmental impact, the extraction of AXT emerged as the most environmentally damaging process across all categories (between 25 and 99%), followed by chitin recovery (between 0 and 28%) and decolouration (between 0 and 24%), being terrestrial ecotoxicity and global warming the categories most affected (as reported on Appendix J, Table J1).

Through the analysis of Figure 17, in which the impact of the different activities related to each process step on global warming and terrestrial ecotoxicity are displayed, it can be concluded that the environmental burdens associated to AXT extraction were mainly related to the use of a DES based on menthol and myristic acid.

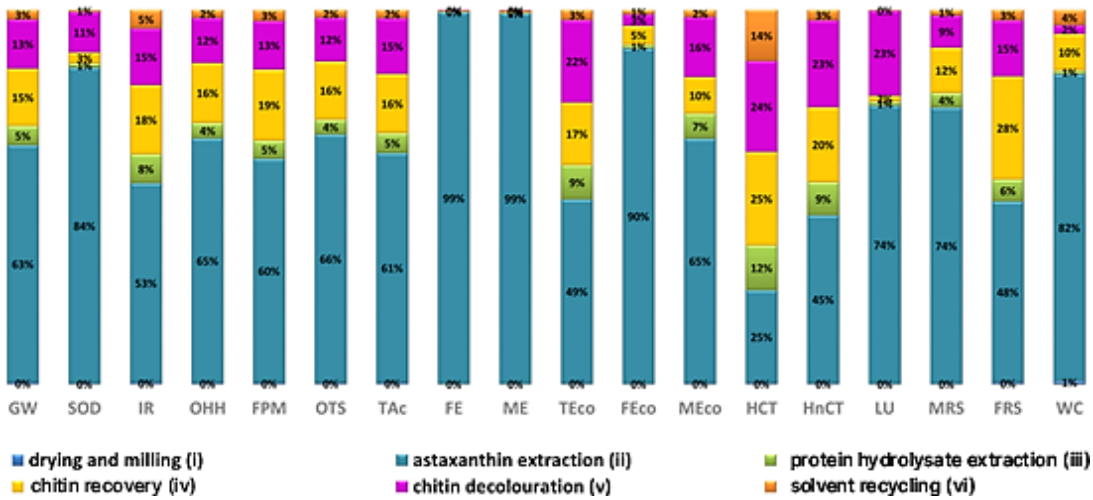

Figure 16. Relative contributions of each process step involved in the valorisation of crab shell residues to the 18 environmental impact categories. Absolute values are reported on Appendix J, Table J1.

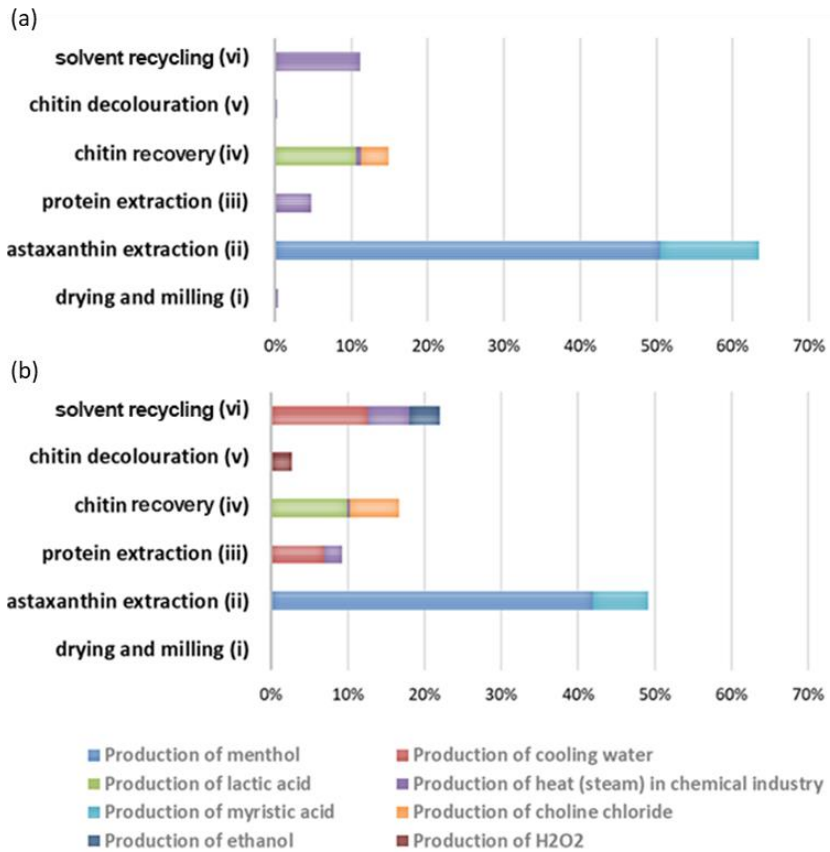

Figure 17. Most significant cradle-to-gate effects on (a) global warming and (b) terrestrial ecotoxicity of using/performing the different activities involved in the valorisation of crab shell residues (a cut-off of approximately 4% was used in (a) and 3% in (b)).

Regarding the steps involved in the recovery and decolouration of chitin, it is possible to conclude that the main contributing factors towards a higher environmental impact were the use of lactic acid and choline chloride during the chitin recovery step, or the use of hydrogen peroxide during chitin decolouration (Figure 17). The solvent recycling and the drying and milling were the steps that contributed the least to the overall process' impact, which in the first case was mainly due to the production of heat or cooling water and ethanol; while the impact of the latter was solely related to the use of heat energy during the drying process. For both processes, when moving towards commercial scale implementation, it would be possible to considerably reduce their environmental footprint if considering heat integration and ethanol recycling.

It is important to note that the accuracy of these results is extremely dependent on the data sources used to model the process, which in the case of menthol, myristic acid, or choline chloride, was performed by using the data available for similar systems from the ecoinvent database (Appendix G, Table G2). Therefore, the results presented might have been compromised by the data used and might be less accurate or possibly overestimated. Ideally, the complete value chain of these chemicals should be modelled. However, minimal public data exist on current industrial processes used to produce these compounds, which hinders their accurate modelling from a cradle-to-grave or -gate approach.

It is also important to mention that the aim of these LCA studies was not to provide exact values, but rather performing a hotspot analysis, thus providing a benchmark to be able to compare the impacts and shifts of the different processes' contributions at an early-stage design, therefore rendering essential insights for process design, development, and implementation at commercial stages.

Altogether, these LCA results suggest that the proposed processes for the valorisation of both sardine and crab shell residues might potentially become competitive, from an environmental perspective, after addressing some of the most critical points. Additionally, it is possible that the environmental burden of these processes could be significantly reduced when implemented at industrial scale due to the beneficial effect of economies of scale, the possibility of energy integration and water recycling and re-use, the replacement or reduction of fossil resources with renewable energy, the increased equipment efficiency

and reduction of resources and solvents/chemicals, the possible recycling and re-use of some of the solvents/chemicals, among others. Furthermore, comparisons with the LCA studies available in the literature are difficult and often inaccurate given the wide range of methodologies and approaches that can be applied (which have a direct impact on the results obtained). In this way, it would be important to model the most commonly used industrial processes for comparison purposes.

5. Conclusions

Overall, this chapter discussed the sustainability of the processes proposed for sardine and crab shell waste streams valorisation, as well as their potential to be integrated and scaled-up to commercial scales.

The experimental scale-up (up to 70-fold increase in scale) has demonstrated to have no significant impact on the composition and bioactive potential of the extracts, nor on the purity and physicochemical properties of chitin. However, when the different processes were integrated, some of the products suffered modifications in terms of bioactivity, namely the protein derivative-rich extract recovered from sardine residues; or in terms of purity and properties as was the case of chitin recovered from crab shell biomass. Nevertheless, the protein derivative-rich extract still had an interesting antimicrobial potential, particularly towards *E. coli*; while chitin, although less pure, could still be applied, for example, in agriculture, pollutants removal or water treatment sectors.

The LCA study developed, taking into consideration the results obtained for PI, allowed the identification of the process steps with the highest environmental impact, being these hotspots mainly related to the production of the DES components used for protein derivatives extraction from sardines and AXT extraction from crab shells. Still, this study provided insights on the global environmental impacts of the proposed processes, which are likely to affect the industrial scale processes, and provided a benchmark for future scenario analysis studies that can help improving the overall environmental footprint of the processes.

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CHAPTER 5

Concluding remarks and outlook

CHAPTER 5

Contents

1. General discussion and conclusions

In the last years, food waste conversion into bio-based commodities, chemicals, and energy, through sustainable integrated biorefineries, has shown potential to be applied as an alternative to conventional refining processes, as well as to contribute to the transformation of a linear economy into a circular bioeconomy, in which the use of resources can be minimized[1,2].

The experimental work developed during this PhD thesis represented, therefore, an endeavour towards finding a scientific basis for an efficient valorisation of waste streams resulting from the fish and shellfish processing industries, namely sardine heads and offal and crab shells, within the concepts of biorefinery and circular bioeconomy. In this way, the processes proposed in this research work for the valorisation of these seafood biomasses, aimed at producing bioactive extracts or at recovering specific target molecules, using alternative technologies to the most commonly used conventional petrochemical or volatile organic solvents[3–5].

Factors such as improving the sustainability of fisheries (a sustainable exploitation of the by-products generated would be of major economic and environmental relevance for the fish and shellfish processing industries), preventing the depletion of natural resources, avoiding the disposal of increasing amounts of fish and shellfish residues, promoting the financial return of seafood processing industries, and fostering the application of alternative solvents were the main motivations behind this work. Accordingly, an effort was made to select processing units that could be fully transferable to pilot and industrial scales, even when applying neoteric solvents, making the upscaling of the most promising processes a reliable possibility.

Specifically, the technologies used herein included (i) supercritical carbon dioxide (sc-CO_2) extraction, which is a well-established technology and already widely applied in different industrial processes, such as the extraction and fractionation of numerous molecules and oils from different natural matrices, the decaffeination of coffee beans or black tea leaves, and the removal of pesticides from plant materials[5,6]; (ii) subcritical water (scW), which, despite being a more recent technology, has also shown great potential to be used as solvent, catalyst, and reactant for hydrolytic conversions and extractions[3], particularly by

the chemical, food, and pharmaceutical industries[7,8]; and finally (iii) deep eutectic systems (DES), a rather young technology, which have just recently started being adopted by the industry, particularly by the beauty/health care and natural ingredient sectors for the extraction of active ingredients from plants[9,10], but that has proven to have great potential to be used at commercial scales, to replace or complement some of the already existing industrial processes[11]. Accordingly, (i) sc-CO_2 and betaine/polyol-based DES were used to extract fatty acids and protein derivatives from sardine biomass, respectively; and (ii) terpene/fatty acid-based DES, scW, and choline chloride/organic acid-based DES were applied for the recovery of astaxanthin (AXT), protein hydrolysates, and chitin from crab shell residues, respectively. Overall, each process studied allowed a successful recovery of the target molecules, enabling the attainment of five different products with interesting physicochemical characteristics or *in vitro* health-promoting properties, namely (i) antiproliferative, antioxidant, and anti-inflammatory fatty acid-rich extracts; (ii) antioxidant and antimicrobial protein derivative-rich extracts; (iii) antiproliferative and antimicrobial AXT-rich extracts; (iv) antioxidant protein hydrolysate-rich extracts; and (v) high-purity and porous chitin.

It was shown that the processes developed in this work may be a viable alternative to similar bioactive ingredients already existing in the food, nutraceutical, cosmetic, or even pharmaceutical markets. Furthermore, taking into consideration the work of Liu *et al.*, in which a techno-economic analysis revealed that a minimum cost of co-product separation is one of the basic requirements for the success of biomass valorisation in biorefineries[12,13], the processes developed in this PhD thesis were designed to contain as few separation steps and downstream operations as possible. For this reason, the DES used in this work were not applied exclusively as extraction solvents, but also as part of the end-product. Similarly to what was previously demonstrated by other authors[14], the betaine/polyol- and the terpene/fatty acid-based DES have shown bioactive potential (antimicrobial activity in the first case, and antiproliferative and antimicrobial effects in the latter), which might have enhanced the bioactivity of the extracts as a whole, namely the protein derivative- and the AXT-rich extracts, respectively, through synergistic or additive effects. In addition, these extracts have also shown an increased bioactive response when compared to the extracts

obtained through conventional methods, possibly due to the selectivity of DES and also their presence in the final product.

It is also important to highlight that this thesis provided valuable knowledge regarding the physicochemical (such as density, viscosity, or polarity data) and toxicological properties (namely *in vitro* cytotoxicity and phytotoxicity) of the DES studied (ChCl/organic acid-based DES were only evaluated in terms of phytotoxicity, since their cytotoxicity and physicochemical properties had already been explored by other authors[15–19]), which might be important if their application at industrial scales is envisioned. The results obtained have also shown that it is possible to modulate these properties by adjusting the hydrogen bond donor, the water content, or the operating temperature (specifically for density and viscosity).

The sustainability and performance of a biorefining process can be measured in terms of economic growth, environmental protection, and social development[13,20]. Two of the most commonly used sustainability assessment tools are process integration (PI) and life cycle assessment (LCA)[13], which were applied in this work, as a proof of concept, for the valorisation of sardine and crab shell processing waste streams. Furthermore, to better understand the overall processes, as well as the problems that might arise from an increase in scale, an experimental scale-up was also performed (although still at bench scale) before PI.

Results showed that no significant changes on the composition, bioactive potential, or physicochemical properties of the final products have arisen from the scale-up performed, except in the case of sc-CO_2 extractions, in which slight variations in the yields were noticeable, possibly due to differences in the extraction bed geometry, in biomass aggregation or compaction, or in CO_2 residence times. Although PI allowed to reduce the quantity of feedstocks used (1.3- to 3-fold reduction), in some cases, it caused some modifications in the final products, namely in the antimicrobial effect of protein derivative-rich extracts recovered from sardine residues, which shifted when compared to the isolated process, being more effective towards the Gram-negative than the Gram-positive bacterium tested; and in the purity of chitin recovered from crab shell biomass, which decreased from

98 to 64%, as well as its thermal stability, which also showed a decrease (about 1.8-fold). Nevertheless, these products can still find interesting applications in different markets.
Regarding LCA, it was shown that the application of betaine/polyol- and the terpene/fatty acid-based DES contributed the most to the overall environmental burden of the processes (≥ 91% and ≥ 25%, respectively). Nevertheless, these processes can become competitive if their environmental footprint is improved before larger scales are envisioned, for example through heat integration, increased equipment efficiency and reduction of resources and solvents/chemicals, with replacement or reduction of fossil resources by renewable energy, or by recycling chemicals and water.

The ultimate goal of the research developed within the scope of this thesis is that the processes proposed may reach commercial levels. Nonetheless, the perspective of upscaling these processes comes with challenges. On one hand, it is of the utmost importance to ensure the availability of feedstock over time to meet the markets' demands[13,21], and on the other hand, to guarantee the overall quality and stability of the feedstock supply[13], as both aspects can limit the viability of the processes. In particular, due to a high content of ω-3 polyunsaturated fatty acids, which are known to be highly prone to oxidation[22,23], the sardine residues revealed to be unstable after freeze-drying and grinding, even when stored at -20 °C under a nitrogen atmosphere (as demonstrated on Chapter 2, Part II). However, when the residue's cellular structure was preserved (unground biomass), it was able to maintain its fatty acid composition during storage at -20 °C (for at least 3 months). Moreover, molecules such as carotenoids present in crab shells may also be unstable and susceptible to oxidation[24], resulting in lower pigment concentrations over time. Hence, particular care should be taken to ensure the quality and stability of the feedstock, so that the quality and stability of the final product can be guaranteed as well.

Overall, this PhD thesis revealed that sardine heads and offal and brown crab shells, which are some of the major by-products from fish and shellfish processing industry, can be a valuable source of chitin and bioactive extracts rich in fatty acids, protein derivatives and hydrolysates, and AXT. The promising bioactive properties of these extracts or the features presented by chitin make them potential ingredients to be explored in functional foods,

nutraceuticals, cosmetics, or pharmaceutical products. Therefore, the results from this experimental work will hopefully contribute to the sustainability of seafood processing companies, through the development of value-added products with high market value, as well as to the future implementation of a seafood waste-based biorefinery and a circular bioeconomy model.

2. Outlook

Even though the results obtained in this work are very encouraging, to be able to persuade the industry to shift from their typical, although extremely optimised and efficient processes, to innovative and more sustainable processes, the latter need to have provided clear evidence of their quality. Therefore, there are still some questions that should be answered before moving towards commercial scales.

(i) Will the prospects of using these particular biomasses in a biorefinery be limited by the quantity of the available feedstock supply?

A lack of biomass availability may strongly limit the production[13]. Therefore, it would be important to understand if the proposed processes would still be viable after evaluating the amount of fish and shellfish by-products generated in Portugal or in specific Portuguese regions.

(ii) How can the variability of the composition of the residues be dealt with?

Considering this potential problem, and based on the properties/specifications defined as endpoint for each final product, a detailed characterization (including seasonal changes in composition), as well as stability studies, should be performed to validate the quality of the biomasses over time, and thresholds should be defined to be able to ensure constant product quality.

(iii) Could the processes proposed herein be applied to other biomasses? Would this enable the implementation of an integrated multi-feedstock biorefinery?

As previously mentioned, to limit the biorefinery to a particular biomass may hinder production[13]. Therefore, if other biomasses have the potential to be used for the production of the same products (similarly to the validation performed in Chapter 3, Part I for the AXT

extraction from shrimp shells, mussels, and *Haematococcus pluvialis* with a menthol:myristic acid DES), an integrated multi-feedstock biorefinery could be envisioned, in which the same technologies could be used for the production of value-added products obtained from different waste biomasses[21].

(iv) Can the potential applications of the products obtained be expanded?
If the extracts are further characterized (not only in terms of chemical composition but also in terms of bioactivity, including studies performed at higher trophic levels) and their functional properties eventually evaluated, it might be possible to expand their applications even further.

(v) Will the processes perform similarly at pilot or industrial scales?
Most of the research performed on the different pathways for food waste valorisation has been conducted at laboratory scale[21]. However, in this work, the fact that the processes have been developed at a low technology readiness level (TRL 4, *i.e.*, processes have been validated in laboratory environment)[25] may limit their full industrial production application. From the experimental scale-up performed in Chapter 4 (up to 70-fold increase in scale), it is possible to draw some preliminary conclusions, namely that there was no significant impact on the composition and bioactive potential of the extracts obtained, nor on the purity and physicochemical properties of chitin. Nevertheless, further research is needed to validate the performance of the processes at larger scales, as well as to standardize the procedures applied.

(vi) Can the environmental impact of the processes be further improved?
As mentioned in Chapter 4, the accuracy of LCA results is dependent on the data sources used to model the process, which in the case of menthol, myristic acid, or choline chloride, was performed by using the data available for similar systems from the ecoinvent database. Regarding betaine, the database has provided a conservative model, associating its environmental burdens to sugar beet cultivation and sugar production, which, in the author's opinion, could be considered as double taxation, since betaine is produced from sugar beet molasses, a by-product from the production of sugar. Therefore, their environmental footprint should be attributed to the production of sugar and not to the

production of betaine. Within this context, it would be important to develop reliable models for the production of these particular chemicals to improve the precision of the results. Furthermore, a scenario analysis should also be performed to determine if the use of different chemicals would improve the environmental performance of the overall process and result in a different outcome.

(vii) This work has revealed that the processes proposed have the potential to be sustainable from an environmental point of view after specific issues are addressed, but are these processes sustainable from an economic and social point of view?

Most of the studies reported in the literature, evaluate the sustainability of a process through a single-criterion analysis, while the possible combined effects of multiple criteria are often neglected[13]. Therefore, having an integrated approach when analysing the sustainability of a process is of the utmost importance.

The high costs associated to a new process, even if it is extremely efficient and yields high value-added products, is probably the factor that most quickly makes its implementation unfeasible. Therefore, since it was not possible to perform a techno-economic analysis (TEA) of the different processes during this thesis due to time constraints, a collaboration was established with Instituto Superior Técnico (Universidade de Lisboa) and Technical University of Denmark, so that a detailed economic study may be performed in the near future. Furthermore, an integration of LCA and TEA results is also planned, being a publication foreseen.

This PhD thesis has given an important contribution towards the future development of an integrated seafood waste-based biorefinery and the implementation of a circular bioeconomy, being a step forward towards an efficient valorisation of currently undervalued marine-based waste streams, a decrease in waste disposal, and a more conscientious resource use. New scientific developments are expected in the next years, which should be key to foster the proposal and enforcement of new regulations and policies that can drive the implementation of such biorefineries.

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APPENDIX

APPENDIX A – Fatty acid profiles of sardine lipid-rich extracts

Table A1. Fatty acid profiles of sardine supercritical CO_2 (sc-CO_2) extracts quantified by gas chromatography-flame ionization detection (GC-FID) ($mg_{fatty\ acid}/100\ g_{dry\ residue}$).

	35 °C			55 °C			75 °C		
Fatty acid (FA)	**300 bar**	**425 bar**	**550 bar**	**300 bar**	**425 bar**	**550 bar**	**300 bar**	**425 bar**	**550 bar**
C12:0	0.0	24.9	112.5	22.8	28.6	146.5	27.4	34.3	29.6
C12:1 (9)	0.0	0.0	30.3	0.0	0.0	0.0	0.0	0.0	0.0
C13:0	4.4	0.0	29.8	7.1	9.3	0.0	8.0	8.6	8.4
iC14:0	3.7	0.0	0.0	0.0	6.9	0.0	0.0	8.5	6.0
C14:0	970.7	1234.3	2184.0	1032.9	1507.5	2254.3	1284.1	1504.2	1433.1
C14:1 (9)	31.2	38.4	77.2	35.7	46.4	73.2	50.8	55.0	47.4
iC15:0	12.0	15.5	0.0	11.6	18.3	0.0	23.8	19.4	18.4
C15:0	74.7	88.7	157.9	81.2	105.8	168.9	102.0	106.6	105.7
C15:1 (10)	13.2	14.3	39.4	12.5	17.0	0.0	13.5	14.0	17.6
C16:0	3061.7	3541.2	4942.9	3320.4	3936.6	4976.8	3466.6	3968.6	3943.5
C16:1 (7)	78.2	99.3	179.5	100.4	101.9	130.1	82.8	115.7	112.7
C16:1 *trans*(9)	31.7	38.2	98.7	36.8	41.1	0.0	37.9	50.8	47.8
C16:1 (9)	814.7	939.5	1354.4	847.1	1076.4	1371.0	913.3	1034.8	1045.5
C16:2 (9, 12)	69.9	80.6	100.0	63.6	68.8	85.4	65.2	83.6	70.8
iC17:0	4.5	25.4	30.9	15.3	20.6	0.0	14.5	20.6	18.2
aiC17:0	66.1	92.0	95.8	69.3	83.5	88.7	74.1	90.8	84.0
C16:4 (4, 7, 10, 13)	63.0	83.0	273.4	72.9	75.1	85.4	65.4	91.3	77.4
C17:0	69.8	85.7	0.0	68.0	100.5	83.8	78.9	87.4	82.7
C17:1 (7)	24.9	29.3	0.0	25.2	32.4	68.2	25.3	62.8	31.1
C17:1 (10)	37.8	51.1	63.0	47.3	69.4	83.6	50.6	13.2	62.0
C18:0	506.3	571.4	652.5	588.6	573.9	727.4	526.9	639.6	633.8
C18:1 (6)	12.6	17.6	40.6	14.6	14.6	0.0	0.0	18.3	16.2
C18:1 (9)	1360.3	1499.0	1619.7	1547.8	1499.1	1648.1	1359.5	1663.4	1605.3
C18:1 (11)	289.7	337.8	359.2	361.9	308.0	347.7	315.0	371.8	370.0
C18:2 *trans*(9), *trans*(12)	23.5	35.3	0.0	34.3	29.7	0.0	25.2	32.7	30.5
C18:2 (9, 12)	151.7	191.5	223.2	180.0	179.4	255.9	167.1	213.2	171.6
C19:0	27.7	49.2	18.0	32.3	31.0	0.0	35.6	55.8	28.8
γ-C18:3 (6, 9, 12)	16.6	19.2	0.0	14.2	25.6	0.0	17.3	19.4	13.6
α-C18:3 (9, 12, 15)	105.3	123.9	121.4	119.4	126.0	145.5	105.0	127.6	124.5
C18:4 (6, 9, 12, 15)	238.4	293.2	326.8	261.3	288.3	282.6	227.1	291.2	279.0
C20:0	41.6	39.6	0.0	48.1	36.1	0.0	38.6	48.5	46.9
C20:1 *trans*(11)*	4.9	0.0	0.0	0.0	101.8	0.0	82.1	0.0	0.0
C20:1 (9)	746.2	823.8	698.0	906.0	618.8	755.6	677.2	888.5	887.8
C20:1 (11)	29.0	36.1	29.8	40.3	34.4	116.4	32.5	35.6	41.4
C20:2 (11, 14)	28.3	40.0	37.2	29.4	27.7	0.0	30.6	34.5	33.7
C20:3 (8, 11, 14)	6.6	20.8	45.3	21.9	17.2	0.0	22.7	16.7	18.8
C20:4 (5, 8, 11, 14)	54.6	65.8	94.2	59.7	54.9	0.0	48.1	65.4	62.9
C20:3 (11, 14, 17)	17.2	21.1	0.0	15.6	16.4	0.0	12.3	15.0	18.5
C20:4 (8, 11, 14, 17)	0.0	0.0	0.0	102.4	0.0	0.0	78.6	0.0	102.8
C22:0	89.4	106.2	0.0	0.0	92.2	81.9	0.0	108.1	0.0
C20:5 (5, 8, 11, 14, 17)	1044.7	1056.0	739.2	1011.4	1060.9	991.5	779.5	997.7	1088.0
C22:1 (11)	852.0	1067.8	1294.7	1206.9	810.8	1134.4	999.1	1262.3	1113.9
C22:1 (13)	7.1	9.2	0.0	16.1	7.4	0.0	10.2	11.8	11.9
C22:2 (13, 16)	33.0	37.3	0.0	0.0	34.0	0.0	0.0	29.1	0.0
C22:3 (13, 16, 19)	14.4	14.8	0.0	0.0	13.0	0.0	0.0	17.3	34.4
C24:0	17.0	0.0	0.0	0.0	11.0	0.0	0.0	0.0	12.0
C22:5 (7, 10, 13, 16, 19)	151.6	147.5	161.2	174.2	145.9	210.7	136.7	180.8	168.6
C22:6 (4, 7, 10, 13, 16, 19)	804.5	897.9	773.7	865.6	752.2	972.5	666.9	917.5	897.2
Saturated FA (SFA)	4949.8	5874.0	8224.3	5297.6	6561.9	8528.4	5680.6	6700.9	6451.1
Monounsaturated FA (MUFA)	4333.4	5001.4	5884.6	5198.7	4779.7	5728.3	4649.9	5597.9	5410.6
Polyunsaturated FA (PUFA)	2823.3	3127.7	2895.3	3025.8	2914.9	3029.5	2447.7	3133.0	3192.3
ω-3 PUFA	2424.7	2622.5	2395.6	2622.7	2464.6	2688.2	2071.6	2621.1	2756.0
ω-6 PUFA	328.7	424.6	399.8	339.6	381.5	255.9	310.9	428.3	365.5
TOTAL FA	12106.4	14003.1	17004.2	13522.1	14256.5	17286.2	12778.2	15431.8	15054.1

* tentatively identified

Table A2. Fatty acid profiles of sardine sc-CO_2 extracts quantified by GC-FID ($mg_{fatty\ acid}/g_{extract}$).

	35 °C			55 °C			75 °C		
FA	**300 bar**	**425 bar**	**550 bar**	**300 bar**	**425 bar**	**550 bar**	**300 bar**	**425 bar**	**550 bar**
C12:0	0.0	1.6	5.8	1.5	1.8	7.5	1.9	2.0	1.7
C12:1 (9)	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0
C13:0	0.3	0.0	1.5	0.5	0.6	0.0	0.5	0.5	0.5
iC14:0	0.3	0.0	0.0	0.0	0.4	0.0	0.0	0.5	0.4
C14:0	71.5	78.9	112.7	67.6	92.9	114.8	88.2	86.0	84.3
C14:1 (9)	2.3	2.5	4.0	2.3	2.9	3.7	3.5	3.1	2.8
iC15:0	0.9	1.0	0.0	0.8	1.1	0.0	1.6	1.1	1.1
C15:0	5.5	5.7	8.1	5.3	6.5	8.6	7.0	6.1	6.2
C15:1 (10)	1.0	0.9	2.0	0.8	1.0	0.0	0.9	0.8	1.0
C16:0	225.4	226.3	255.0	217.2	242.6	253.4	238.2	226.9	232.0
C16:1 (7)	5.8	6.3	9.3	6.6	6.3	6.6	5.7	6.6	6.6
C16:1 *trans*(9)	2.3	2.4	5.1	2.4	2.5	0.0	2.6	2.9	2.8
C16:1 (9)	60.0	60.0	69.9	55.4	66.3	69.8	62.8	59.2	61.5
C16:2 (9, 12)	5.1	5.1	5.2	4.2	4.2	4.3	4.5	4.8	4.2
iC17:0	0.3	1.6	1.6	1.0	1.3	0.0	1.0	1.2	1.1
aiC17:0	4.9	5.9	4.9	4.5	5.1	4.5	5.1	5.2	4.9
C16:4 (4, 7, 10, 13)	4.6	5.3	14.1	4.8	4.6	4.3	4.5	5.2	4.6
C17:0	5.1	5.5	0.0	4.4	6.2	4.3	5.4	5.0	4.9
C17:1 (7)	1.8	1.9	0.0	1.7	2.0	3.5	1.7	3.6	1.8
C17:1 (10)	2.8	3.3	3.2	3.1	4.3	4.3	3.5	0.8	3.6
C18:0	37.3	36.5	33.7	38.5	35.4	37.0	36.2	36.6	37.3
C18:1 (6)	0.9	1.1	2.1	1.0	0.9	0.0	0.0	1.0	1.0
C18:1 (9)	100.2	95.8	83.6	101.2	92.4	83.9	93.4	95.1	94.4
C18:1 (11)	21.3	21.6	18.5	23.7	19.0	17.7	21.6	21.3	21.8
C18:2 *trans*(9), *trans*(12)	1.7	2.3	0.0	2.2	1.8	0.0	1.7	1.9	1.8
C18:2 (9, 12)	11.2	12.2	11.5	11.8	11.1	13.0	11.5	12.2	10.1
C19:0	2.0	3.1	0.9	2.1	1.9	0.0	2.4	3.2	1.7
γ-C18:3 (6, 9, 12)	1.2	1.2	0.0	0.9	1.6	0.0	1.2	1.1	0.8
α-C18:3 (9, 12, 15)	7.8	7.9	6.3	7.8	7.8	7.4	7.2	7.3	7.3
C18:4 (6, 9, 12, 15)	17.6	18.7	16.9	17.1	17.8	14.4	15.6	16.7	16.4
C20:0	3.1	2.5	0.0	3.1	2.2	0.0	2.7	2.8	2.8
C20:1 *trans*(11)*	0.4	0.0	0.0	0.0	6.3	0.0	5.6	0.0	0.0
C20:1 (9)	54.9	52.6	36.0	59.3	38.1	38.5	46.5	50.8	52.2
C20:1 (11)	2.1	2.3	1.5	2.6	2.1	5.9	2.2	2.0	2.4
C20:2 (11, 14)	2.1	2.6	1.9	1.9	1.7	0.0	2.1	2.0	2.0
C20:3 (8, 11, 14)	0.5	1.3	2.3	1.4	1.1	0.0	1.6	1.0	1.1
C20:4 (5, 8, 11, 14)	4.0	4.2	4.9	3.9	3.4	0.0	3.3	3.7	3.7
C20:3 (11, 14, 17)	1.3	1.3	0.0	1.0	1.0	0.0	0.8	0.9	1.1
C20:4 (8, 11, 14, 17)	0.0	0.0	0.0	6.7	0.0	0.0	5.4	0.0	6.0
C22:0	6.6	6.8	0.0	0.0	5.7	4.2	0.0	6.2	0.0
C20:5 (5, 8, 11, 14, 17)	76.9	67.5	38.1	66.2	65.4	50.5	53.6	57.1	64.0
C22:1 (11)	62.7	68.2	66.8	78.9	50.0	57.8	68.7	72.2	65.5
C22:1 (13)	0.5	0.6	0.0	1.1	0.5	0.0	0.7	0.7	0.7
C22:2 (13, 16)	2.4	2.4	0.0	0.0	2.1	0.0	0.0	1.7	0.0
C22:3 (13, 16, 19)	1.1	0.9	0.0	0.0	0.8	0.0	0.0	1.0	2.0
C24:0	1.3	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.7
C22:5 (7, 10, 13, 16, 19)	11.2	9.4	8.3	11.4	9.0	10.7	9.4	10.3	9.9
C22:6 (4, 7, 10, 13, 16, 19)	59.2	57.4	39.9	56.6	46.4	49.5	45.8	52.5	52.8
SFA	364.4	375.3	424.3	346.5	404.4	434.2	390.4	383.2	379.5
MUFA	319.1	319.6	303.6	340.1	294.6	291.7	319.5	320.1	318.3
PUFA	207.9	199.9	149.4	197.9	179.7	154.2	168.2	179.2	187.8
ω-3 PUFA	178.5	167.6	123.6	171.6	151.9	136.9	142.4	149.9	162.1
ω-6 PUFA	24.2	27.1	20.6	22.2	23.5	13.0	21.4	24.5	21.5
TOTAL FA	891.4	894.8	877.2	884.5	878.7	880.1	878.1	882.4	885.7

* tentatively identified

Table A3. Fatty acid profiles of sardine Bligh and Dyer (B&D) extracts at day 0 and 35 quantified by GC-FID.

	B&D (day 0)		B&D (day 35)	
FA	**$mg_{FA}/100\ g_{dry\ residue}$**	**$mg_{FA}/g_{extract}$**	**$mg_{FA}/100\ g_{dry\ residue}$**	**$mg_{FA}/g_{extract}$**
C12:0	23.1	1.1	43.7	1.5
C12:1 (9)	5.2	0.2	6.9	0.2
C13:0	6.8	0.3	0.0	0.0
C14:0	1207.7	57.0	2201.0	75.1
C14:1 (9)	38.8	1.8	97.2	3.3
iC15:0	15.8	0.7	45.1	1.5
C15:0	95.6	4.5	309.1	10.5
C15:1 (10)	17.2	0.8	48.2	1.6
C16:0	3876.0	183.0	6979.8	238.2
C16:1 (7)	101.2	4.8	312.0	10.6
C16:1 *trans*(9)	38.3	1.8	108.2	3.7
C16:1 (9)	1033.2	48.8	1135.5	38.7
C16:2 (9, 12)	71.5	3.4	102.1	3.5
iC17:0	21.7	1.0	97.6	3.3
aiC17:0	96.3	4.5	55.1	1.9
C16:4 (4, 7, 10, 13)	95.0	4.5	227.9	7.8
C17:0	100.3	4.7	320.6	10.9
C17:1 (7)	35.7	1.7	151.0	5.2
C17:1 (10)	78.4	3.7	37.2	1.3
C18:0	791.3	37.4	1867.2	63.7
C18:1 (6)	17.2	0.8	0.0	0.0
C18:1 (9)	1936.7	91.4	4198.4	143.3
C18:1 (11)	400.8	18.9	501.1	17.1
C18:2 *trans*(9), *trans*(12)	31.8	1.5	83.0	2.8
C18:2 (9, 12)	230.6	10.9	396.4	13.5
C19:0	41.9	2.0	52.6	1.8
γ-C18:3 (6, 9, 12)	26.3	1.2	0.0	0.0
α-C18:3 (9, 12, 15)	177.7	8.4	177.4	6.1
C18:4 (6, 9, 12, 15)	440.4	20.8	297.0	10.1
C20:0	60.8	2.9	98.2	3.4
C20:1 *trans*(11)*	0.0	0.0	169.0	5.8
C20:1 (9)	1128.0	53.2	1021.7	34.9
C20:1 (11)	42.4	2.0	105.9	3.6
C20:2 (11, 14)	51.7	2.4	81.3	2.8
C20:3 (8, 11, 14)	31.6	1.5	10.7	0.4
C20:4 (5, 8, 11, 14)	132.4	6.2	93.6	3.2
C20:3 (11, 14, 17)	25.5	1.2	34.6	1.2
C22:0	183.5	8.7	0.0	0.0
C20:5 (5, 8, 11, 14, 17)	2720.4	128.4	814.4	27.8
C22:1 (11)	1048.1	49.5	1514.8	51.7
C22:1 (13)	19.8	0.9	0.0	0.0
C22:5 (7, 10, 13, 16, 19)	342.4	16.2	111.6	3.8
C22:6 (4, 7, 10, 13, 16, 19)	2402.4	113.4	1761.8	60.1
SFA	6520.8	307.8	12070.1	411.9
MUFA	5940.8	280.4	9407.1	321.0
PUFA	6779.7	320.0	4191.6	143.0
ω-3 PUFA	6203.7	292.8	3424.6	116.9
ω-6 PUFA	504.5	23.8	664.9	22.7
TOTAL FA	19241.3	908.3	25668.8	876.0

* tentatively identified

APPENDIX B – Principal component analysis of sardine fatty acid-rich extracts

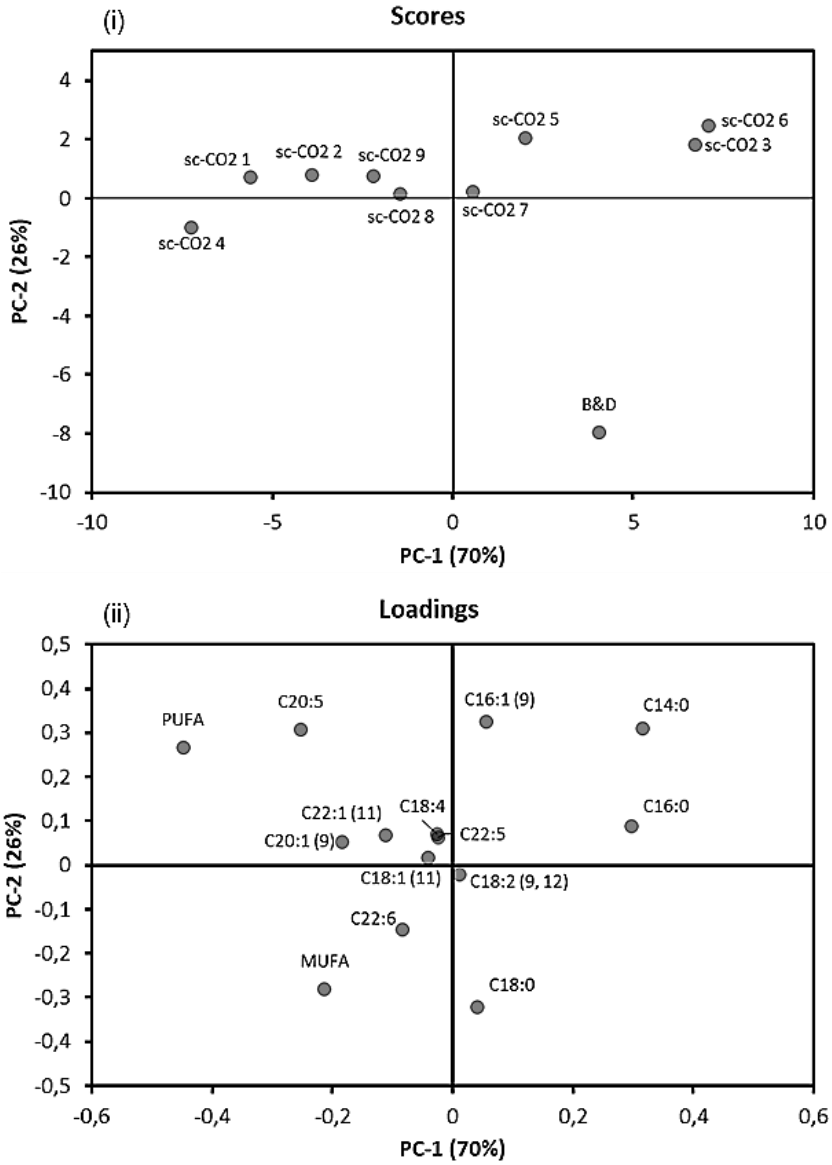

Figure B1. Distribution of the variables regarding Bligh and Dyer (B&D) and supercritical CO_2 (sc-CO_2) extracts composition as (i) scores and (ii) loadings defined by principal component 1 (PC-1) and 2 (PC-2). (sc-CO_2 1) 35 °C, 300 bar; (sc-CO_2 2) 35 °C, 425 bar; (sc-CO_2 3) 35 °C, 550 bar; (sc-CO_2 4) 55 °C, 300 bar; (sc-CO_2 5) 55 °C, 425 bar; (sc-CO_2 6) 55 °C, 550 bar; (sc-CO_2 7) 75 °C, 300 bar; (sc-CO_2 8) 75 °C, 425 bar; (sc-CO_2 9) 75 °C, 550 bar. (MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids).

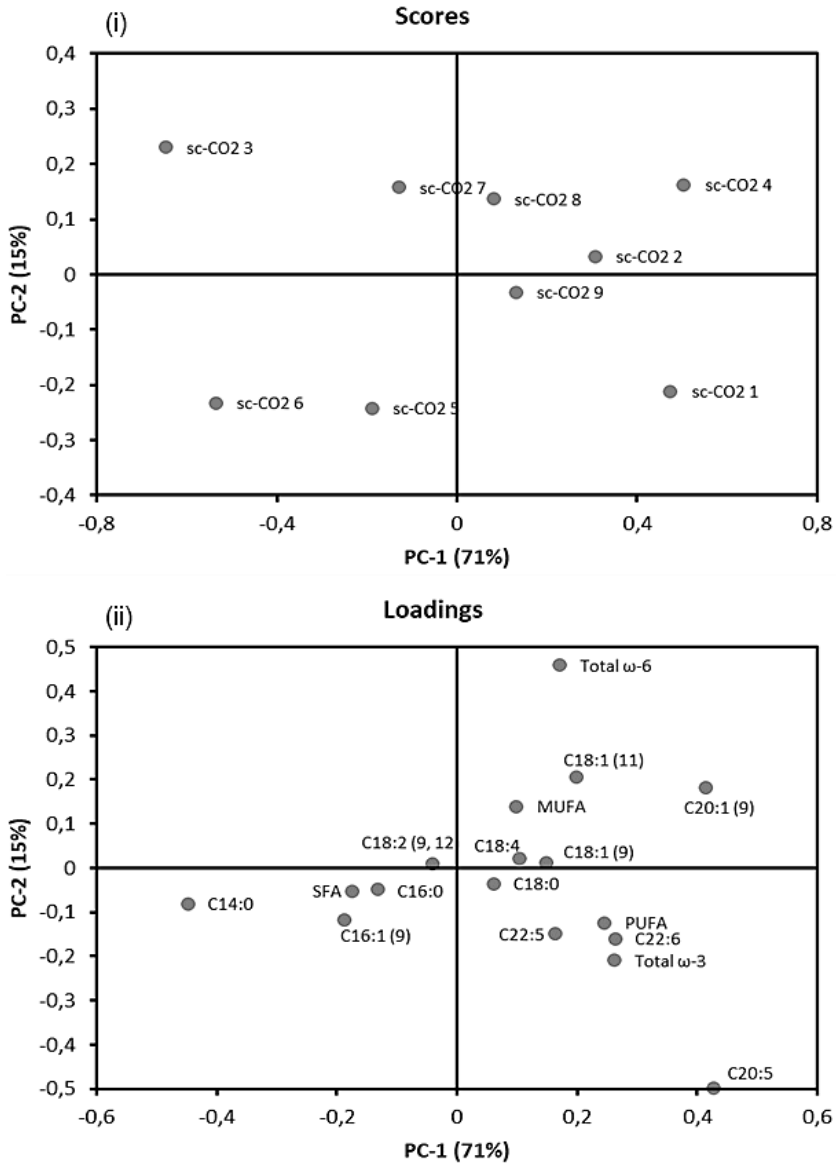

Figure B2. Distribution of the variables regarding sc-CO_2 extracts composition as (i) scores and (ii) loadings defined by PC-1 and 2 PC-2. (sc-CO_2 1) 35 °C, 300 bar; (sc-CO_2 2) 35 °C, 425 bar; (sc-CO_2 3) 35 °C, 550 bar; (sc-CO_2 4) 55 °C, 300 bar; (sc-CO_2 5) 55 °C, 425 bar; (sc-CO_2 6) 55 °C, 550 bar; (sc-CO_2 7) 75 °C, 300 bar; (sc-CO_2 8) 75 °C, 425 bar; (sc-CO_2 9) 75 °C, 550 bar. (SFA, saturated fatty acids).

APPENDIX C – Dose-response curves of terpene/fatty acid-based deep eutectic systems (DES) and respective isolated components obtained on Caco-2 and HT-29 cell lines

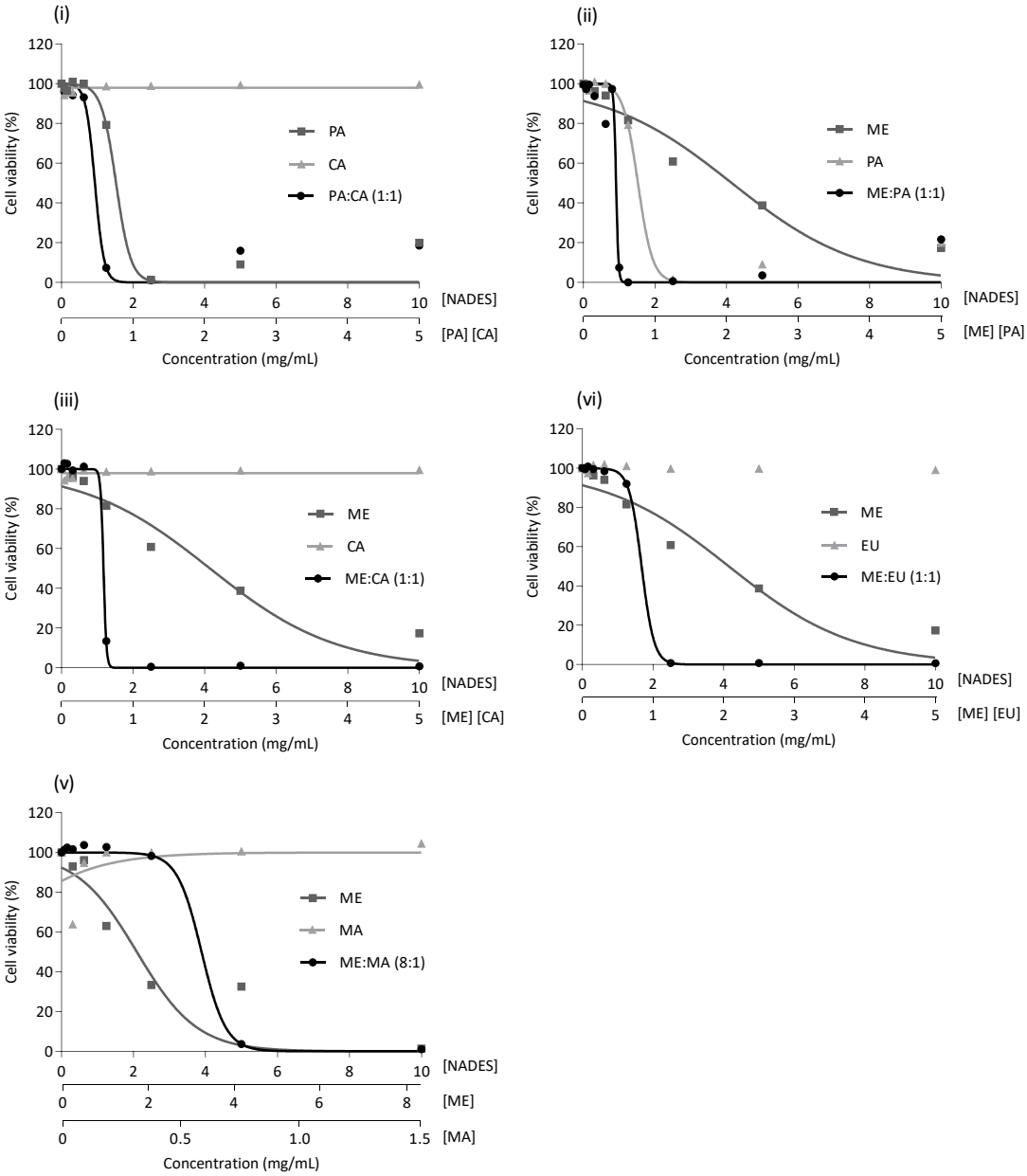

Figure C1. Dose-response curves of the cytotoxic effect induced by DES and respective isolated components evaluated on Caco-2 cells, after 24 h incubation. (i) perillyl alcohol (PA), camphor (CA), and PA:CA (1:1); (ii) menthol (ME), PA, and ME:PA (1:1); (iii) ME, CA, and ME:CA (1:1); (iv) ME, eucalyptol (EU), and ME:EU (1:1); and (v) ME, myristic acid (MA), and ME:MA (8:1).

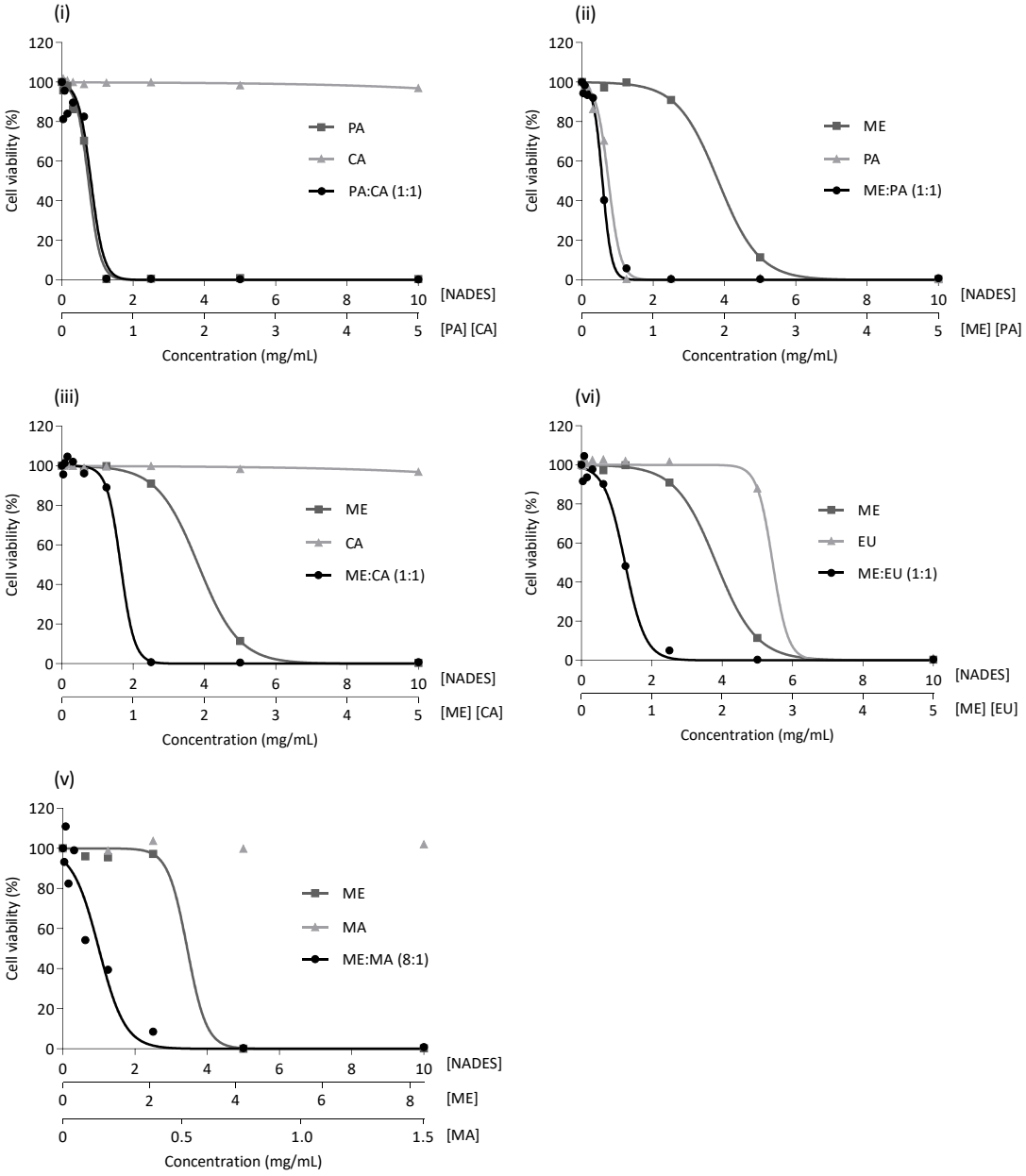

Figure C2. Dose-response curves of the antiproliferative effect induced by DES and respective isolated components evaluated on HT-29 cells, after 24 h incubation. (i) PA, CA, and PA:CA (1:1); (ii) ME, PA, and ME:PA (1:1); (iii) ME, CA, and ME:CA (1:1); (iv) ME, EU, and ME:EU (1:1); and (v) ME, MA, and ME:MA (8:1).

APPENDIX D – Free amino acid profile of crab shell protein hydrolysate-rich extracts

Table D1. Free amino acids content ($mg_{amino\ acid}/g_{dry\ residue}$) of subcritical water (scW) final extracts quantified by gas chromatography-flame ionization detection (GC-FID). (scW 1) 150 °C, 1:10 g/mL, 6 °C/min; (scW 2) 200 °C, 1:10 g/mL, 6 °C/min; (scW 3) 250 °C, 1:10 g/mL, 6 °C/min; (scW 4) 200 °C, 1:15 g/mL, 6 °C/min; (scW 5) 200 °C, 1:15 g/mL, 6 °C/min; (scW 6) 200 °C, 1:10 g/mL, 3 °C/min.

Amino acid	**scW 1**	**scW 2**	**scW 3**	**scW 4**	**scW 5**	**scW 6**
Alanine (Ala)	1.14	1.52	3.02	1.08	1.72	1.59
Aspartic acid (Asp)	0.20	0.52	0.29	0.49	0.43	0.53
Cysteine (Cys)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glutamic acid (Glu)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycine (Gly)	3.73	3.48	3.33	2.29	3.55	3.70
Histidine (His)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isoleucine (Ile)	0.39	0.43	0.56	0.34	0.42	0.49
Leucine (Leu)	0.32	0.51	1.02	0.42	0.47	0.57
Lysine (Lys)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methionine (Met)	0.11	0.19	0.30	0.16	0.20	0.22
Phenylalanine (Phe)	0.06	0.42	0.82	0.32	0.41	0.48
Proline (Pro)	0.56	0.82	1.14	0.54	0.94	0.88
Serine (Ser)	0.18	0.35	0.27	0.31	0.31	0.41
Threonine (Thr)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tryptophan (Trp)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyrosine (Tyr)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine (Val)	0.58	0.80	1.91	0.63	0.78	0.85
Total	7.26	9.02	12.66	6.60	9.24	9.72

n.d.: not detected

Table D2. Free amino acids content (relative mass % in the extract) of scW final extracts quantified by GC-FID. (scW 1) 150 °C, 1:10 g/mL, 6 °C/min; (scW 2) 200 °C, 1:10 g/mL, 6 °C/min; (scW 3) 250 °C, 1:10 g/mL, 6 °C/min; (scW 4) 200 °C, 1:15 g/mL, 6 °C/min; (scW 5) 200 °C, 1:15 g/mL, 6 °C/min; (scW 6) 200 °C, 1:10 g/mL, 3 °C/min.

Amino acid	**scW 1**	**scW 2**	**scW 3**	**scW 4**	**scW 5**	**scW 6**
Ala	15.7	16.8	23.8	16.3	18.6	16.4
Asp	2.7	5.7	2.3	7.4	4.6	5.5
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glu	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gly	51.4	38.6	26.3	34.7	38.4	38.1
His	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ile	5.3	4.8	4.5	5.2	4.6	5.0
Leu	4.4	5.7	8.0	6.3	5.1	5.9
Lys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Met	1.5	2.1	2.4	2.5	2.1	2.3
Phe	0.9	4.6	6.5	4.9	4.5	4.9
Pro	7.7	9.0	9.0	8.2	10.2	9.1
Ser	2.5	3.8	2.1	4.8	3.3	4.2
Thr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Val	8.0	8.8	15.1	9.6	8.5	8.7

n.d.: not detected

APPENDIX E – Cytotoxic effects of crab shell protein hydrolysate-rich extracts on Caco-2 cells

Table E1. Half maximal effective concentrations (EC50) ($mg_{extract}$/mL) obtained for scW final extracts in Caco-2 cells, after an incubation period of 24 h. (scW 1) 150 °C, 1:10 g/mL, 6 °C/min; (scW 2) 200 °C, 1:10 g/mL, 6 °C/min; (scW 3) 250 °C, 1:10 g/mL, 6 °C/min; (scW 4) 200 °C, 1:15 g/mL, 6 °C/min; (scW 5) 200 °C, 1:15 g/mL, 6 °C/min; (scW 6) 200 °C, 1:10 g/mL, 3 °C/min.

Samples	**EC50**
scW 1	> 20
scW 2	> 27
scW 3	4.76 ± 0.05
scW 4	> 13
scW 5	> 56
scW 6	> 26

APPENDIX F – Scanning electron microscopy (SEM) images of crab shells and chitin

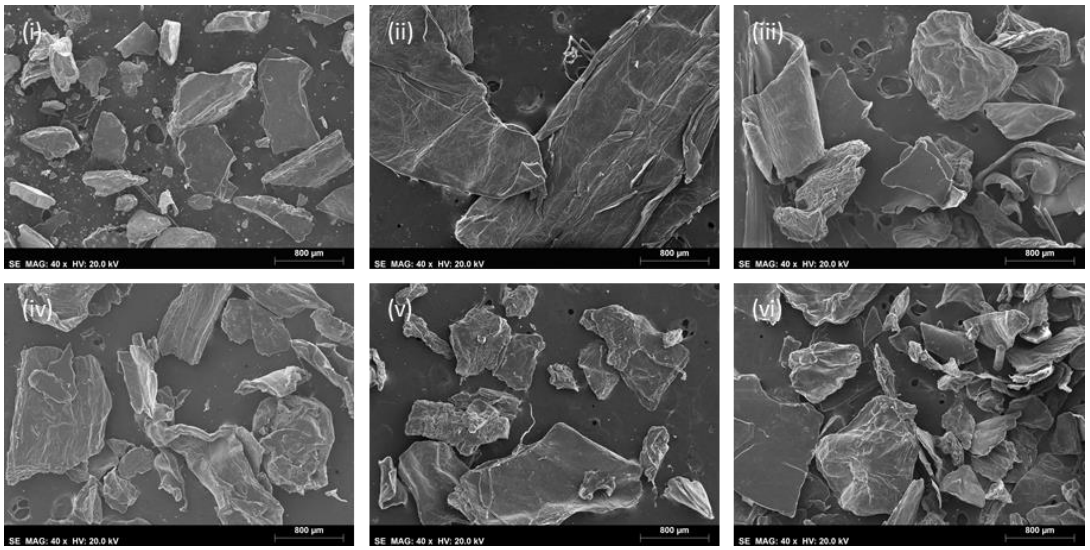

Figure F1. SEM images of (i) shell residues, (ii) chitin STD, and chitin recovered from crab shell biomass by (iii) conventional hydrolysis, (iv) choline chloride:malonic acid (1:2), (v) choline chloride:malic acid (1:2), and (vi) choline chloride:lactic acid (1:1) treatment (obtained at the processing conditions summarized on Table 4 of Chapter 3, part III).

APPENDIX G – Data sources, modelling, and assumptions used in life cycle assessment

Table G1. Summary of the data sources and modelling of sardine heads and offal processes.

Sardine residue		Agri-footprint 5.0 [1]. Offal from fishery used as proxy
Energy	Heating (steam)	Ecoinvent database[2]
	Cooling water	Ecoinvent database[2]
Chemicals	Betaine	Ecoinvent database[3]. Obtained from sugar beet molasses (waste stream from sugar factory)
	Propylene Glycol	Ecoinvent database[4]
	CO_2, liquid	Ecoinvent database[4]
	Water	Ecoinvent database[4]
Wastewater treatment	Water, organics	Ecoinvent database[5]. Modelled as wastewater from potato starch production and used as proxy

Table G2. Summary of the data sources and modelling of brown crab shell processes.

Crab shell residue	Astaxanthin	80.5% carbon, 10.72% oxygen based on empirical formula ($C_{40}H_{52}O_4$)[6]
	Protein	47% carbon, 15% nitrogen[7]
	Chitin	47% carbon, 7% nitrogen, based on empirical formula ($C_8H_{13}O_5N$)[8]
	Minerals	Ecoinvent database[9]
	Lipids	77% carbon[7]
	Water	Ecoinvent database[4]
Energy	Heating (steam)	Ecoinvent database[2]
	Cooling water	Ecoinvent database[2]
Chemicals	Menthol	Ecoinvent database[10]. Produced from vegetable oil (soybean oil) as proxy
	Myristic Acid	Ecoinvent database[4]. Saturated fatty acid present in palm kernel oil. The oil composition was taken into account and used as proxy
	Water	Ecoinvent database[4]
	Choline Chloride	Ecoinvent database[11]. Organic salt used in animal feed and as a wood preservative used as proxy
	Lactic acid	Ecoinvent database[4]
	Ethanol	Ecoinvent database[4]. Produced from wood fermentation
	Hydrogen peroxide	Ecoinvent database[4]
Wastewater treatment	Water, organics, hydrogen peroxide	Ecoinvent database[5]. Modelled as wastewater from potato starch production and used as proxy

Table G3. Sardine heads and offal processes assumptions.

The residue could be dried as opposed to freeze-dried

Table G4. Brown crab shell processes assumptions.

The residue could be dried as opposed to freeze-dried
The residual water in crab shells was not soluble in menthol:myristic acid deep eutectic system (DES) (Extractor 1 and Filter 1) and, therefore, remained in the raw material (stream 10)
Nitrogen could be fully recirculated in Extractor 2
Only proteins were extracted in Extractor 2
All water could be evaporated from the protein-rich extract (Dryer 2)
Residual lipids were soluble in choline chloride:lactic acid (ChCl:LA) DES, being removed from the shell residues in Extractor 3
No colour was removed from chitin during extraction with chloride:lactic acid DES (Extractor 3)
No chitin was lost in Filters 3, 4, 6, and 7
Chitin could not be completely dried (Dryer 4), containing 2.2% of water. This value was extrapolated from experimental data obtained for crab shells
The recycling of ChCl:LA DES could be performed as previously described by Hong *et al.* and Feng *et al.*, *i.e.*, the water could be evaporated from the DES under reduced pressure (Evaporator 1); the calcium carbonate contained in the DES could be precipitated by adding ethanol to the mixture at a mass ratio of 1:10 (DES:ethanol) (Mixer 6) and recovered after filtration (Filter 7); and the DES could be regenerated by evaporation of the ethanol under reduced pressure (Evaporator 2)[12,13]
All calcium carbonate contained in the crab shell residue could be obtained by the above-mentioned procedure
The percentage of DES that was regenerated was about 84%. This value was extrapolated from the work of Feng *et al.* regarding the regeneration of a ChCl:DL-malic acid (1:2) DES[13]
The percentage of ethanol retained in the filter was about 0.7%. This value was extrapolated from experimental data obtained for water

APPENDIX H – Fatty acid profiles of sardine lipid-rich extracts

Table H1. Fatty acid profiles of sardine supercritical CO_2 (sc-CO_2) extracts obtained at 35 °C and 550 bar, quantified by gas chromatography-flame ionization detection (GC-FID) ($mg_{fatty\ acid}/100\ g_{dry\ residue}$).

Fatty acid (FA)	10 g | 5 g/min	50 g | 25 g/min
C12:0	57.6	73.6
C13:0	15.4	20.2
C14:0	2488.9	3173.2
C14:1 (9)	9.8	14.7
iC15:0	66.9	88.7
aiC15:0	32.7	40.9
C15:0	199.6	257.4
C15:1 (10)	34.9	48.0
C16:0	5600.8	7193.1
C16:1 (7)	198.6	268.8
C16:1 *trans*(9)	66.1	91.3
C16:1 (9)	2289.8	3074.3
C16:2 (9, 12)	62.3	85.9
iC17:0	54.1	96.4
aiC17:0	134.6	178.3
C16:4 (4, 7, 10, 13)	210.2	324.2
C17:0	163.3	177.7
C17:1 (7)	190.8	261.4
C17:1 (10)	0.0	53.6
C18:0	1103.6	1328.5
C18:1 *trans*(9)	38.1	46.8
C18:1 (9)	2268.6	2873.3
C18:1 (11)	884.1	1103.7
C18:2 *trans*(9), *trans*(12)	30.2	34.9
C18:2 (9, 12)	213.1	274.3
C19:0	90.8	116.8
γ-C18:3 (6, 9, 12)	28.9	51.4
α-C18:3 (9, 12, 15)	125.4	173.2
C18:4 (6, 9, 12, 15)	370.8	486.1
C20:0	146.9	166.7
C20:1 (7)	81.4	116.4
C20:1 (9)	544.5	657.6
C20:1 (11)	68.3	101.1
C20:2 (11, 14)	43.3	56.2
C20:3 (8, 11, 14)	24.7	66.6
C20:4 (5, 8, 11, 14)	123.0	152.7
C20:3 (11, 14, 17)	22.1	24.7
C20:5 (5, 8, 11, 14, 17)	2617.9	3152.0
C22:1 (11)	424.4	491.7
C22:1 (13)	69.3	112.4
C22:3 (13, 16, 19)	6.1	16.3
C22:4 (7, 10, 13, 16)	25.5	28.3
C24:0	25.2	20.0
C22:5 (7, 10, 13, 16, 19)	225.0	262.9
C22:6 (4, 7, 10, 13, 16, 19)	1776.6	1984.5
Saturated FA (SFA)	13459.6	13465.6
Monounsaturated FA (MUFA)	7168.6	9315.3
Polyunsaturated FA (PUFA)	5858.0	7027.8
ω-3 PUFA	5348.0	6407.7
ω-6 PUFA	494.6	680.7
TOTAL FA	26486.1	29808.7

Table H2. Fatty acid profile of sardine sc-CO_2 extracts obtained at 35 °C and 550 bar, quantified by GC-FID ($mg_{fatty\ acid}$ /$g_{extract}$).

FA	10 g | 5 g/min	50 g | 25 g/min
C12:0	2.1	2.2
C13:0	0.6	0.6
C14:0	92.4	92.8
C14:1 (9)	0.4	0.4
iC15:0	2.5	2.6
aiC15:0	1.2	1.2
C15:0	7.4	7.5
C15:1 (10)	1.3	1.4
C16:0	207.9	210.3
C16:1 (7)	7.4	7.9
C16:1 *trans*(9)	2.5	2.7
C16:1 (9)	85.0	89.9
C16:2 (9, 12)	2.3	2.5
iC17:0	2.0	2.8
aiC17:0	5.0	5.2
C16:4 (4, 7, 10, 13)	7.8	9.5
C17:0	6.1	5.2
C17:1 (7)	7.1	7.6
C17:1 (10)	0.0	1.6
C18:0	41.0	38.8
C18:1 *trans*(9)	1.4	1.4
C18:1 (9)	84.2	84.0
C18:1 (11)	32.8	32.3
C18:2 *trans*(9), *trans*(12)	1.1	1.0
C18:2 (9, 12)	7.9	8.0
C19:0	3.4	3.4
γ-C18:3 (6, 9, 12)	1.1	1.5
α-C18:3 (9, 12, 15)	4.7	5.1
C18:4 (6, 9, 12, 15)	13.8	14.2
C20:0	5.5	4.9
C20:1 (7)	3.0	3.4
C20:1 (9)	20.2	19.2
C20:1 (11)	2.5	3.0
C20:2 (11, 14)	1.6	1.6
C20:3 (8, 11, 14)	0.9	1.9
C20:4 (5, 8, 11, 14)	4.6	4.5
C20:3 (11, 14, 17)	0.8	0.7
C20:5 (5, 8, 11, 14, 17)	97.2	92.1
C22:1 (11)	15.8	14.4
C22:1 (13)	2.6	3.3
C22:3 (13, 16, 19)	0.2	0.5
C22:4 (7, 10, 13, 16)	0.9	0.8
C24:0	0.9	0.6
C22:5 (7, 10, 13, 16, 19)	8.4	7.7
C22:6 (4, 7, 10, 13, 16, 19)	65.9	58.0
SFA	377.8	378.0
MUFA	266.1	272.3
PUFA	217.4	205.4
ω-3 PUFA	198.5	187.3
ω-6 PUFA	18.4	19.9
TOTAL FA	861.3	855.8

APPENDIX I – Energy requirements for the integrated processes

Table I1. Primary energy breakdown for the processes involving both raw materials, per process stage and corresponding processing units. (The electricity requirements were not measured nor quantified).

Sardine residues				Crab shell residues			
		Heating (kJ)	**Cooling water (kJ)**			**Heating (kJ)**	**Cooling water (kJ)**
Drying and milling (i)	Dryer 1	251.8	-	**Drying and milling (i)**	Dryer 1	129.8	-
	Cutter 1	-	-		Cutter 1	-	-
	Total	251.8	-		Total	129.8	-
Fatty acid extraction (ii)	Extractor 1	184.5	665.2	**AXT extraction (ii)**	Mixer 1	13.3	-
	Total	184.5	665.2		Extractor 1	2.58	-
Protein extraction (iii)	Mixer 1	30.5	-		Filter 1	-	-
	Extractor 2	3.4	-		Total	15.9	-
	Filter 1	-	-	**Protein extraction (iii)**	Extractor 2	520.0	520.0
	Total	33.9	-		Filter 2	-	-
Total		475.1	665.2		Dryer 2	1200.5	-
					Total	1720.6	-
				Chitin recovery (iv)	Dryer 3	55.3	-
					Mixer 2	61.8	-
					Extractor 3	133.7	-
					Filter 3	-	-
					Mixer 3	-	-
					Filter 4	-	-
					Total	250.8	-
				Chitin decolouration (v)	Mixer 4	17.5	-
					Filter 5	-	-
					Mixer 5	-	-
					Filter 6	-	-
					Dryer 4	40.7	-
					Total	58.2	
				Solvent recycling (vi)	Evaporator 1	2666.8	-
					Mixer 6	-	-
					Filter 7	-	-
					Evaporator 2	1419.9	937.4
					Total	4086.7	937.4
				Total		6339.8	1457.5

APPENDIX J – Absolute values for the impact assessment (ReCiPe method at the midpoint level (Hierarchist approach))

Table J1. Absolute values for the life cycle impact assessment of the processes involving both raw materials (ReCiPe midpoint (H)). (GW, global warming; SOD, stratospheric ozone depletion; IR, ionizing radiation; OHH, ozone formation, human health; FPM, fine particulate matter formation; OTS, ozone formation, terrestrial ecosystems; TAc, terrestrial acidification; FE, freshwater eutrophication; ME, marine eutrophication; TEco, terrestrial ecotoxicity; FEco, freshwater ecotoxicity; MEco, marine ecotoxicity; HCT, human carcinogenic toxicity; HnCT, human non-carcinogenic toxicity; LU, land use; MRS, mineral resource scarcity; FRS, fossil resource scarcity; WC, water consumption; DM, drying and milling; FAE, fatty acid extraction; PE, protein extraction (derivatives in the case of sardine and hydrolysates in the case of crab shell residues); AE, astaxanthin extraction; CR, chitin recovery; CD, chitin decolouration; SR, solvent recycling).

	Sardine residues				**Crab shell residues**						
Impact category	**Total**	**DM (i)**	**FAE (ii)**	**PE (iii)**	**Total**	**DM (i)**	**AE (ii)**	**PE (iii)**	**CR (iv)**	**CD (v)**	**SR (vi)**
GW ($kg_{CO2\ eq}$)	**1.9E+00**	5.0E-02	1.0E-01	1.8E+00	**3.8E+00**	1.4E-02	2.4E+00	2.0E-01	5.9E-01	5.0E-01	9.8E-02
SOD ($kg_{CFC\text{-}11\ eq}$)	**2.0E-05**	1.2E-08	4.5E-08	2.0E-05	**5.2E-06**	3.4E-09	4.4E-06	6.3E-08	1.5E-07	5.6E-07	2.8E-08
IR ($kBq_{Co\text{-}60\ eq}$)	**6.8E-03**	1.4E-04	4.5E-04	6.2E-03	**9.2E-03**	3.7E-05	4.9E-03	7.1E-04	1.7E-03	1.4E-03	4.6E-04
OHH ($kg_{NOx\ eq}$)	**5.2E-03**	6.2E-05	3.6E-04	4.8E-03	**6.8E-03**	1.7E-05	4.5E-03	2.9E-04	1.1E-03	8.3E-04	1.5E-04
FPM ($kg_{PM2.5\ eq}$)	**2.3E-03**	4.1E-05	1.2E-04	2.1E-03	**4.1E-03**	1.1E-05	2.5E-03	2.0E-04	7.8E-04	5.3E-04	1.2E-04
OTS ($kg_{NOx\ eq}$)	**5.4E-03**	6.3E-05	3.6E-04	5.0E-03	**7.4E-03**	1.7E-05	4.9E-03	3.0E-04	1.1E-03	8.6E-04	1.5E-04
TAc ($kg_{SO2\ eq}$)	**6.8E-03**	1.1E-04	2.6E-04	6.4E-03	**9.3E-03**	3.1E-05	5.7E-03	5.0E-04	1.5E-03	1.4E-03	2.1E-04
FE ($kg_{P\ eq}$)	**2.4E-04**	7.5E-07	1.6E-06	2.4E-04	**7.7E-03**	2.1E-07	7.7E-03	4.7E-06	2.6E-05	1.3E-05	3.0E-06
ME ($kg_{N\ eq}$)	**4.3E-03**	2.7E-08	5.5E-07	4.3E-03	**2.1E-03**	7.3E-09	2.0E-03	7.8E-07	1.0E-05	4.8E-06	2.8E-06
TEco ($kg_{1.4\text{-}DCB}$)	**1.0E+01**	7.7E-02	5.9E-01	9.4E+00	**1.2E+01**	2.1E-02	5.9E+00	1.1E+00	2.0E+00	2.6E+00	3.3E-01
FEco ($kg_{1.4\text{-}DCB}$)	**4.8E-03**	7.7E-06	5.9E-05	4.7E-03	**7.3E-03**	2.1E-06	6.5E-03	7.6E-05	3.6E-04	2.2E-04	8.6E-05
MEco ($kg_{1.4\text{-}DCB}$)	**9.5E-03**	1.0E-04	3.7E-04	9.0E-03	**1.4E-02**	2.8E-05	9.3E-03	9.6E-04	1.4E-03	2.3E-03	2.7E-04
HCT ($kg_{1.4\text{-}DCB}$)	**1.3E-02**	7.8E-05	1.1E-03	1.2E-02	**1.5E-02**	2.1E-05	3.8E-03	1.8E-03	3.8E-03	3.7E-03	2.1E-03
HnCT ($kg_{1.4\text{-}DCB}$)	**8.4E-01**	3.1E-03	2.8E-02	8.1E-01	**6.1E-01**	8.3E-04	2.7E-01	5.4E-02	1.2E-01	1.4E-01	1.7E-02
LU ($m^2_{an.\ cropland\ eq}$)	**3.2E+00**	1.2E-03	6.5E-04	3.2E+00	**9.4E-01**	3.2E-04	7.0E-01	1.0E-02	1.5E-02	2.1E-01	1.9E-03
MRS ($kg_{Cu\ eq}$)	**1.3E-02**	2.1E-05	4.3E-04	1.2E-02	**1.7E-02**	5.7E-06	1.3E-02	6.9E-04	2.1E-03	1.5E-03	2.6E-04
FRS ($kg_{oil\ eq}$)	**5.0E-01**	1.7E-02	2.9E-02	4.5E-01	**1.1E+00**	4.6E-03	5.3E-01	6.5E-02	3.0E-01	1.6E-01	3.1E-02
WC (m^3)	**3.1E-01**	8.7E-05	1.6E-04	3.1E-01	**1.0E-01**	9.3E-04	8.4E-02	1.1E-03	9.9E-03	2.4E-03	4.2E-03

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