Universidade do Minho
Escola de Engenharia

Pedro Miguel Ferreira Santos

Green processing for bioresources valorization

January 2021

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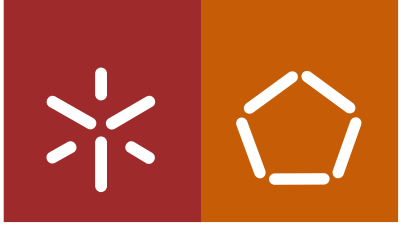
UMinho | 2021



Universidade do Minho
Escola de Engenharia

Pedro Miguel Ferreira Santos

Green processing for bioresources valorization

Doctoral Thesis
Food Science and Technology and Nutrition

Work developed under supervision of
Professor Doctor José António Couto Teixeira
Doctor Cristina Maria Ribeiro Rocha

January 2021

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

ACKNOWLEDGEMENTS

Na realização do meu trabalho de doutoramento, contei com o apoio direto ou indireto de múltiplas pessoas e instituições às quais estou profundamente grato. Sem esses contributos esta tese não teria sido possível. Contudo, e correndo o risco de injustamente não mencionar algum dos contributos quero deixar expresso os meus agradecimentos:

Aos meus orientadores, Professor José António Teixeira e Doutora Cristina Rocha, pela disponibilidade e acompanhamento constante, pelo incentivo e pela orientação prestada em todo momento. Não posso deixar de agradecer por tudo que me transmitiram ao longo deste tempo. Aqui lhes exprimo a minha gratidão.

A todas as pessoas que me acolheram durante as minhas estadias por Itália, aos membros da EVRA Italia e aos colegas da Università Cattolica del Sacro Cuore, foi um privilégio ter trabalhado convosco. Grazie e auguri a tutti.

Aos meus amigos que me acompanham cada dia nesta etapa, à Zlatina, Elisita, Carlitos, Nuno, C. Milho, Joana, Gabi, Veronica, Elisa, Jorge, Ana, pelos conselhos, ajuda e pelos momentos vividos ao longo destes anos. Convosco tenho muitos contos de histórias, espero levar-vos para o resto da vida, tanto no pessoal como no profissional (grandes atletas!).

Aos meus colegas e amigos do CEB que ao logo de 4 anos me acompanharam e me ouviram (e muito) nos laboratórios, corredores e noutros recantos do edifício 7. A eles, a todos e cada um, deixo um grande obrigado. Desejo a todos o melhor dos sucessos!

Quero agradecer também os momentos de "tesouraria e secretariado" quando as meninas da secretaria (Sandra, Isabel, Sónia e Maritza) me deixavam lanchar e desabafar no seu espaço (e pela ajuda na papelada). Às técnicas, Maura, Aline, Diana`s, Nicolina, Vitória, e também à Eng. Madalena e ao Sr. Santos pela disponibilidade e ajuda quando necessitava. A todos os colegas dos laboratórios onde me espalhei (LIP, LF, LTL, LEQ) e a todos os membros do Centro de Engenharia Biológica.

Aos meus queridos amigos e amigas de sempre e para sempre, não só pelo apoio, mas acima de tudo pela amizade.

Por último quero agradecer a todos os meus familiares, principalmente aos meus pais, Corina e José que foram e são uma ajuda preciosa desde sempre na minha vida.

UM BEM-HAJA A TODOS!

FCT Fundação para a Ciência e a Tecnologia
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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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RESUMO

Processamento verde para valorização de biorrecursos

Atualmente, o esgotamento de recursos, o acúmulo de resíduos e as mudanças climáticas são os principais fatores que impulsionam a necessidade de práticas sustentáveis. Algumas das estratégias para a redução do impacto ambiental gerado são o reaproveitamento de biorresíduos industriais, bem como o aumento do uso de recursos subexplorados por processos efetivos/intensificados para obtenção de produtos sustentáveis, ecológicos, seguros e de alta qualidade.

A presente tese teve como objetivo desenvolver metodologias eficientes e ecologicamente corretas envolvendo a aplicação de campos elétricos e enzimas, além de solventes de baixo custo e não tóxicos para a valorização de biorecursos subvalorizados, como casca de *Pinus pinaster* e microalga *Spirulina platensis*.

Os extratos de casca de pinheiro (PBE) apresentaram alta atividade antioxidante, antibacteriana e anti-hiperglicêmica, com baixa citotoxicidade e atividade seletiva contra células cancerosas, pois foram afetados negativamente. Essas propriedades foram atribuídas ao seu perfil fenólico e flavonóide. Além disso, o Aquecimento Óhmico (OH) evidenciou um aumento na extração de compostos fenólicos antioxidantes sem degradação. Os resultados também indicam extração preferencial sob o solvente apropriado, presumivelmente devido à ação do campo elétrico agindo em sinergia com o calor e solventes, promovendo efeitos de eletroporação da membrana. A microencapsulação por *spray-drying* de PBE utilizando maltodextrina foi eficaz na proteção de compostos fenólicos durante as condições gastrointestinais, controlando sua liberação e aumentando os seus benefícios para a saúde, diminuindo a produção de espécies reativas de oxigênio implicadas no processo de estresse oxidativo associado a algumas patologias.

A *Spirulina platensis* é interessante para a indústria alimentar devido à sua composição geral e alto teor em C-ficocianina (corante azul natural). A presente tese mostrou que campos elétricos moderados associados à temperatura promovem efeitos de desintegração celular e eletroporação melhorando a extração e estabilidade de compostos intracelulares da *Spirulina*, tendo também um efeito positivo no rendimento de extração de C-ficocianina com tempos de tratamento mais curtos. Uma extração sequencial em múltiplos estágios avaliada por extração térmica convencional, OH, tratamento enzimático (EAE), e suas combinações, evidenciou que a extração combinada de OH e EAE permitiu a recuperação seletiva de ficobiliproteínas na etapa de extração aquosa. Pigmentos, como clorofila e carotenóides, foram extraídos seletivamente com etanol (95%), bem como a fração lipídica da microalga.

De modo geral, a tecnologia OH tem potencial para ser uma alternativa "verde", melhorando a extração de compostos intracelulares, além de reduzir o tempo de processo, solventes e consumo de energia, oferecendo a possibilidade de ajustar a seletividade e estabilidade dos compostos extraídos. Além disso, processos integrados que abordem simultaneamente diferentes frações, em direção a uma economia de “desperdício zero”, são desejáveis e podem até ser benéficos para a eficácia de cada fração individual. Os biocompostos obtidos podem ser usados como aditivo tecnológico natural e/ou ingredientes funcionais para aplicações alimentares, cosméticas e farmacêuticas.

Palavras-chave: Aquecimento ôhmico; Compostos (Bio)funcionais; Microencapsulação; Recursos subexplorados; Sustentabilidade.

ABSTRACT

Green processing for bioresources valorization

Nowadays, resources depletion, waste accumulation and climate change are key factors driving the need for sustainable practices. Some of the strategies for the reduction of generated environmental impact are the reuse of industrial biowastes, as well as increasing the use of underexploited resources by effective/intensified processes to obtain sustainable, ecological, safe and high quality products.

The present thesis aimed to develop efficient and environmentally friendly methodologies involving the application of electric fields and enzymes, as well as low cost and non-toxic solvents for the valorization of undervalued bioresources, as *Pinus pinaster* bark and *Spirulina platensis* microalgae.

Pine bark extracts (PBE) presented high antioxidant, antibacterial and antihyperglycemic activities, with low cytotoxicity and a selective activity against cancer cells as these were negatively affected. These properties were ascribed to their phenolic and flavonoid profile. Moreover, Ohmic Heating (OH) evidenced an increased in the extraction of antioxidant phenolic compounds with no degradation. Results also indicate preferential extraction under the appropriate solvent, presumably due to the action of electric field effects acting in synergy with heat and solvents, promoting membrane electroporation effects. Spray-drying microencapsulation of PBE using maltodextrin was effective in protecting phenolic compounds during gastrointestinal conditions, controlling their delivery and enhancing its health benefits, decreasing the production of reactive oxygen species (ROS) implicated in the process of oxidative stress associated with some pathologies.

Spirulina platensis is interesting for the food industry due to its overall composition and high content in C-phycocyanin (natural blue colorant). The present thesis showed that moderated electric fields associated with temperature promotes cell structure disintegration and electroporation effects improving the extraction and stability of intracellular compounds from *Spirulina*, having also a positive effect on the extraction yield of C-phycocyanin at shorter treatment times. A sequential multi-stage extraction evaluated by conventional thermal extraction, OH, enzymatic treatment (EAE), and their combinations evidenced that the combined OH and EAE extraction allowed selective recovery of phycobiliproteins in the aqueous extraction step. Pigments, such as chlorophyll and carotenoids were selectively extracted with 95% ethanol, as well as the lipid fraction of the microalgae.

Overall, OH technology holds the potential to be a "green" alternative, improving intracellular compounds extraction, in addition to reducing the process time, solvents and energy consumption, offering the possibility of tuning the selectivity and stability of the extracted compounds. Moreover, integrated processes simultaneously addressing different fractions, towards a “zero waste” economy, are desirable and may be even beneficial to the effectiveness of each individual fraction. The obtained biocompounds can be used as natural technological additive and/or functional ingredient for food, cosmetic and pharmaceutical applications.

Keywords: (Bio)Functional compounds; Microencapsulation; Ohmic heating; Sustainability; Underexploited resources.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

AA	Antioxidant activity
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AC	Periodically reverses direction
ANOVA	Analysis of variance
APC	Allophycocyanin
ATR	Attenuated total reflectance
CCD	Central composite design
CD	Circular dichroism
CE_q	Catechin equivalents
CE	Conventional extraction
Chl-a	Chlorophyll-a
Chl-b	Chlorophyll-b
DAD	Diode Array Detector
DC	Unidirectional flow
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl
EAE	Enzyme-assisted extraction
EE	Encapsulation efficiency
EU	European Union
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic acid equivalents
GC	Gas chromatography
GID	Gastrointestinal digestion
GLcE	Glucose equivalents
GRAS	Generally recognized as safe
HPLC	High-performance Liquid Chromatography
HVED	High Voltage Electrical Discharges
IC_{50}	Inhibit 50% of the activity
ICP-AES	Inductively coupled plasma atomic emission spectrometry

ILs	Ionic liquids
LPBE	Lyophilized pine bark extracts
MC	Moisture content
MD	Maltodextrin
MEF	Moderate electric fields
MHA	Mueller-Hinton Agar
MW	Microwaves
NADES	Natural deep eutectic solvents
NREL	National Renewable Energy Laboratory
OH	Ohmic heating
PB	Pine bark
PBE	Pine bark extracts
PBS	Phosphate Buffer Solution
PC	C-phycocyanin
PE	Phycoerythrin
PEF	Pulsed Electric Fields
pNPG	p-nitrophenyl-R-D-glucopyranoside
POH	Pulsed Ohmic Heating
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RSM	Response surface methodology
RT	Room temperature
SD	Standard deviation
SEM	Scanning Electron Microscopy
TC	Total carbohydrates
TPC	Total phenolic content
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
TROLOX	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSE	Solvent solid-liquid extraction
UHPLC	Ultra-high-performance liquid chromatography
US	Ultrasound

SCIENTIFIC OUTPUT

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below.

The results presented in this thesis have been partially published elsewhere.

Papers accepted in peer-reviewed journals:

Electric field-based technologies for valorization of bioresources. Bioresource Technology, 254, 325-339. **2018**. DOI: 10.1016/j.biortech.2018.01.068

Moderate Electric Fields as a Potential Tool for Sustainable Recovery of Phenolic Compounds from *Pinus pinaster* Bark. ACS Sustainable Chem. Eng., 7, 9, 8816-8826. **2019**. DOI: 10.1021/acssuschemeng.9b00780

Influence of thermal and electrical effects of ohmic heating on C-phycocyanin properties and biocompounds recovery from *Spirulina Platensis*. LWT - Food Science and Technology, 128. **2020**. DOI: 10.1016/j.lwt.2020.109491

Unravelling the Biological Potential of *Pinus pinaster* Bark Extracts. Antioxidants, 9, 334. **2020**. DOI: 10.3390/antiox9040334

Green and Sustainable Valorization of Bioactive Phenolic Compounds from *Pinus* By-Products. Molecules, 25(12), 2931. **2020**. DOI: 10.3390/molecules25122931

Papers submitted to peer-reviewed journals:

Encapsulated pine bark polyphenolic extract during gastrointestinal digestion: Bioaccessibility, bioactivity and oxidative stress prevention. Foods (submitted)

Sequential multi-stage extraction of biocompounds from *Spirulina platensis*: combined effect of ohmic heating and enzymatic treatment. Innovative Food Science and Emerging Technologies (submitted)

Conference poster:

Antioxidant activity of *Pinus pinaster* bark: comparison between conventional and ohmic heating extraction". 31st EFFoST International conference. Sitges, Espanha. **2017**.

Selection of the solvent and extraction conditions for maximum recovery of phenolic compounds from *Pinus pinaster* bark. 2nd International Biorestec Conference, Sitges, Spain, **2018**

Biocompounds recovery from *Spirulina* by conventional and ohmic heating methodologies: chemical and biological properties. MicroBiotec'19 - Congress of Microbiology and Biotechnology, Coimbra, Portugal, **2019**

Optimization of water and ethanol relation in conventional and ohmic heating extraction of phenolic compounds from *Pinus pinaster* bark. Congreso CYTA/CESIA – Impulsando la Investigación y la Innovación, León, Spain, **2019**

Oral communications:

Valorization of aqueous and ethanolic extracts from *Pinus pinaster* bark: chemical and biological properties. MicroBiotec'19 - Congress of Microbiology and Biotechnology, Coimbra, Portugal, **2019**

Ohmic heating-assisted extraction of *Pinus pinaster* bark compounds: a qualitative and quantitative assessment. IFT19 Annual Meeting & Food Expo, New Orleans, LA, USA, **2019**

CHAPTER I

MOTIVATION AND OUTLINE

This chapter introduces the background information about this work, as well as its objectives.

The outline of the thesis and its outputs are also presented.

1.1 CONTEXT AND MOTIVATION

Agroforestry and food industries are an important part of the manufacturing industry and their growth can help to achieve the objectives of European Union (EU) industrial policy, acting in different strategic areas, such as increasing energy efficiency, deploying renewable sources, circular economy, bioeconomy and natural carbon sinks ("2050 Long-term strategy | Energy," n.d.; European Commission., 2019). Moreover, the development of these industries should also be in line the 17 sustainable development goals by 2030 dictated by the United Nations; in particular, the agroindustry can directly impact on at least 4 of these goals related to the use of clean energy, industry innovation, responsible consumption and production, and climate action (United Nations., n.d.).

Nowadays, 5 billion tons of biomass residues from agroforestry and food industries are estimated worldwide and represent an emission for 3.3 billion tonnes of carbon dioxide each year (Naidu et al., 2018; Torres-Valenzuela et al., 2019). In the EU, the total annual biowaste is estimated at around 100 million tonnes, generating a negative ecological impact (Jablonský et al., 2018).

One of the strategies for the reduction of generated environmental impact is the reuse of industrial biomass residues (or biowastes) to obtain new natural ingredients. Concomitantly, the growing interest in the development of effective/intensified processes and application of green technologies to obtain sustainable, ecological, safe and high quality products has become a reality (Chemat et al., 2019; Cvjetko Bubalo et al., 2018). This idea is in close association with the principles governing the concept of Green Chemistry, which are mainly aimed at reducing wastes and promoting a more efficient use of energy and resources (Herrero and Ibañez, 2018).

Resources depletion, waste accumulation and climate change are key factors driving the need for sustainable practices. The decrease in the use of "non-recyclable" fossil derivatives and the increase in the use of biowastes, renewable raw materials and underexploited resources (*e.g.,* lignocellulosic residues and algae biomass) by effective processes are in the sights of the EU and the world, contributing to the reduction of the negative impact of processes in the environment and the fight against climate changes ("2050 Long-term strategy | Energy," n.d.).

Therefore, this work tries to address some of these issues through the extraction and characterization of bioactive compounds from the bark of *Pinus pinaster* and *Spirulina platensis* to potentiate new functionalities, opening the possible application of these extracts or biomolecules in the food, nutraceutical, cosmetic and pharmaceutical industry.

1.2 RESEARCH AIMS

The main objective of this thesis was the valorization of agroforestry biowastes and underexploited resources through the obtaining of high added-value products using environmentally friendly practices. The work focused on the recovery of high value biomolecules from *Pinus pinaster* bark and from microalgae *Spirulina platensis* using various technologies (conventional heating, ohmic heating (OH) and enzymatic extraction). The main components of this thesis were to find out how the applied electric fields and/or enzyme promote the diffusion of compounds from the cells of pine bark (PB) and *Spirulina*, as well as to understand possible modifications in the characteristics of biomolecules extracted (such as the type of compounds and their properties) and evaluation of these biomolecules functional properties, including bioactivity and bioaccessibility (bioavailability), as well as their protection by encapsulation.

To achieve the main objective, this thesis was focused on:

1. The chemical/proximal characterization of *Pinus pinaster* bark and *Spirulina platensis.*
2. The application of the conventional heating, OH technology and enzymatic treatments for the recovery of high added-value products from PB and *Spirulina*. This implies the design and optimization of these processes for the treatment of these bioresources.
3. Characterization and separation of bioactive and functional molecules (such as phenolic compounds, phycobiliproteins, lipids and pigments).
4. Study of the permeabilization level of cells membranes and the profile of extracted biomolecules occurring during application of treatments.
5. Evaluation of stability and bioavailability of the biomolecules, mainly phenolic compounds and C-phycocyanin.
6. Optimisation of the encapsulation of bioactive compounds from PB extract and evaluation of stability and bioavailability of the encapsulated compounds.

1.3 OUTLINE OF THE THESIS

To address the above-mentioned objectives, this thesis has been structured in ten chapters as follows:

CHAPTER I – The current chapter presents the context, motivation and the research goals of this thesis. The structure and the scientific outputs are also outlined.

CHAPTER II – This chapter comprises a comprehensive review of the current demand for more sustainable processes for enhancing the biotechnological potential of natural resources. The main motivations for this work together with the objectives and the stucture are highlighted.

The different sections of **Experimental Results** are presented from Chapter III to Chapter VII.

CHAPTER III – It presents the study of solid-liquid conventional extraction of bioactive compounds from *Pinus pinaster* bark and unravel the biological potential (antioxidant, antimicrobial, antidiabetic) and *in vitro* cell viability of PBE. In addition, polyphenolic extracts were characterized by colorimetric methods, liquid chromatography and spectroscopy.

CHAPTER IV – In this chapter, a study was designed to evaluate the aptness of OH-assisted extraction combined with different solvents to obtain extracts with high content of antioxidant phenolic compounds from *P. pinaster* bark. Results of OH-assisted extraction were also compared with a conventional heating performed under identical conditions. The final aim was to achieve a feasible, low-cost, low environmental impact and selective extraction process.

CHAPTER V – This section describes the optimization of PBE encapsulation process by spray-drying using maltodextrin (MD) as coating material and to determine its influence on the bioaccessibility and bioactivity (antioxidant, antibacterial and anticancer) of the phenolic compounds during gastrointestinal digestion.

CHAPTER VI – This chapter describes the influence of thermal and electrical effects of OH on C-phycocyanin properties and other biocompounds recovery from cyanobacterium *Spirulina platensis.*

CHAPTER VII – In this chapter, a study was designed to evaluate the possible sequential multi-stage extraction of biocompounds from *Spirulina platensis* by OH and enzymatic treatments as green technologies.

CHAPTER VIII – This chapter comprises the general conclusions and provides future perspectives.

1.4 REFERENCES

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Chemat, F., Abert-Vian, M., Fabiano-Tixier, A.S., Strube, J., Uhlenbrock, L., Gunjevic, V., Cravotto, G., 2019. Green extraction of natural products. Origins, current status, and future challenges. TrAC Trends Anal. Chem. 118, 248–263. https://doi.org/10.1016/J.TRAC.2019.05.037

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CHAPTER II

GENERAL INTRODUCTION

This chapter was based on the following review papers:

Cristina M.R. Rocha, Zlatina Genisheva, **Pedro Ferreira-Santos**, Rui Rodrigues, António A. Vicente, José A. Teixeira, Ricardo N. Pereira, **2018**. Electric field-based technologies for valorization of bioresources. *Bioresource Technology*, 254, 325–339. doi.org/10.1016/j.biortech.2018.01.068

Pedro Ferreira-Santos, Elisa Zanuso, Zlatina Genisheva, Cristina M. R. Rocha, José A. Teixeira, **2020**. Green and sustainable valorization of bioactive phenolic compounds from pine by-products. *Molecules*, 25 (12), 2931. doi.org/10.3390/molecules25122931.

2.1 UNDERVALUED BIORESOURCES AND BIOREFINERY APPROACH

In past decades, researchers have focused on the discovery of high valuable compounds from natural resources to replace the synthetic compounds used in daily life. Numerous products can be obtained and/or valorized from different sources (e.g. agro-food wastes, forestry by-products and micro and macro-algae). Exploitable compounds or fractions may include proteins and peptides, polysaccharides or oligosaccharides, fibers, gum exudates, lipids, polyphenols, carotenoids and other secondary metabolites with highly-valued bioactivity. A full (bio)chemical and nutritional characterization and the identification of the relevant fractions for each resource is the first step in most bioresources' valorization strategy. Most of the compounds or fractions of interest are intracellular, and appropriate strategies for extraction, separation and further processing of the different exploitable fractions need to be designed to allow a financially and environmentally sustainable valorization of relevant by-products or biowastes.

Concomitantly, resources depletion, waste accumulation and climate change are a combination of forces driving the need for sustainable practices we are facing nowadays. Additionally, urbanization and population growth are generating a continuous rise in the world energy demand. With the energy demand increasing, the necessity of detachment from fossil fuels and the transition to renewable resources is mandatory to reduce the environmental problems. Energy resources as biomass, wind and solar energy can meet the energy requirements if large scale technologies are well developed (Gielen et al., 2016). In this sense, biorefinery has come as the analogous of fossil fuel refinery. The biorefinery term dates back to 1980. Since then, several definitions have been considered. These definitions are based on the type of feedstock used, type of processes and type of products obtained (Wenger and Stern, 2019). In general, the biorefinery concept is the synergy of technologies that convert biomass into their building blocks to produce a variety of products, including biofuels, chemicals and high added value compounds. Hereof, the development of sustainable process involves not only the use of biomass but also implies reducing the use of harmful chemicals, transition to greener processes, efficient use of energy and elimination of wastes (Herrero and Ibañez, 2018). In the last decade, the concept of biorefinery has been widely applied to different matrixes for a complete reuse of biorecourses. In this sense, in the continuation of this work will be approached two types of biomass, one lignocellulosic and a microalgae, for obtaining of high added value compounds with potential for industrial application.

2.1.1 Lignocellulosic biomass

Currently, lignocellulosic biomass has been largely studied as potential substrate in fermentation processes or thermochemical processes. Nevertheless, innovative and new emerging technologies are being studied to increase the recovery of high-value compounds of interest, particularly bioactive ones. The recovery of these of high-value compounds is linked to the biorefinery concept and the green chemistry principles. Lignocellulosic biomass is mostly considered as a residue from crops as straw, sugarcane bagasse, corn stover and wood waste. Cellulose is the major component of the lignocellulosic materials, followed by hemicellulose and lignin. Cellulose is the world's most abundant biopolymer made up of glucose units. Applications of cellulose extracted from lignocellulosics include the manufacture of cellulosic fiber and nanocrystalline cellulose in a wide range of industries such as automotive, textile and medicine due to the strength on its structure, availability, modifiable surface, renewability and low cost (Brinchi et al., 2013; Nuruddin et al., 2016). Hemicellulose is the second most abundant polysaccharide in lignocellulosic biomass mainly composed by monomeric units as xylose, mannose, arabinose, glucose, galactose and acids as uranic acid (Luo et al., 2019). Hemicellulose and specific target products from hemicellulose are used in a variety of areas as food, medicine, and chemicals due to the biocompatibility and bioactivity properties they show (Luo et al., 2019). Pine sawdust has been used to produce levulinic, formic and acetic acid and furfural form hemicellulose extracted by steam explosion (Clauser et al., 2018). Also, pinewood (*Pinus eldarica)* pretreated with dilute sodium hydroxide was used to produce ethanol from the pretreated solid where the solubilized hemicellulose fraction was used to produce biogas (Safari et al., 2017). The third main component of lignocellulosic materials is lignin, an amorphous phenolic polymer which provides mechanical strength and rigidity to plants (Charisteidis et al., 2019). In the biorefinery process, lignin cannot be used as substrate for fermentation as it contains no sugars. Therefore, a wide area of research is on lignin valorization. Lignin is mainly used to generate heat and electricity due to the high heating capacity though other applications are possible, including the use as precursor for carbon fiber synthesis, resins and low–molecular weight aromatic and phenolic compounds (Ragauskas et al., 2014; Yuan et al., 2013). Nowadays, the modern polymer industry from natural sources of aromatic compounds is limited due to the high prices of the final product. Here, lignin plays an important role since phenolic compounds can be obtained from lignin deconstruction (Paone et al., 2019).

Historically, wood has been a major energy source for human being. Forest biomass is the most abundant feedstock on earth, representing 89.3% of the total biomass (Chen et al., 2017). In Europe, forest area is one of the most important renewable resources, representing near 5% of the world's forest and covering 43% of its land, comprising close to 182 million hectares of forest. Forest is also considered as a resource for improving life quality and job generation ("Forests, forestry and logging - Statistics Explained," n.d.). Wood biomass can be densified into solid fuels, as pellets, or converted into heat, electricity, biofuels, and other bioproducts through a variety of chemical, thermochemical, and biochemical processes (Cambero et al., 2016). On the other hand, lumber industry generates a considerable amount of waste which includes leaves, barks, sawdust, chips, cones, resins and branches. These residues are not usually well valued and are thrown away, burned or used for animal bedding, although they can be a profitable source of high added value compounds (Vega et al., 2019). Therein, wood biomass residues have an increased overall value due to the metabolites that are present in lower abundance compared with cellulose, hemicellulose and lignin. These extractive compounds combine alkaloids, waxes, essential oils, pectins, resins and phenolic compounds (Belt et al., 2017) and are of great importance considering the wide industry applications. More precisely, the antioxidant, antimicrobial, anti-inflammatory and antitumoral effects they show makes them suitable for application in food and pharmaceutical industries (Tungmunnithum et al., 2018). Overall, it is important to reduce and give a "second life" to these agroforestry residues, moving to "zero waste". **Figure 1** shows the schematic representation of a lignocellulosic biorefinery process, using as an example the pine by-product.

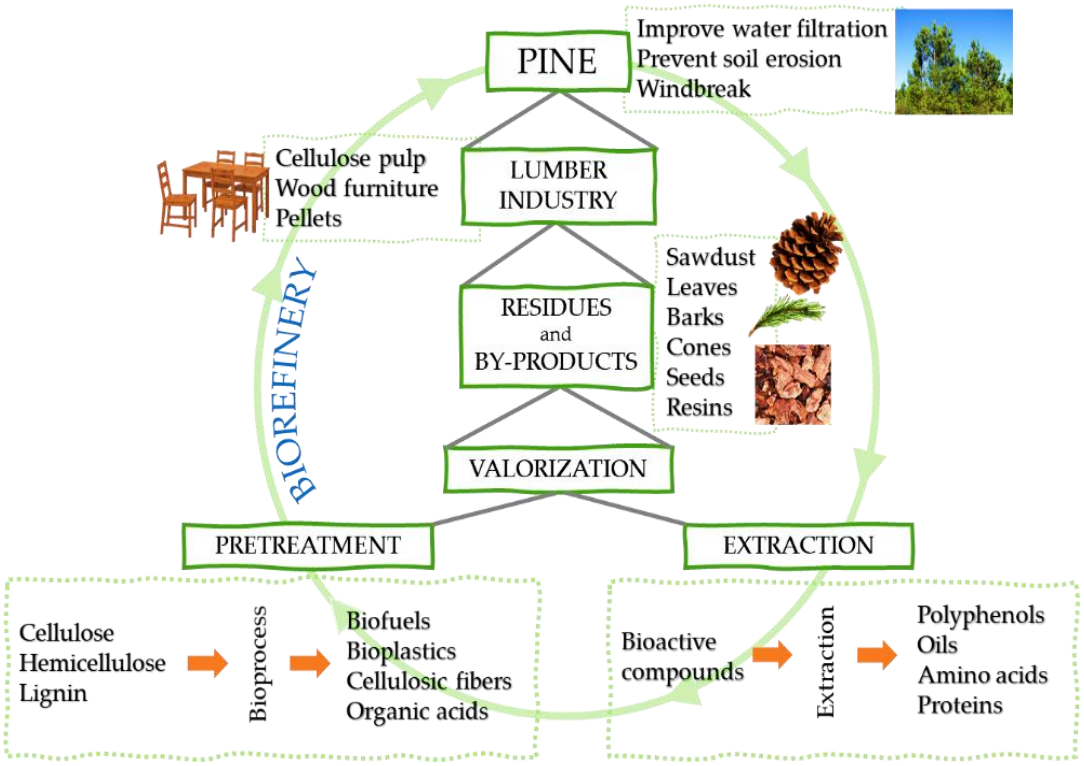

Figure 1. Example of lignocellulosic biomass valorization under biorefinery concept.

2.1.1.1 Pine as feedstocks

Part of this work is mainly focused on the valorization of the *Pinus* species (and its by-products), which are evergreen trees of resinous conifers group from the *Pinaceae* family. In the EU, there are more than 14 different species, representing one of the largest forest occupations ("Species - EUFORGEN European forest genetic resources programme," n.d.). In Portugal, pine forests are the third forest formation after eucalyptus and cork oaks, with an area of approximately 1 million hectares, representing an important part of the total forest, about 23% ("IFN6 — ICNF," n.d.).

The chemical composition of pine and its constituents (wood, bark, leaves, cones, seeds, and resin) varies depending on the *Pinus* tree and also on many other factors, such as genotypic, ecological, and seasonal, among others (Tümen et al., 2018). The methodology used for the determination of chemical composition of plant resources is also a factor to consider, since different methods lead to different results (del Río et al., 2019).

The general chemical/nutritional composition of pine by-products has been described by several authors (wood (Akgül et al., 2007; Chaula et al., 2014; Cotana et al., 2014; Gulsoy and Ozturk, 2016; Räisänen and Athanassiadis, 2013; Shemfe et al., 2015), bark (Fradinho et al., 2002;

Miranda et al., 2017, 2012; Vieito et al., 2019), needles (Khan et al., 2019; Ramay and Yalçın, 2019), cones (Dönmez et al., 2012; Gulsoy and Ozturk, 2016), seeds (nuts) (Cheikh-Rouhou et al., 2006; López-Mata, 2001; Tukan et al., 2013) and resin or oleoresin (Rodrigues-Corrêa et al., 2012; Wiyono et al., 2006)) and is summarized in **Figure 2**.

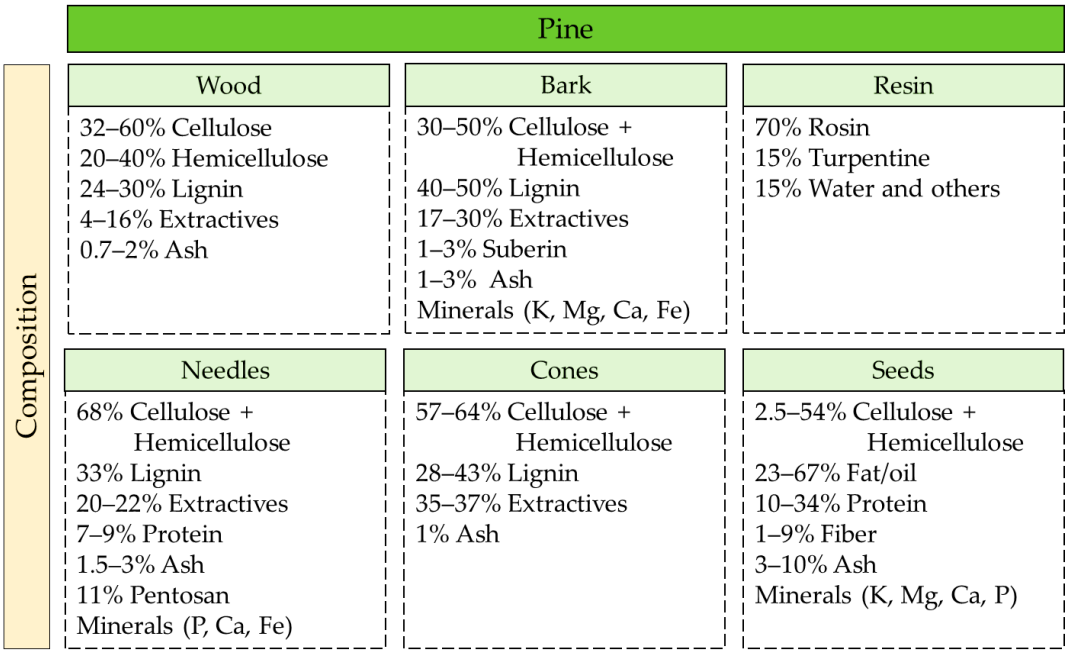

Figure 2. General chemical/nutritional composition of pine by-products.

Pinus plant is very important economically, considered a good feedstocks for bioeconomy (see **Figure 1**) (Mitchell et al., 2016). In its natural environment, it has an important protective function, such as improving water infiltration, preventing soil erosion on dry slopes and serving as a windbreak ("Species - EUFORGEN European forest genetic resources programme," n.d.). Trees are also used as ornamental plants in urban and industrial contexts. Other uses include Christmas trees and fuelwood. Interestingly, in a study by Ehn and co-workers (Ehn et al., 2014), it has been found that pine forest aroma (for its content in volatile compounds, terpenes) can limit climate change, preventing the global warming.

The main industrial activities are related to the usage of pine wood and wooden products, including sawmills, wood panels, cellulose pulp and paper production, wood fuels, carpentry, packing and wood furniture ("Species - EUFORGEN European forest genetic resources programme," n.d.). These feedstock, their components and by–products are considered a good source for wood biorefineries, transforming the lignocellulosic fractions into biofuels, chemical products and composite materials, as previously mentioned (Mitchell et al., 2016).

Pine bark, the by-product obtained in larger quantities that is produced when wood is transformed, is almost exclusively used as fuel, being also subjected to composting to filling substrate in nurseries, utilized for cover in public gardens, or simply thrown away on landscapes (Fradinho et al., 2002). Nowadays, this by-product has been used as low-cost and green alternatives waste–based biosorbents for the removal of a wide range of water pollutants (B. Silva et al., 2020).

The pine leaves (needles) are normally used in agriculture to enrich the soil, and the seeds are used for human consumption because they are highly nutritious and much appreciated by consumers in cooked/prepared dishes (food industry) or simply as edible pine nuts. The resins, a product resulting from the exploitation of these species, are more regularly used as a sealant, glue, varnish and also as a solvent and paint thinner (turpentine oil) (Kim et al., 2019; Mitchell et al., 2016; Wiyono et al., 2006).

In addition to the "traditional" uses of these by-products, it is important to take advantage of these bioresources to create high value-added products.

Currently, the agroforestry by-products have been increasingly exploited to isolate biocompounds with high industrial interest. Studies using natural matrices as a potential source of bioactive compounds have been published in recent decades (Chemat et al., 2015; Cvjetko Bubalo et al., 2018; Fidelis et al., 2019; Lourenço et al., 2019; Maqsood et al., 2019; Shi et al., 2005; Soquetta et al., 2018). For instance, in a new review paper (Tanase et al., 2019), the authors report that, in addition to fruits and vegetables, tree barks are rich in phenolic compounds with excellent biological properties (such as antioxidant, immunostimulatory, anticancer, antibacterial, anti-inflamatory, antimutagenic, *etc.*) and may used to obtain functional ingredients.

PB is one of the most sought-after sources of antioxidant biocompounds of natural origin. The extracts obtained from this by–product are mostly composed of phenolic compounds with high biological activity (Ku et al., 2007; Mellouk et al., 2016; Miranda et al., 2012; Sharma et al., 2016; Shi et al., 2005; Yesil-Celiktas et al., 2009). Nowadays, there are numerous studies reporting the applicability of pine bioactive extracts in various areas, such as health care, food, agro–chemical and others (Iravani and Zolfaghari, 2011; Maimoona et al., 2011; Mármol et al., 2019). One of the most promising applications for these extracts is in the preservation and enrichment of foods, thus replacing synthetic antioxidants, as well as a nutraceutical, cosmeceutical or pharmaceutical.

The pine wood/sawdust extractives, rich in phenolic antioxidant compounds, have a potential for food and pharmaceutical applications, such as preservatives or nutraceuticals (Chaula et al., 2014; Meullemiestre et al., 2016).

Pine tars, by-product of pine wood and bark, are known to contain tricyclic diterpenoid resin acids, tricyclic diterpene hydrocarbons, alkylphenanthrenes and fatty acids. This water resistant by-product has a wide range of applications, for example, as a multipurpose adhesive, sealant and in medicine (Egenberg et al., 2002; Stacey et al., 2020).

Knowing the chemical composition and physicochemical properties, pine seeds or nuts appear to have a positive effect on human health (Cheikh-Rouhou et al., 2006; Tukan et al., 2013). The seed lipids, rich in linoleic acid, have a beneficial effect on blood pressure and cholesterol. The fatty acid composition and the relatively high polyphenol content present high protection against oxidative stress. In this sense, pine seeds can potentially be used in the food industry and other non-food industries, such as pharmaceutical and cosmetics (Hoon et al., 2015; Hou et al., 2018; Lin et al., 2017).

Oleoresins are widely used in the synthesis of perfumed compounds for cosmetics, essences as additives for food and beverages, food protection (antimicrobial), bioinsecticides (high repellent activity), tapping green chemicals, biofuels and carbon sequestration from multipurpose trees (Kim et al., 2019; Mitchell et al., 2016; Rodrigues-Corrêa et al., 2012; Wiyono et al., 2006).

Interestingly, this search for functional extracts, new natural molecules and the creation of new high value–added products has increased the use/study of agroforestry by-products and residues, including pine bark, sawdust, leaves, seeds and resin. This makes it possible to potentially bring these "wastes" back to the market.

2.1.1.2 Pine (bio)functional compounds

Lately, a lot of studies refer to the wood bark as an important source of polyphenols with a potential biological effect (Tanase et al., 2019). Polyphenols are chemical compounds distributed in herb plants, vegetables, fruits and other photosynthetic organisms (such as microlagae) with a wide range of applicability. Currently, more than 8000 phenolic compounds are known, and among them, 4000 flavonoids have been identified [68]. They have developed an array of defenses to protect the chloroplast from reactive oxygen species (ROS) and also protect the photosynthetic organisms against insects and other animals (Kepekçi and Saygideger, 2012). Among natural antioxidants, phenolic antioxidants are in the forefront as they have the structural requirements of free radical scavengers.

Extracts from pine contain considerable amount of flavonoids and condensed tannins (Chupin et al., 2015). The amount of these active constituents varies depending on the pine specie and

geographical location of growth (Yesil-Celiktas et al., 2009). Moreover, the solvent polarity and different methods of extraction used contribute for the different content in natural antioxidant and antioxidant activity (AA) of the extracts (Hadzri et al., 2014). It is very important to ensure the chemical stability of polyphenols during the extraction processes, using mild extraction methods. The emergent technologies are important options to be considered (Liazid et al., 2010).

As previously mentioned, different parts of the pine can be used for the extraction of polyphenols compounds (needles, seeds, bark and cone), but the most studied is the pine bark. In Europe there are fourteen different known pine species. Although all extracts from pine regardless the solvent, the method, the plant part or pine specie used, have high amounts of total polyphenols, there are differences in the concentrations and type of the individual compounds as well as in the strength of the bioactivities (**Table 1**). This is due to the natural variability such as genotype, differences in growing and harvesting conditions, climate, soil type, etc. In a comparative study of three different species of PBE (*P. pinea*, *P. pinaster* and *P. halepensis*), it was found that all extracts had induced cell-cycle arrest and apoptosis in Caco-2 cells (human colorectal adenocarcinoma). However, the extracts were different in terms of individual polyphenol compounds and strength of the bioactivities. The extract of *P. pinaster* was the one with the highest biological activity and the one with the highest amount of procyanidin B2. The most abundant compounds in the pine samples were taxifolin and catechin. Procyanidin A2 was only present in samples of *P. halepensis.* Procyanidin B1 was found in *P. pinea* in concentrations two times higher compared to the other two species of pine in the study. Extracts of *P. pinaster* had the highest antioxidant capacity, while *P. halepensis*, had the lowest antioxidant capacity (Gascón et al., 2018).

Barks of various pine species from different regions of Turkey (*P. pinea*, *P. sylvestris*, *P. nigra*) and Germany (*P. parfliora*, *P. ponderosa*, *P. sylvestris*, *P. nigra*) were compared in terms of their flavonoids content and AA (Yesil-Celiktas et al., 2009). The highest AA was achieved by *P. pinea* (81.0%) while *P. parfliora* showed the lowest activity (31.9%). Moreover, a very good correlation was found between the AA of the extracts and its total phenolic compounds, *i.e.* species *P. pinea* had the highest total phenol content, while *P. parfliora* had the lowest total phenol content. The main polyphenol compounds found in all extracts were catechin, catechin gallate, epicatechin and taxifolin. The *P. pinea* had the highest amount of individual phenolic compounds.

Different methods (maceration with magnetic stirring, ultrasound-assisted extraction, microwave-assisted extraction, and extraction with pressurized liquids) were used to obtain the extracts of seeds from *P. pinaster*. Direct relationship was found between the AA and total polyphenol content

of pine seeds extracts. Moreover, it was concluded that high extraction temperatures in any of the methods used led to a decrease in bioactivities (Liazid et al., 2010).

Needle and twig extracts of five different pine species (*P. brutia*, *P. halepensis*, *P. nigra*, *P. pinea*, and *P. sylvestris*) together with Pycnogenol®, a PB commercial extract, besides the strong AA, presented cholinesterase inhibitory potential (Ustun et al., 2012). Extract from *Pinus brutia* bark had 3.3-fold more total catechins and 9.8-fold more taxifolin than Pycnogenol®, showing strong anti-inflammatory activity (Ince et al., 2009). A total of 17 phenolic compounds (mainly flavonoids) were identified in needles of four pine species, *P. peuce*, *P. nigra*, *P. mugo* and *P. sylvestris* from the Macedonian flora (Karapandzova et al., 2015). Authors concluded that there are no differences between the studied species of pine in terms of polyphenols. Taxifolin and quercetin were not found in any of the Macedonian pine species.

Moreover, the impact of the particle size of PB (between 0.05 mm and 1 mm) in the extracts was evaluated (Chupin et al., 2015). Mass transfer kinetics and the access of the solvent to the soluble components depends on the particle size (Meullemiestre et al., 2016). The particle size have direct effect on the amount of the polyphenol extracted. The smaller the particle size is, the more extracts are obtained (best results were registered for size 0.4 mm). However, there is no impact on the nature of the extract and on the types of the compounds extracted (Chupin et al., 2015). There is a lower limit of the particle size beyond which the quantity of extracted polyphenols decreased. It was registered that very fine particles stayed in suspension at the surface of the solvent and therefore were not subjected to proper extraction (Meullemiestre et al., 2016).

The extraction of polyphenols would also depend on the solid/liquid ratio. Meullemiestre et al. (Meullemiestre et al., 2016) found the optimum ratio to be about 6 g of dry material/100 mL; when concentrations were higher than 7.5 g of dry material/100 mL the maritime pine wood absorbed all available liquid.

There are not many studies showing identification and even less studies showing quantification of polyphenols in extracts from *Pine species* (**Table 1**). The extracts are obtained mostly from the pine bark, and fewer from needles. The chemical composition of the extracts depends on the type of pine used (specie, location), on the part of the plant, on the method of extraction and on the solvent. For example the main group of polyphenols compounds found in the *P. sylvestris* is the group of stilbenes, while in *P. pinaster* the main group of compounds are the flavonoids (Venugopal et al., 2015).

As one can see from **Table 1**, the main polyphenol compounds found in extracts from pine needles are *p*-coumaric acid and epicatechin, in pine seeds eriodictyol and taxifolin, and in PB catechin, gallic acid and taxifolin. In the following text we summarize the bioactivities of the individual compound found in the extracts of pine species. However, we want to draw the attention of the reader that, in terms of expressing biological activities the polyphenols act as group of compounds rather than individual compounds, and synergistic and/or antagonist or simply different effects may be found.

The *p*-coumaric acid together with ferulic and caffeic acids are the most common hydroxycinnamic acids in pine-based extracts. The hydroxylation of *p*-coumaric acid results in the formation of ferulic acid. While the oxymethylation of *p*-coumaric acid produces caffeic acid, respectively. These phenolic acids are used as precursors in the synthesis of lignins and other phenolics (Kumar and Goel, 2019). Taofiq *et al.* (Taofiq et al., 2019) conducted a study on individual compounds as possible ingredients in cosmeceutical formulations. The authors concluded that *p*-coumaric, protocatechuic and cinnamic acids displayed anti-tyrosinase, antimicrobial and anti-inflammatory activities, showing their potential for the cosmeceutical industry. Caffeic acid and, at a higher degree, ferulic acid proved to protect the skin against UVB-induced erythema. Besides as antioxidants, these two hydroxycinnamic acids can be used as photoprotectors in skin cosmetics (Saija et al., 2000).

The protocatechuic, vanillic, and syringic acids are the three commonly found hydroxybenzoic acids (Kumar and Goel, 2019). This tree hydroxybenzoic acids were also found in pine extracts (**Table 1**). Vanillic acid demonstrated anti-inflammatory activity with neuroprotective activity and was found to be a promising candidate for preventing and/or delaying the onset and progression of ischemic injury and vascular dementia (Khoshnam et al., 2018). In other cases the use of a mixture of phenolic compounds rather than the individual compounds exhibit stronger activities. For example the combined use of syringic acid, resveratrol and gallic acid, in rats, revealed antioxidant and cardio protective activities (Sammeturi et al., 2019). Resveratrol also showed anticancer effect when examined in lung, prostate, breast, skin and gastrointestinal cancers (Sajadimajd et al., 2020). Resveratrol is the most known constituent of wines and grapes, but was also found in PBE. It was also proven that resveratrol has anti-inflammatory capacity especially in the skeletal muscle, but is less active in liver (Szkudelska et al., 2020). Moreover, gallic and *p*-coumaric acids were considered as promising adjuvant agents against the progression of neurodegeneration in the brain by diabetes (Abdel-Moneim et al., 2017). Rosmarinic acid is known to have a number of potentially

beneficial biological effects and is an acid ester of caffeic acid and 3(3,4-dihydroxyphenyl)lactic acid. The use of rosmarinic acid in gelatin edible film showed long-term antibacterial activity. Rosmarinic acid edible films may have promising application in the fields of food and pharmaceutical packaging, as they showed a good antibacterial activity even after 3 months of storage (Ge et al., 2018). Rosmarinic acid was found in extracts of PB in concentrations between 0.4 mg/g and 0.8 mg/g (Ferreira-Santos et al., 2019a). This acid was found to be the predominant compound of *Salvia* species. Strong correlations between the rosmarinic acid contents and bioactivites of *Salvia* samples were established (Adımcılar et al., 2019). Moreover this acid demonstrated potent antiviral properties (Chung et al., 2015). 3,4 dihydroxybenzoic acid is universal in the Angiosperm plants, as it is constituent of lignin. It is a strong antioxidant, as well as, neuroprotective against Aβ-induced neuronal damage (Ban et al., 2007). This acid can be used in formulations for phytonematode control, as it showed nematicidal activity against juveniles of *M. incognita* (Nguyen et al., 2013).

As we can see from the **Table 1**, in different pine extracts many catechin compounds with diphenylpropane (C_6–C_3–C_6–) skeleton were found. Epigallocatechin, epigallocatechin gallate and epicatechin are the main constituents of the leaves of *Camellia sinensis* (the tea plant). While catechin gallate is a minor polyphenolic constituent in green tea, 1.28% (by weight) of the total catechin content in green tea (Babich et al., 2007). These catechins are responsible for the astringent and bitter taste of the green tea (Pauli et al., 2014). Catechin is the main phenolic compound present in *P. pinaster* bark extract followed by epicatechin and epicatechin gallate (Chupin et al., 2013). Gallocatechin was found also in Norway spruce and confirmed to be a strong inhibitor of melanin biosynthesis, however there is little information on the biological activities of this compound (Hammerbacher et al., 2018). All these catechins have strong antioxidants, and anticancer activities against different types of cancer (Babich et al., 2007). Catechins have received considerable attention, as promising candidates for development of therapeutic agents.

Taxifolin, as an individual compound, is extensively studied. It was found in the pine extracts of seeds and bark, as in bark it is present in much higher concentrations than in seeds (Gascón et al., 2018; Lantto et al., 2009; Vieito et al., 2019). For instance, this compound was recovered from *P. nigra* bark (Ghoreishi et al., 2016) with a maximum extraction recovery of 34 %. Taxifolin was detected as the major compound in other needle leaved trees like the Japanese larch, *Larix kaempferi* (Muramatsu et al., 2019). Its main bioactivities are antioxidant, anticancer and anti-inflammatory (Gascón et al., 2018; Talmaciu et al., 2016). Quercetin is a taxifolin related flavonoids

found in onions, and showed anti-inflammatory, antimicrobial and anticancer properties (*in vitro* and *in vivo*) (Świeca et al., 2013). Quercetin rich extracts from onion skin can be used in functional bread production (Świeca et al., 2013).

According to Lantto and co-workers (Lantto et al., 2009), eriodictyol was one of the main compounds found in extracts of Siberian pine bark. Eriodictyol, as taxifolin can be find in citrus fruits. It has showed antioxidant and anti-inflammatory activities. Recent findings indicated that eriodictyol might be a new preventative agent for osteoarthritis (Wang et al., 2019). Another promising therapeutic agent for the treatment of Osteoarthritis is the ellagic acid (Lin et al., 2020). It is found in high concentrations in the ethanolic extracts of *P. pinaster* (Gascón et al., 2018; Vieito et al., 2019), also is constituent in the fruit peel of berries and nuts (Lin et al., 2020).

Apigenin is found abundantly in herbs, fruits and vegetables (peppermint, grape fruit, parsley). It has potent antioxidant, anti-inflammatory, and anticancer properties (Silvan and Manoharan, 2013). Gascon el al. (Gascón et al., 2018) found three procyanidin compounds in PBE: A2, B1 and B2. The activities of procyanidins depend on their structure, especially on their degree of polymerization. Procyanidin B2 is one of the most active molecules within the procyanidins, as well as the most studied. It is also found in cocoa and grape seeds. The three compounds have AA, B-type procyanidins have also neuroprotective activity. The richest set of bioactivities agglomerates for the procyanidins B2, but as we mentioned this is also the most studied one.

There are several PB commercial extracts: Oligopin®, Pycnogenol® and Flavangenol®. Pycnogenol® is the most known and most studied one. Its extraction involves standardized consecutive steps using water and ethanol as solvents. It is a polyphenol rich extract prepared from *P. pinaster* (French maritime pine). The main constituents are procyanidins (85%), flavonoids (catechin, taxifolin), as well as some phenolic acids in minor amounts (gallic, caffeic, and ferulic acid) (Ustun et al., 2012). This extract proved to have excellent antioxidant properties that can promote various health properties such as: cardioprotective, anticancer, antihypertensive and anti-inflammatory (Braga et al., 2008; Ince et al., 2009). In another study, the clinical efficiency of Pycnogenol® in the management, treatment and control of chronic venous insufficiency and venous microangiopathy was proven (Cesarone et al., 2010). This extract showed also anti-diabetic property as the supplementation of Pycnogenol® to conventional diabetes treatment lowered glucose levels and improved endothelial function (Liu et al., 2004). Oligopin® is another extract obtained from the pine tree *P. pinaster* from a specific location in France (Landes of Gascony). Its production includes two extraction steps and one purification step. This methodology ensure that

the obtained extract has a specific and constant composition. The main compounds found in Oligopin® are flavonoids (catechin and taxifolin) and acids (ferulic, gallic, caffeic, *p*-coumaric and protocatechic) (Segal et al., 2018).

Like a final remark, the individual polyphenol compounds found in the extracts of PB have diverse bioactivities that go in the encounter of the mentioned previously bioactivities of the hole extracts like antioxidant, anticancer, cardioprotective, antidiabetic, anti-inflammatory, *etc*. Pine extracts have active ingredients that are useful for the food industry as supplements or natural pigments, for food preservation and as active food packaging. In the cosmetic formulations they can be used for protecting the skin against oxygen reactive species, formed by pollution, stress or ultraviolet reaction.

Table 1. Individual phenolic compounds found in pine by-products and their reported bioactivities.

Name	Chemical Formula	Concentration range (mg/g)	Bioactivities	Reference
		NEEDLES		
epicatechin	$C_{15}H_{14}O_6$	1.5	Antioxidant	(Pauli et al., 2014; Yen et al., 2008)
p-coumaric acid	$C_9H_8O_3$	2.3	antioxidant, anti-inflammatory, hepatoprotective and renoprotective, anti-neurodegenerative, anti-cholesterolemic, improve insulin resistance, anti-tyrosinase, antimicrobial	(Abdel-Moneim et al., 2017; Latha and Daisy, 2011; Taofiq et al., 2019; Yen et al., 2008)
		SEEDS		
protocatechuic acid	$C_7H_6O_4$	0.5	anti-tyrosinase, antimicrobial and anti-inflammatory activities	(Lantto et al., 2009; Taofiq et al., 2019)
catechin	$C_{15}H_{14}O_6$	0.5	hepatoprotective activity	(Lantto et al., 2009; Zhang et al., 2019)
epigallocatechin gallate	$C_{22}H_{18}O_{11}$	0.5	antimicrobial, antioxidante, photoprotective	(Lantto et al., 2009; Zhang et al., 2019)
vanillic acid	$C_8H_8O_4$	0.9	anti-inflammatory, neuroprotective	(Khoshnam et al., 2018; Lantto et al., 2009)
syringic acid	$C_9H_{10}O_5$	1.0	cardioprotective, antioxidante, antimicrobial, anti-inflammatory, neuro and hepato-protective activities	(Lantto et al., 2009; Sammeturi et al., 2019)
epicatechin	$C_{15}H_{14}O_6$	1.3	Antioxidant	(Lantto et al., 2009)
taxifolin	$C_{15}H_{14}O_7$	1.7	antioxidant, anticancer, anti-inflammatory	(Lantto et al., 2009)
cinnamic acid	$C_9H_8O_2$	0.1	anti-tyrosinase, antimicrobial and anti-inflammatory	(Lantto et al., 2009; Taofiq et al., 2019)

eriodictyol	$C_{15}H_{12}O_6$	3.8	anti-inflammatory	(Lantto et al., 2009; Wang et al., 2018)
m-coumaric acid	$C_9H_8O_3$	Traces	not found	(Lantto et al., 2009)
BARK				
gallic acid	$C_7H_6O_5$	traces – 5.5	anti-inflammatory, antihyperlipidemic, antioxidant, antitumor, antihyperglycemic, and anti-neurodegenerative, cardioprotective	(Abdel-Moneim et al., 2017; Chupin et al., 2013; Latha and Daisy, 2011; Sammeturi et al., 2019; Vieito et al., 2019)
gallocatechin	$C_{15}H_{14}O_7$	0.07 – 0.95	inhibitor of melanin biosynthesis	(Hammerbacher et al., 2018)
epicatechin	$C_{15}H_{14}O_6$	0.06 – 1.9	Antioxidant	(Chupin et al., 2013; Yesil-Celiktas et al., 2009)
epicatechin gallate	$C_{22}H_{18}O_{10}$	0.3 – 0.9	Antioxidant	(Chupin et al., 2013)
catechin	$C_{15}H_{14}O_6$	0.095 – 7.7	antioxidant, anticancer, cardioprotective, antifungal	(Chupin et al., 2013; Gascón et al., 2018; Hammerbacher et al., 2018; Yesil-Celiktas et al., 2009)
vanillic acid	$C_8H_8O_4$	0.02 – 0.07	neuroprotective, anti-inflammatory	(Khoshnam et al., 2018)
caffeic acid	$C_9H_8O_4$	0.03 – 0.2	antioxidant, photoprotective	(Iravani and Zolfaghari, 2014; Saija et al., 2000)
rosmaniric acid	$C_{18}H_{16}O_8$	0.4 – 0.8	antioxidant, antidiabetic, antibacterial, antiviral	(Adımcılar et al., 2019; Chung et al., 2015)
catechin gallate	$C_{22}H_{18}O_{10}$	0.002 – 1.5	antioxidant, anticancer	(Babich et al., 2007; Yesil-Celiktas et al., 2009)
taxifolin	$C_{15}H_{12}O_7$	0.01 – 4.7	antioxidant, anticancer, anti-inflammatory	(Gascón et al., 2018; Iravani and Zolfaghari, 2014; Talmaciu et al., 2016; Vieito et al., 2019)

3,4 dihydroxy-benzoic acid	$C_9H_{10}O_4$	0.08 – 0.8	neuroprotective, antioxidante, nematicidal activity	(Ban et al., 2007)
ellagic acid	$C_{14}H_6O_8$	0.4 – 4.0	anti-inflammatory, antioxidant	(Lin et al., 2009)
apigenin	$C_{15}H_{10}O_5$	0.3 – 0.5	anticancer, antioxidant, anti-inflammatory	(Silvan and Manoharan, 2013)
resveratrol	$C_{14}H_{12}O_3$	0.03 – 0.4	antioxidant, anti-cancer, cardioprotective, anti-inflammatory	(Sammeturi et al., 2019; Szkudelska et al., 2020)
ferulic acid	$C_{10}H_{10}O_4$	0.06 – 0.5	antioxidant, photoprotective	(Iravani and Zolfaghari, 2014; Saija et al., 2000; Vieito et al., 2019)
p-coumaric acid	$C_9H_8O_3$	n.q.	antioxidant, anti-inflammatory, hepatoprotective and renoprotective, anti-neurodegenerative, anti-cholesterolemic, improve insulin resistance, anti-tyrosinase, antimicrobial	(Abdel-Moneim et al., 2017; Sasidharan et al., 2011; Taofiq et al., 2019)
quercetin	$C_{15}H_{10}O_7$	0.06 – 1.1	inflammatory, antimicrobial, anticancer	(Vieito et al., 2019)
procyanidin A2	$C_{30}H_{24}O_{12}$	n.q.	Antioxidant	(Gascón et al., 2018)
procyanidin B1	$C_{30}H_{26}O_{12}$	n.q.	antioxidant, neuroprotective, anti-proliferative activity	(Gascón et al., 2018)
procyanidin B2	$C_{30}H_{26}O_{12}$	n.q.	antioxidant, anti-inflammatory, cardioprotective, neuroprotective, anti-proliferative activity	(Gascón et al., 2018)

n.q. – not quantified.

2.1.2 Microalgae biomass

Recently, microalgae use have emerged in response to the uprising energy crisis, depletion of natural sources and climate change. Nowadays, microalgae are classified as potential candidates in biorefinery processes because they are capable of producing multiple high added-value products, contributing to the circular economy. They are considered as renewable sources of biomass, which is beneficial in terms of rapid growth under optimal conditions, decreased competition with food industry and composition that are selective. Moreover, a highly contentious issue of great concern is the argument that emissions of carbon dioxide (CO_2) from fossil fuel use, especially from coal combustion, are main responsible for global climate change. As a result of studies during the past five decades, emissions of CO_2 have become an important issue with respect to global climate change because atmospheric CO_2 concentrations increased significantly in the last century and have continued to rise at an increasing rate (Geada et al., 2018; Jacob-Lopes et al., 2015).

Therefore, various CO_2 sequestration techniques have been developed and the various technologies for CO_2 capture and storage need to be evaluated from the point of view of obtaining carbon credits, aimed at stabilizing emissions of the pollutant (i.e. from exhaust gases originated by fossil fuel combustion) and/or wastewater treatment processes (Geada et al., 2018). Of these techniques, CO_2 capture by photosynthetic organisms such as microalgae/cyanobacteria shows good potential in view of the economic advantages it presents, rate of CO_2 capture, and the speed with which the technology can be introduced to the industrial community.

Furthermore, microalgae have great potential in generating energy from biotechnological processes using renewable sources and without compromising food security and agriculture. Microalgae have been of major interest in biofuel production as well as in the feed, chemical and pharmaceutical sectors (Geada et al., 2018). Depending on the species and growth conditions, microalgae can be selected to produce a wide variety and abundance of lipids, proteins, phycocolloids, carbohydrates, vitamins and feedstocks important for biofuels and production of nutraceuticals (Chia et al., 2019; Geada et al., 2018).

Figure 3 shows the schematic representation of a typical "microalgae biorefinery" and applications of a fully integrated microalgae biomass cultivation processing system. Microalgae biorefining involves assessment and use of different technologies to obtain different types of bioproducts from biomass, which can be marketed and used to solve specific problems in many different areas. Finally, there must be the safe and inexpensive disposal of all waste products generated during the

process. Therefore, a portion of the residual biomass can go to an anaerobic digester to generate biogas, and the rest can be used as nutrients to feed the photobioreactor again.

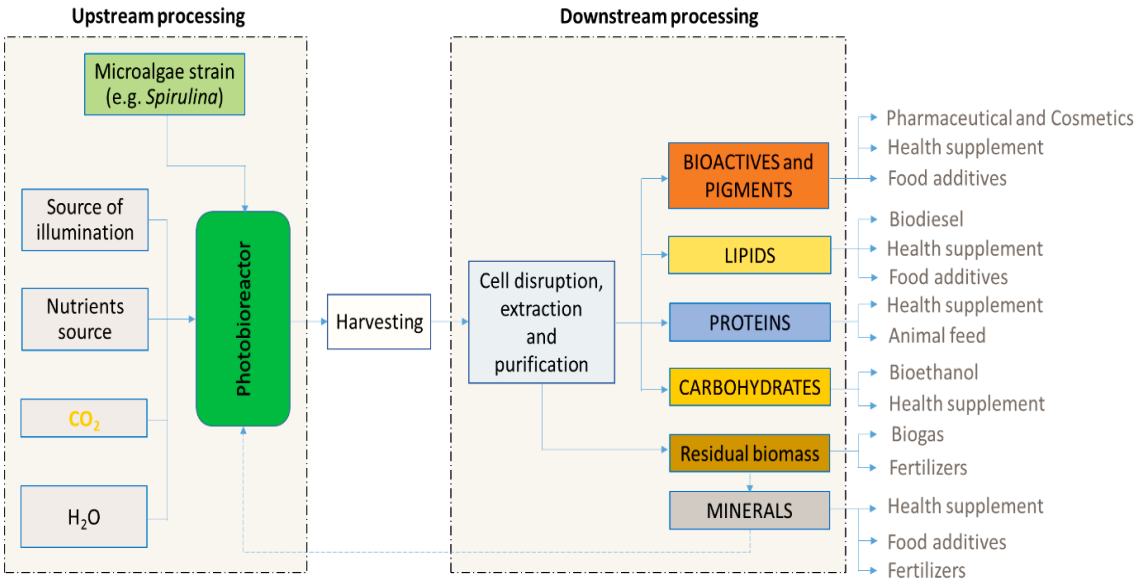

Figure 3. Example of microalgae biomass valorization under biorefinery concept.

2.1.2.1 *Spirulina* as feedstocks

The blue-green microalgae *Spirulina* (*Arthrospira*) has been used in human nutrition for centuries. *Spirulina* is a multicellular filamentous and helical photosynthetic cyanobacteria (Phormidiaceae family), naturally growing in alkaline brackish and saline waters. Nutritional analysis has revealed its exceptional properties, so it is referred as "super-food" and sold as a dried powder, flakes, or capsules (Stanic-Vucinic et al., 2018). *Spirulina* is a natural source highly rich in proteins (55–75 %), as well as an excellent source of carbohydrates (≈20 %), lipids (4-13%), essential amino acids (≈15 %) with particularly high contents of valine and leucine, vitamins (A, K, and vitamin B complex), macro- and micro-elements (calcium, potassium, magnesium, iron, iodine, selenium, chromium, zinc, and manganese), essential fatty acids, including γ-linoleic acid, glycolipids, lipopolysaccharides, and sulfolipids. Moreover, *Spirulina* is especially rich in a variety of pigments, such as chlorophylls, carotenoids, and phycobilins (phycobiliproteins) (Jacob-Lopes et al., 2015; Ljubic et al., 2018; Mata et al., 2020; Soni et al., 2017; Sotiroudis and Sotiroudis, 2013; Vernès et al., 2015).

Studies published in the last few decades, have revealed potentially beneficial effects of *Spirulina* on human health. Health benefits mainly arise from the antioxidant effect of microalgae as a whole, or from its individual ingredients, such as phycobiliproteins, essential fatty acids, phenolic

compounds, among others. The potential health effects include antioxidant immunostimulant, anti-inflammatory, anticancer, antiviral and antibacterial activities, as well as positive effects against malnutrition, obesity, diabetes, hyperlipidemia, cancer, radiation damage and anemia (Hoseini et al., 2013; Lafarga et al., 2020; Lupatini et al., 2017). Therefore, the biomass of this rich source of biocompounds is employed as feed and food additives in many industries (e.g. agriculture, food, pharmaceutics and cosmetics) (**Figure 3**) (Chia et al., 2019).

It is important to mention that dried biomass or derived products from *Spirulina* are "generally recognized as safe" (GRAS) for human consumption by the Food and Drug Administration (FDA) of United States, and commercialised in the EU as "One of the best protein source", should not exceed the daily dosage of 30g (Lafarga et al., 2020; Sotiroudis and Sotiroudis, 2013). Also, World Health Organization has described *Spirulina* as Mankind's best health product. According to UNESCO, Spirulina is most ideal food for tomorrow. According to NASA and European Space Agency, it is one of the primary foods that can be cultivated in long-term space missions. Intergovernmental institution permitted for the use of micro-algae *Spirulina* against Malnutrition (IIMSAM) (Soni et al., 2017).

2.1.2.2 *Spirulina* (bio)functional compounds

As mentioned above, *Spirulina* is highly rich in functional biocompounds, especially in a variety of pigments. For this reason, researchers and industry seek feasible alternatives to extract from this microalgae specific biocompounds.

Phycobiliproteins are easily isolated as a pigment protein complex, which are soluble in water and very fluorescent, and absorb radiation in visible spectrum regions where chlorophyll a has low absorption, mainly in the green and yellow zones of light spectrum. It is a stable protein and contains multiple chromophore prosthetic groups responsible for the fluorescent properties of this protein in cyanobacteria (Pagels et al., 2019). These brightly colored pigment proteins can be classified into three main types, namely: C-phycocyanin (PC) (dark blue), phycoerythrin (PE) (deep red) and allophycocyanin (APC) (blue-green) (Vernès et al., 2015). Quantitatively, phycocyanin is the most abundant phycobiliprotein in cyanobacteria, followed by APC and PE.

Studies on phycobiliproteins have shown the potential of these compounds as antioxidant, anticancer, antiviral, antimicrobial, anti-inflammatory, among others (**Table 2**). Commercially, phycobiliproteins are high-value natural products from cyanobacteria which have attracted attention for their potential use in biotechnological applications, such as nutraceuticals, pharmaceuticals,

cosmetic and food industries as well as in biomedical and clinical research (Manirafasha et al., 2016). The use of phycobiliproteins as non-toxic and non-carcinogenic natural food colorants is gaining importance worldwide in the view of the potential toxicity and carcinogenicity of the synthetic food colorants, moreover their therapeutic value has also been demonstrated (Manirafasha et al., 2016; Pagels et al., 2019; Stanic-Vucinic et al., 2018).

In addition to these compounds, also chlorophylls, carotenoids and phenolics present in *Spirulina* are of high interest due to their biological properties.

Chlorophyll-a is well known as the major pigments that converts photons into chemical energy. Chlorophyll-b has an indirect role in photosynthesis as an accessory pigment that transfers the energy it absorbs to chlorophyll-a. In the centre of the chlorophyll molecule, Mg^{2+} has ionic and hydrophilic properties, surrounded by hydrophobic porphyrin rings with polar carbonyl group (Hynstova et al., 2018). This chlorophyll complex molecule is positioned inside a water-soluble chlorophyll-binding protein. The difference between chlorophyll-b and chlorophyll-a is attributed to one –CHO functional group on the porphyrin ring making it readily more soluble than chlorophyll-a (Sumanta et al., 2014). The use of this lipophilic compound is authorized in food applications such as colouring in the manufacture of cold drinks, ice creams, among others ("Directive 94/36/CE of the European Parliament and the Council, 30 June 1994, about Dyes used in Food Products.," n.d.). As early as the 1800's, chlorophylls were described as therapeutic agents, with anti–inflammatory and chemopreventive activities, among others (see **Table 2**).

Carotenoids are also lipophilic pigments produced by plants and some microorganisms. These pigments result in a variety of colours observed in a large number of foods. Carotenoids are classified to carotenoid hydrocarbons, also known as carotenes, and oxygenated carotenoids, also known as xanthophylls. Carotenes consist only of carbon and hydrogen (e.g., lycopene, α-carotene and β-carotene). Xanthophylls contain oxygen and may carry any of several functional groups (e.g. hydroxy group in lutein and zeaxanthin, carboxylic acid group in torularhodin, oxo group in canthaxanthin, methoxy group in spirilloxanthin and epoxy group in violaxanthin). Carotenoids play a role in the regulation of plant growth and development, serving as photo-protectors, as photosynthesis pigments, as hormone precursors and as attractants for other species in the processes of pollination and distribution of seeds. The most interesting feature of the carotenoid structure is characteristic to the central part of the molecule where there is an alternating arrangement of single and double bonds. This arrangement of double bonds facilitates the

movement of π-electrons along the entire polyene chain, and gives carotenoids their unique chemistry and light-absorbing properties (Hynstova et al., 2018).

In humans, some carotenoids are pro-vitamin A (precursors transformed into vitamin A) that play role in prevention of nyctalopia (night blindness disease), skin health, and tooth and bone development. Nearly 700 naturally occurring carotenoids have been described. Of the 50 compounds exhibiting pro-vitamin A activity, three are the most important for human diet, i.e. α-carotene, β-cryptoxanthin and β-carotene. The consumption of carotenoids, and/or pro-vitamin A compounds, has been linked with a number of health benefits, including cancer chemoprotection, prevention of cardiovascular and metabolic diseases (e.g. cholesterol, hypertension, diabetes, *etc.*) and degenerative diseases (e.g., Alzheimer's Disease) (Amorim-Carrilho et al., 2014; Ferreira-Santos et al., 2018, 2020). Almost all carotenoids, to a greater or lesser degree, show scavenging properties against excessive numbers of free radicals (Santocono et al., 2007). This antioxidant capacity has been the most investigated and it has been suggested as the main mechanism of action of the carotenoids (Amorim-Carrilho et al., 2014).

In a recent study, authors showed that some microalgae/cyanobacteria, such as *Chlorella vulgaris* and *Spirulina platensis,* contain high concentrations of chlorophylls, mainly chlorophyll a, and carotenoids (zeaxanthin, lutein, astaxanthin, canthaxanthin, β-carotene) (Hynstova et al., 2018).

Currently, the production of natural colorants from microalgae/cyanobacteria is a very important field of research that has direct impact on industries. On the other hand, photosynthetic pigments such as chlorophylls and carotenoids, and phenolics are highly susceptible to degradation when exposed to light, oxygen, moisture conditions and high temperatures. Therefore, considerations for natural pigments in industry usually focus on preventing the breakdown of these compounds during the steps of harvesting, extraction, storage and processing (Hynstova et al., 2018).

Phenolic compounds, as mentioned before, are parts of a complex defense mechanism against a wide range of stresses in photosynthetic organisms. Phenolic compounds function as effective antioxidants due to their ability to donate hydrogen from hydroxyl groups positioned along the aromatic ring to terminate free radical oxidation of lipids and other biomolecules (Kepekçi and Saygideger, 2012). Although microalgae have received much attention as potential sources of natural antioxidants, there is limited information on the correlation between the antioxidant potential and the phenolic content of microalgae (Hajimahmoodi et al., 2010).

Spirulina, like other microalgae, has a high capacity for the production of various efficient protective chemicals against oxidative and radical stresses. These capabilities are a consequence of their

phototrophic life that caused them to be permanently exposed to high oxygen and radical stresses. Among the environmental factors that influence phenolic metabolism, light has a particularly strong effect. The biosynthesis of phenolic compounds was proposed as an "energy over flow" mechanism by diverting photosynthate and cellular reducing power into stable product pools (Hajimahmoodi et al., 2010).

Previous studies carried out by other authors have shown that *Spirulina* contains phenolic compounds with a high antioxidant capacity (Goiris et al., 2014; Machado et al., 2019; Seghiri et al., 2019). Casazza *et al.* (Casazza et al., 2015) reported that the maximum polyphenol yield was 33.2 mgGAE/g *Spirulina*, and other authors showed that phenolic acids are the main group of phenolics present in *Spirulina* (see **Table 2**) (Goiris et al., 2014; Machado et al., 2019; Seghiri et al., 2019).

On the other hand, *Spirulina* contains considerable amounts of lipids and polysaccharides (mentioned above), considered to be compounds of high functional and technological interest.

Microalgae possess a versatile metabolic capacity that can be transformed into valuable products through various processing routes. Some microalgae species as *Spirulina*, *Chlorella*, among others, have a high carbohydrate content that mainly comes from starch in chloroplasts and cellulose cell walls (Jacob-Lopes et al., 2015). Carbohydrate-rich *Spirulina* biomass (52%) were mostly evaluated for bioethanol production and were found to provide good yields (27.71 mL/100 g biomass) (Jacob-Lopes et al., 2015; Mata et al., 2020). Concomitantly, the polysaccharides from *Spirulina* microalgae have significant antioxidant and anticancer properties, as well as improve the enzymatic activity of the cell nucleus and synthesis of DNA repair, besides being a beneficial species to the immune system (immunomodulatory properties) (Andrade et al., 2018; Chaiklahan et al., 2013; Pugh et al., 2001). Rhamnose was found as the main compound ≈52.3% of total polysaccharides produced by *Spirulina* microalgae (Pugh et al., 2001). Similarly, the extracts of *Spirulina* polysaccharides were characterized, in which rhamnose represented ≈49.7% of total polysaccharides (Majdoub et al., 2009). Other monosaccharides such as fructose, galactose, xylose and glucose are present in the composition of spirulina, but in small amounts.

Microalgae lipids are categorized into two groups, one mostly used for transformation in biofuel and one for food supplements, with carbon numbers of between 14–20 and 20 carbons respectively (Andrade et al., 2018). *Spirulina* microalgae are a source of polyunsaturated fatty acids (PUFAs), ≈30% of total fatty acids composition, especially of the omega-6 family (ω6), such as γ-linolenic acid and linoleic acid (15–23%). Other fatty acids, such as palmitic and oleic acid are

found in the biomass of spirulina (see **Table 2**) (Andrade et al., 2018; Chaiklahan et al., 2008; Harun et al., 2009; Mata et al., 2020). Omega 3 and 6 families are essential fatty acids that cannot be synthesized by humans, so they must be ingested from food or supplements. Moreover, omega 3 and 6 families are intimately related to health maintenance and disease prevention, in which *Spirulina* microalgae are a promising source of ω6 (Andrade et al., 2018).

Table 2. Individual (bio)compounds found in *Spirulina platensis* and their reported bioactivities.

Compounds		Concentration Range	Bioactivities	References
Phycobiliproteins (µg/g)	Phycocyanin	28 510–103 007	Antioxidant, anticancer, antidiabetic, neuroprotectives, antiviral, antimicrobial and immunomodulating	Andrade et al., 2019, 2018; Jaeschke et al., 2019; Manirafasha et al., 2016; Pan-utai and Iamtham, 2019; Vernès et al., 2019
	Allophycocyanin	8 320–25 020		
	Phycoerythrin	4 250–10 280		
Chlorophyll (µg/g)	Chl-a	1 000–20 000	Anti–inflammatory, antimutagenic, chemoprotective, antioxidant and antimicrobial	Andrade et al., 2018; Hayes and Ferruzzi, 2020; Lafarga et al., 2020; Rangel-Yagui et al., 2004
	Chl-b			
Carotenoids (µg/g)	Zeaxanthin	28–1465	Antioxidant, anticancer, antidiabetic	Hynstova et al., 2018; Ljubic et al., 2018
	Lutein	118–1 031		
	Astaxanthin	96–719		
	Canthaxanthin	438–646		
	β-carotene	87–1 745		
Phenolics (µg/g)	Gallic acid	2.3–17.74	Antioxidant, antimicrobial, anticancer, anti–inflammatory	Abd El-Baky et al., 2009; Goiris et al., 2014; Machado et al., 2019; Seghiri et al., 2019
	Chlorogenic acid	0.86–19.27		
	Cinnamic acid	-		
	Hydroxybenzoic acid	54.66–687		
	Quimic acid	844		
	Caffeic acid	47.02		
	Vanillic acid	16.24		
	Ferulic acid	0.48		
	p-Coumaric acid	0.92		
	Phloroglucinol	51		
	Protocatechuic acid	11.06		

	Apigenin	-		
	Catechin	585		
	Succinic acid	1 123		
	Citric acid	64.06		
Fatty acids (% of total fatty acids)	Palmitic acid	30–50	Anti-hypercholesterolemic, anti-hypertensive, anticancer, anti–inflammatory, antioxidant	Andrade et al., 2018; Chaiklahan et al., 2008; Harun et al., 2009; Mata et al., 2020
	Stearic acid	1–5		
	Oleic acid	5–20		
	Linoleic acid	15–23		
	Gamma-linoleic acid	14–20		

2.2 PROCESSES FOR BIORESOURCES VALORIZATION

The recovery of bioactive and functional biomolecules from natural bioresources is an important step to enable the reuse of natural resources for subsequent application in pharmaceutical and cosmetic products, food enrichment and preservatives, dietary supplements and nutraceuticals.

The extraction process of natural biomolecules or extracts depends on several factors, including the applied extraction technique, the parameters associated with the technique (such as temperature, time and the extraction solvent), and the raw materials composition (Soquetta et al., 2018). It is known that the main biomolecules are metabolites present in the intracellular medium (cell vacuoles) (Silva et al., 2017). Therefore, it is also important to promote the opening of pores or even the rupture of the cell wall to facilitate the release of the compounds into the extraction medium.

In this sense, it is important to study all variables of the process in order to maximize the potential of the extraction method, developing a highly efficient process (Chemat et al., 2019). On the other hand, all variables in the process have to make it possible to obtain a safe and high quality final product (eco–extract or purified biocompounds), in addition to maximizing the extraction of the compounds of interest. **Figure 4** illustrates the main principles of an efficient extraction process, following the concept of green extraction.

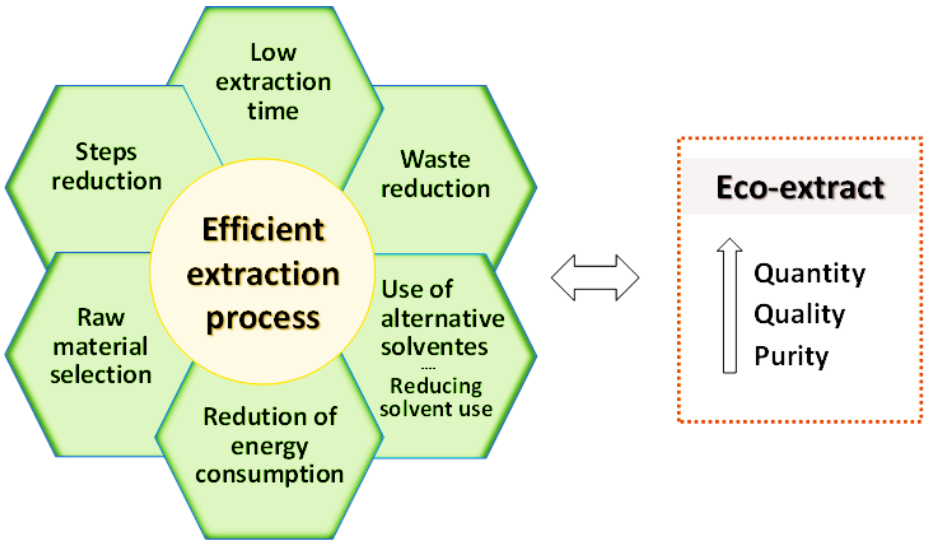

Figure 4. Principles of efficient process for obtaining natural extracts.

2.2.1 Extraction solvents

The reduction use of hazardous solvents is also considered one of the priorities of the EU policy for the 2010 to 2050 period (Cvjetko Bubalo et al., 2018). Nowadays, extraction using conventional organic solvents is the most commonly used procedure to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. The efficiency of the extraction methods depends on the choice of the solvent, since solvents with different polarities are needed for the isolation of compounds with different chemical constitution. In addition, it is difficult to define a single method for the efficient extraction of all compounds since the polarities of the molecules to be extracted vary (Soquetta et al., 2018).

A suitable solvent has to be able to obtain safe and high quality extracts and to preserve the biological effects of the extracted compounds without exhibiting toxicity when consumed. Furthermore, it should be recyclable and reusable, preventing negative environmental effects. Other parameters, such as flammability, explosiveness, volatility, mass transfer and (in)ability to dissociate the complex extract should be considered (Lourenço et al., 2019). The extraction yield depends not only on the solvent used but also on several other factors such as sample/solvent ratio, temperature, extraction time, stirring and raw material composition (da Silva et al., 2016).

Conventional solvents from "non-natural"/petroleum resources, such as methanol, ethanol, acetone, ethyl acetate, dichloromethane, hexane, *etc*. and their aqueous solutions have been used for the extraction of bioactive compounds from natural bioresources. Several studies have been done demonstrating the importance of these solvents in the recovery of natural molecules and active extracts from different natural bioresources (Chia et al., 2019; Lourenço et al., 2019; Marcheafave et al., 2019; Mokrani and Madani, 2016; Venkatesan et al., 2019), including the lignocellulosic by-products (Fierascu et al., 2019) and microalgae ('t Lam et al., 2017; Lee et al., 2017; Machado et al., 2019; Papadaki et al., 2017; Vernès et al., 2015). Researchers studied the influence of these different solvents in obtaining antioxidant phenolic compounds from pine by-products, and depending on the solvent used the extracted fraction (extract composition) is different (Ferreira-Santos et al., 2019a; Hadzri et al., 2014; Venkatesan et al., 2019). For example, in a work by Venkatesan and collaborators (Venkatesan et al., 2019), the impact of different extraction solvents (such as ethanol, methanol, isopropanol, acetonitrile and acetone) was analyzed to obtain phenolic extracts with AA from *Pinus densiflora* bark. Their results showed that low concentrations of ethanol and acetonitrile are favorable for the extraction of phenolics with high AA. In another study, using *Pinus niruri*, methanol was more efficient than other solvents such as ethanol, hexane

and ethyl acetate, showing enhanced extraction rate of phenolic and flavonoid compounds with higher biological activities (Hadzri et al., 2014). In the case of microalgae, the use of different solvents makes the process selective for the extraction of different families of compounds, for example, water to extract phycobilinoproteins and ethanol to extract more apolar compounds such as chlorophylls, carotenoids, *etc*. (Zhang et al., 2020).

It is known that water is an efficient solvent for the extraction of various compounds, due to its properties and thanks to the fact that water is easily available, safe, non-toxic, non-flammable, and environmentally friendly (Chemat et al., 2019). In this sense, it is considered the cleanest/greenest solvent (apart from the use of no solvent, which is the greenest), according to the principles of green chemistry (Chemat et al., 2015; Prat et al., 2015). However, it is not suitable for the extraction of less polar substances.

Other possible environmental friendly solvents' option is to replace petroleum-based solvents by "bio-solvents". For instance bioethanol can be produced from bioresources, by fermentation. This solvent of second generation could be made cost-competitive by the development of biorefinery-based processes for the integral use of lignocellulosic biomass, substituting ethanol obtained from petroleum derivatives (Domínguez et al., 2017).

As alternatives to conventional solvents, the use of green solvents like ionic liquids (ILs) and natural deep eutectic solvents (NADES) is emerging, in order to make the extraction process eco-friendly and more effective (Liu et al., 2018). In general, ILs and NADES are derived from cheap, abundant, low toxic, and biodegradable natural components (Cvjetko Bubalo et al., 2018; Jablonský et al., 2018). NADES can be defined as "mixtures of pure naturally occurring compounds that present an eutectic point temperature below an ideal liquid mixture" (Jablonský et al., 2018; Martins et al., 2019). ILs are liquid molten salts at temperatures below 100 °C composed by cations and organic or inorganic anions with exclusive and adjusted physicochemical properties (Ventura et al., 2017). However, lack of information on the biological activity and toxicity of the obtained extracts limits the use and industrial applications of ILs and NADES (Cvjetko Bubalo et al., 2018), leading to these solvents not being regulated by the FDA (Martins et al., 2017). Furthermore, though they can be tuned for enhanced affinity towards the compound of interest, their separation from the final mixture may be hindered by the high boiling point characteristic of these solvents.

Murador and collaborators (Murador et al., 2019) summarize the main chemical constituents of these ILs and NADES and mention some works where they are applied in the extraction of antioxidant compounds, such as phenolic compounds, carotenoids, proteins, among others.

Specifically, ILs and NADES have been applied to the phenolic compounds and other antioxidant compounds extraction from lignocellulosic biomass and agri-food wastes (da Costa Lopes et al., 2016; Ivanović et al., 2018; Jablonský et al., 2018; Passos et al., 2014; Torres-Valenzuela et al., 2019; Ventura et al., 2017), and lipids, carbohydrates, proteins and carotenoids from microalgae (Lu et al., 2016; Orr and Rehmann, 2016; Shankar et al., 2017; Suarez Garcia et al., 2018).

In the case of pine plants as feedstock, the process of extracting bioactive compounds with added-value (such as phenolics) using these green solvents is not widely explored. In a recent study, ILs were combined with enzymes and microwave technology to promote cell wall disruption for the extraction of essential oil and procyanidins from pine cones of *Pinus koraiensis* (Hou et al., 2019). However, there are no reports using ILs and NADES as alternative solvents for extraction of bioactive molecules of other parts of pine plant, despite the advantage they showed for obtaining functional compounds in other lignocellulosic residues.

2.2.2 Extraction technologies

Traditionally, solvent solid-liquid extraction (TSE) is used for most fractioning processes. The correct choice of solvents to achieve good extraction yields with a high concentration in the target compound depends on the target's solute solubility and polarity. This choice includes frequently organic compounds such as dichloromethane, ethanol and methanol. Heat and/or agitation are usually side-by-side with TSE, both to increase the solute's solubility and increase the mass transfer rate, though minimum damage to the target compound has to be assured (e.g. avoiding oxidation and/or thermal degradation). Besides issues such as the molecular affinity between solvent and solute and mass transfer, other factors should not be overlooked such as the need for a co-solvent, environmental safety, human toxicity and financial feasibility. Traditional water or organic solvent extractions are time-consuming processes that often require high solvent and energy consumptions and generate large amounts of waste.

Issues such as growing environmental concerns and petroleum shortage as well as increasing oil price instability caused by geopolitical conflicts (causing increased costs of chemicals and energy) have boosted the search for alternative environmentally friendly extraction and fractioning methodologies, aiming at reducing energy and chemicals consumption, waste generation and operational time, while increasing overall yield, selectivity and quality of the extract. Studied alternative technologies include accelerated solvent extraction, subcritical water extraction, pulsed electric fields, supercritical fluid extraction, enzyme-assisted extraction or digestion and extrusion.

In addition, the trend is to use the least solvent possible, and ideally to move towards solvent-free technologies. These may include cold pressing, enzyme-assisted cold pressing, extrusion, solvent-free microwave-assisted extraction, instant controlled pressure drop or the use of electrotechnologies, such as the case of pulsed electric fields (PEF) (Chemat et al., 2015) or alternating moderate electric fields (MEF) combined with OH.

Process intensification towards improved sustainability, efficiency and environmental performance could be a highly beneficial approach to this kind of processes. In fact, a deep knowledge of the spatial, thermodynamic, functional and temporal domains can be used to obtain the best extraction possible for each system using four approaches, respectively: structure, energy, synergy and time (Van Gerven and Stankiewicz, 2009). In particular, instead of the conventional conductive heating with a steam boiler, a large variety of other forms and sources of energy can be considered for process intensification, including ultrasound (US), light, microwaves (MW) and electric fields (Stefanidis et al., 2014).

The focus of this thesis will be the on use of electrotechnologies in the extraction and bioprocessing of biocompounds or fractions from underused bioresources, aiming at their valorization. Electric fields processing is particularly interesting due to its versatility, easy scale-up, membrane electroporation effects and energetic efficiency. Though both pulsed and non-pulsed electric technologies will be addressed, special emphasis will be put in OH and its MEF, due to their more innovative character and emergent potential applications to this end.

2.2.2.1 Electrotechnologies

To achieve the goals implicit in the concepts of circular economy and biorefinery, not only production methods and strategies have to be rethought, but also the development of new approaches and technological solutions is a fundamental requirement. Many technologies have been developed or applied in the context of bioprocessing and despite some being available for a considerable time, their industrial application has been impaired by limitations as diverse as high costs, operational problems or lack of control and knowledge of all important variables (Galanakis, 2013).

Driven by technological advances or by changes in social-economic circumstances, some of these technologies show steady growth in interest and applications, in both research and industry. These so-called “novel” or “emergent” technologies hold the potential to change the paradigm and revolutionize the bioprocessing industry (Golberg et al., 2016).

A branch of the novel and emergent bioprocess technologies is represented by the electro-technologies, which are based on the application of electric current in biomaterials with technological purposes (Kotnik et al., 2015; Lebovka et al., 2008; Sastry, 2008). The concept of applying an external electric field (EF) to promote or assist bioprocesses has been recognized as soon as electricity became a viable technology. However only more recently, with the advances in material sciences, power generators and process variables understanding and control, it was possible to push electricity-based technologies as a viable bioprocessing alternative (Sastry, 2008). These technologies have thrived over the last 30 years and found diverse applications, as is the case of bioresources valorization (Puértolas and Barba, 2016).

The application of an EF on a biological or bio-based system will result on dissipation of heat, since the system will act as a semi-conductor. Often designated as OH, this is explained by the Joule effect and provides a fast and homogeneous heating rate along with high energetic efficiencies (Pereira and Vicente, 2010). Other consequence of the EF presence is electroporation, as the exposure of cells to an external EF results on the formation of a transmembrane potential. When this potential overcomes a value between 0.2 and 1 V, electropermeabilization of the membrane is induced. The temporal nature (i.e. temporary or permanent) and extent of the permeabilization is dependent of variables such as EF intensity, exposure time, and medium composition, among others (Mahnič-Kalamiza et al., 2014). The presence of the EF also results on charge-related phenomena, as almost all natural occurring molecules have a built-in electric charge. Electrophoresis or dielectrophoresis may occur when these molecules are subjected to continuous or non-uniform EF respectively, enabling to explore electro-kinetic phenomena for focusing, trapping or fractioning biological material (Wong et al., 2004). The application of EF may also result on the occurrence of secondary phenomena such as electrochemical reactions, shock wave formation and light emission (Lebovka et al., 2008).

The use of electrotechnologies brings advantages in different stages of bioresources valorization as they may promote stabilization of the biomaterials, endorse or enhance extraction and diffusion of compounds, assist in separation and fractioning, among others (Puértolas and Barba, 2016; Wong et al., 2004). The success of EF processing in performing one or more of these tasks will be dependent of the operational parameters and specifications applied, resulting on favoring of one or more EF-related effects and ultimately defining the technologies into subcategories (see **Table 3**). As a result, electrotechnologies can be classified according to type of electric flow (i.e. direct or alternating current), application in pulses or not, EF strength (voltage applied by the section length),

extension of heat deposition, among others (**Figure 5**). In this section, the major electrotechnologies applied to extraction and valorization of compounds from bioresources will be defined and addressed according to their specifications and potential applications.

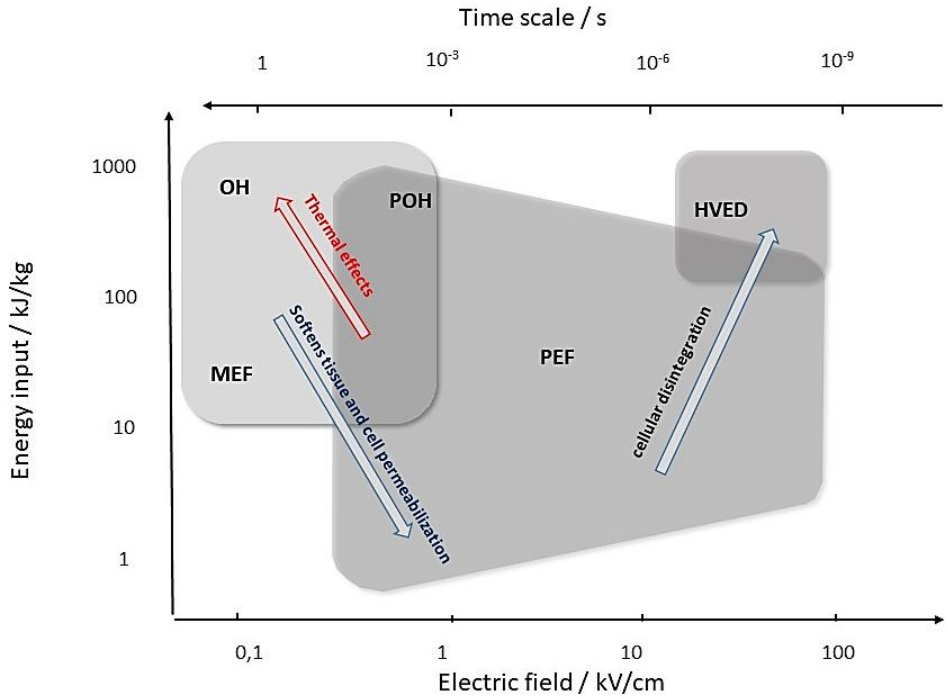

Figure 5. Electrical pulsed and non-pulsed protocols for extraction procedures; moderate electric fields (MEF), ohmic heating (OH), pulsed electric fields (PEF), high voltage electric discharges (HVED); pulsed ohmic heating (POH).

The principle of pulsed electrotechnologies is the application of electric pulses, generally of high intensity, for short periods of time (Mahnič-Kalamiza et al., 2014). Following these principles, techniques such as High Voltage Electrical Discharges (HVED), PEF and Pulsed Ohmic Heating (POH) have been applied on the bioresources valorization (Vorobiev and Lebovka, 2016).

In PEF applications, high voltages (kV range) are applied in pulses of short duration (nano or micro-seconds) with the main objective of causing electropermabilization (Kotnik et al., 2015). When HVED are applied, a direct high voltage is used and high current pulse is applied into a liquid medium, it results on a sudden energy release accompanied by the formation of a plasma channel. Consequently, secondary effects such as shock waves, cavitation, light emission, radical generation will occur as well (Boussetta and Vorobiev, 2014). HVED promotes heat dissipation and electroporation; however, the secondary effects resulting from the high-energy release will have predominance over EF effects (Puértolas and Barba, 2016). High levels of cellular damage or

cellular disintegration can be achieved with this techniques, placing it as an interesting alternative to cell inactivation and extraction of intracellular compounds.

Non-pulsed applications are techniques where the electric current flows on a unidirectional flow (DC) or periodically reverses direction (AC) without interruption for a significant period of time. Despite DC techniques being relevant in bioprocess applications, particularly separation techniques such as electrophoresis, electrofiltration or cross-flow electrofilration, the focus of this work is on extractions and functionalization of bioresources. Therefore, DC process will not be further addressed.

Applications involving AC usually fall under the specification of MEF where a low EF (generally between 1 and 1000 V) and defined wave shape (typically sinusoidal or square) is applied (Sastry, 2008; Varghese et al., 2014). Electric frequency is a relevant parameter in these processes, dictating efficiencies and affecting process reliability. Under low frequencies, electrochemical reactions may be an issue as they can result in the formation of radical species, corrosion and erosion of the electrodes. The use of frequencies above 15-20 kHz eliminates these problems as electrochemical reactions are completely eradicated (Pataro et al., 2014).

Overall, different electrotechnologies have been used for the extraction of biocompounds from diverse raw materials, mainly agro-industrial wastes and microalgae (see **Table 4**).

Table 3. Main novel and emergent electrotechnologies and its main applications.

Electric fields	Technological common names	Main applications
Pulsed	Pulsed Electric Fields (PEF)	Non-thermal inactivation of microbial cells Electroporation of cell membranes Softening tissues and peeling Extraction of thermal labile biocompounds
	Pulsed Ohmic Heating (POH)	Thermal extraction of biocompounds
	High Voltage Electric Discharges (HVED)	Extraction of biocompounds
Non-Pulsed	Ohmic Heating (OH)	Continuous or batch thermal processing High-temperature, short-time pasteurization and sterilization of materials Thermal extraction of biocompounds
	Moderate Electric Fields (MEF)	Non thermal inactivation of microbial cells Extraction of biocompounds
	Electrofiltration	Separation of bioproducts
	Electrophoresis	Protein separation and diagnostics

2.2.2.1.1 Moderate electric fields and ohmic heating

MEF and OH terms are often used in an interchangeable way but it is important to adopt a clear definition. OH will always be a side effect of the application of electric fields on a semi-conductive material: if the medium where MEF is applied has enough conductivity and the process takes place for sufficient time, significant heat deposition will take place through the Joule effect, thus occurring OH. For the purposes of this work OH will be mainly referred to thermal processing, while MEF will be used to give emphasis on electrical effects either when thermal aspects are attenuated or compared in a similar basis. OH resulted possibly in the most successful case of an electrotechnology with industrial application, being significantly widespread and commercially available throughout the food industry as a pasteurization technology (Jaeger et al., 2016). However, under the MEF field range, permeabilization and extraction enhancement processes are still relevant (Lebovka et al., 2005; Pereira et al., 2016a; Sensoy and Sastry, 2004). Processes as the mentioned POH or pure MEF processes, either with OH or without association of thermal effects, are gaining expression on reported applications about the valorization of bioresources.

In the last few years there was an increased interest in using MEF and the corresponding OH for extraction processes. OH presents high heating rates with a precise temperature control allowing mild processing and preserving nutritional, functional and structural properties. Heat is generated inside the material to be heated (Joule effect), the heating process does not depend on heat transfer between phases and interfaces, allowing uniform heating and an extremely rapid heating rate. Furthermore, it also allows heating of large particulates and fluids at comparable rates, as long as their conductivities remain similar. Many studies also suggest that the used MEF have a significant effect on the cell wall permeabilization (Kusnadi and Sastry, 2012). The process has high energy conversion efficiencies resulting in lower operational costs and in a more environmentally-friendly system (Pereira and Vicente, 2010).

As for pulsed electrotechnologies, extraction of anthocyanins from different sources is the most common application of OH as an extraction technology. OH was used in black rice bran with the final goal of preparing a natural food colorant (Loypimai et al., 2015). The treatment with OH increased the anthocyanins content in the extracts four-fold. Extracts obtained with OH contained also the highest amounts of bioactive compounds (α-tocopherol and γ-oryzanol). However, electric conductivity was a critical issue that needed to be adjusted, as it had a low initial value due to the high levels of fat. Pereira and co-authors (Pereira et al., 2016a), studied the effect of OH and MEF intensity in the extraction of polyphenols and anthocyanins from colored potato. Different

operational parameters were considered, such as EF strength, temperature and process time on the extraction yields. Increased extraction yields were registered at 20 V/cm and heating (90 °C). At EF values above a critical value (20 V/cm, in this case), degradation of the anthocyanins occurred. However, when an EF above 20 V/cm was combined with temperatures above 70 °C, an increase of polyphenol extraction yield was noticed. As this increase in polyphenol yield matches the electrical field at which the anthocyanins yield decrease, the authors suggested that this is the point of possible degradation of anthocyanins to its constituent phenolic acids. The previous results coincided with the results published by other authors with other materials. Sarkis *et al.* (2013) (Sarkis et al., 2013) reported the anthocyanin degradation during conventional and OH extractions of blueberry pulp. The degradation of anthocyanins depended on the voltage used and on the solids content. It was concluded that when lower voltage levels were used, the degradation can be lower to that obtained during conventional heating. The use of a high voltage level led to higher anthocyanin degradation. Nevertheless the use of high-temperature short-time treatments can be combined with electric fields, with a good extraction yield of anthocyanins (up to 85%) without affecting their quality profile (Pereira et al., 2016a).

OH has been used for the extraction of essential and fatty oils (Aamir and Jittanit, 2017; Gavahian et al., 2015, 2013, 2012, 2011; Nair et al., 2014) or pectins (Saberian et al., 2017) (see **Table 4**). In all works it was concluded that the OH is the greenest technology for the extraction in terms of energy consumption in comparison to traditional techniques (distillation, hydrodistillation or traditional heating). The use of OH generally reduced the extraction time and energy consumption, though extraction times were sometimes similar to those achieved with conventional methods.

The application of OH was not always successful. For instance, the achieved extraction yields of inulin from Jerusalem artichoke tuber powder were lower than the conventional heating process (Khuenpet et al., 2017), though the energy efficiency claim is still valid.

Application of MEF up to 1000 V/cm, may be achieved at sub-lethal temperatures (< 45 °C), thus without significant OH effect. According to the existing literature there are only few works reporting the use of MEF for extraction of added-value compounds. MEF was used for the extraction of lipids and carotenoids from microalgae (Jaeschke et al., 2016). The results demonstrated that carotenoid extraction was affected by both MEF and ethanol concentration, while lipid extraction was only affected by ethanol concentration. Moreover, analyses of the extract showed that the xanthophylls all-*trans*-lutein and all-*trans*-zeaxanthin were the major carotenoids in the extracts.

MEF was also used for pectin extraction from passion fruit (De Oliveira et al., 2015). Even though the extraction yield of MEF was lower than that of the traditional extraction, the obtained pectin using different extraction methods had also similar values of galacturonic acid and esterification degree.

Though the potential is evident, MEF and OH have only marginally been applied to extract compounds mainly from vegetable tissues, including polysaccharides, essential oils and polyphenols, sugar from sugar beets, potato starch and fruit juice expression (Aamir and Jittanit, 2017; Gavahian et al., 2011; Praporscic et al., 2006, 2005; Saberian et al., 2017; Seidi Damyeh and Niakousari, 2017; Zhu et al., 2015) (**Table 4**) . Extraction procedures have been established case by case, frequently in an empirical way, and the establishment of a correlation between the chemical properties of the extracts e.g. with the intensity, frequency and other parameters associated with the application of the EF is highly desirable. Electroporation effects under MEF are still controversial once cell lysis may also result from thermal permeabilization of the membranes due to local heating, thus more fundamental research about non-thermal effects of MEF is also needed. Nevertheless, even in the cases where no improvement in extraction yields is observed, OH will have higher energetic efficiency, which have a massive importance in thermal processing. Currently, the number of MEF based plants installed worldwide for thermal processing of foods (commonly designated by OH Technology) is increasing, thus it is expected that industrial application of MEF aiming at extraction for waste recovery will be straightforward and, thus, extensively applied as soon as its advantages are perceived as such by the industry.

2.2.2.1.2 Processing and energetic aspects of electrotechnologies

Novel and emerging technologies not only have to prove effective and advantageous at the processing level; they have to be technically feasible, sustainable and economically competitive. The major impairments associated with these technologies are always their implementation, as the associated costs tend to be higher than those of the conventional technologies and there is a lack of operational information and associated costs (Mohamed and Amer Eiss, 2012; Pereira and Vicente, 2010). In order to assess the profitability of a new technology, several aspects have to be taken into account, such as investment in equipment, energetic costs, and operational aspects such as efficiency gains and reduction or elimination of process requirements (e.g. utilization of solvents or mechanical methods to increase extraction).

2.2.2.1.2.1 Processing demands and scaling

In principle, electrotechnologies are almost linearly scalable and involve low maintenance costs, since their application does not involve diffusional processes, mechanical stress or moving parts (Pereira and Vicente, 2010). However, their industrialization offers some challenges, as large volumes processing implies larger treatment chambers or flow rates, larger electrodes surface areas and electrode gaps. Consequently, the demands on the generators increase, implying high energetic capacity, increase of output voltage and higher frequency of pulses (Kotnik et al., 2015). Obtaining power sources capable of high voltage and current output with defined pulse/wave shape and frequency is still a challenge (Golberg et al., 2016). Nonetheless, developments in generators and control systems, along with increasing application of MEF and PEF systems in the food industry, are contributing for cost reduction and increase of reliability of these systems. In addition, optimization of several factors such as materials used, design of electric treatment chambers and continuous operation mode are pushing these processes towards industrial feasibility (Golberg et al., 2016; Mahnič-Kalamiza et al., 2014). In this scenario, HVED is still the less developed technology, because not only it is the most recent, but also it is the more demanding in terms of generator specifications and challenging in terms of materials used and chamber design (Vorobiev and Lebovka, 2013).

2.2.2.1.2.2 Energy consumption and environmental impact

Electrotechnologies presented here are considered environmentally friendly once they may eliminate, or at least diminish, the use of water and thus production of wastewaters (e.g. avoiding the use of steam systems and boilers), and may use a renewable source of energy (e.g. hydroelectric power) to produce electricity. Furthermore, in general, electrical processing needs lower energy consumption compared with conventional pre-treatment methods and extraction technologies. MEF treatments often involve OH as a thermal process and the energy requirements will be dependent of the materials heat capacity and thermal elevation needed. OH energetic efficiency is above 90% and compared with the less efficient conventional thermal processing it can achieve energy savings up to 70% (Pereira and Vicente, 2010; Varghese et al., 2014). When applied to extraction processes, OH has shown energy inputs between 30 and 180 kJ/kg (Pereira et al., 2016b), being significantly higher than PEF and at similar levels of mechanical and enzymatic processing.

The energy input associated with PEF treatment also varies with the material to be treated; commonly it ranges from 1 to 15 kJ/kg in soft tissues such as pulps and peals, and arround100–800 kJ/kg in hard and resistant materials such as seeds. In contrast, conventional treatments (such as mechanical or enzymatic processing) require from 20 to 100 kJ/kg to achieve similar results (Golberg et al., 2016; Puértolas and Barba, 2016; Vorobiev and Lebovka, 2010). For HVED, limited data are available and few industrial or economic studies were performed.

Along with the increase of bioprocessing and recovery efficiency, and in some cases reduction or elimination of solvent use, electrotechnologies are likely to significantly contribute to reduce the use of non-renewable resources, increase the added value of wastes and by-products and to the development of a green, sustainable and circular economy.

Table 4. Main results published on the application of electrotechnologies to improve the extraction processes of valuable compounds.

	Matrix	Extracted compounds	Optimum extraction paratmeters			Reference
			Electrical	Time /Temperature	Solvent	
Ohmic heating (OH)	Gac aril	Essential oil Carotenoids	20 kV; 50 Hz-1MHz	n.a. 50 °C	Hexane	(Aamir and Jittanit, 2017)
	Prangos ferulacea Lindle	Essential oil Terpenic compounds	120 V; 50 Hz	73 min; ≈99°C	Water (hydodestillation)	(Seidi Damyeh and Niakousari, 2016)
	Pulicaria undulata	Essential oil Terpenic compounds	220 V; 50 Hz	61 min; ≈99 °C	Water (hydrodestillation)	(Seidi Damyeh and Niakousari, 2017)
	Myrtus communis	Essential oil	220 V; 50 Hz	≈26 min; ≈99 °C	Water (hydrodestillation)	(Gavahian et al., 2013)
	Thymus vulgaris L.	Essential oil	220 V; 50 Hz	≈24 min; ≈99 °C	Water + 1% NaCl (hydrodestillation)	(Gavahian et al., 2012)
	Peppermint	Essential oil	220 V; 50 Hz	≈20 min; ≈99 °C	Water + 1% NaCl (hydrodestillation)	(Gavahian et al., 2015)
	Shirazi thyme	Essential oil	220 V; 50 Hz	≈32 min; ≈99 °C	Water + 1% NaCl (hydrodestillation)	(Gavahian et al., 2011)
	Oregano	Essential oil	220 V; 50 Hz	31 min; ≈99 °C	Water + 2.85% NaCl (hydrodestillation)	(Hashemi et al., 2017)
	Jerusalem artichoke tuber	Inulin	200 V; 20 kHz	30 min; 75 °C	Water	(Khuenpet et al., 2017)
	Black rice bran	Anthocyanins	50-200 V/cm	n.a.; 105 °C	Water	(Loypimai et al., 2015)
	Colored potato	Polyphenols	25 kHz; 15 V/cm	10 min; 90 °C	Water + KCL	(Pereira et al., 2016a)
	Orange juice	Anthocyanins Pectin	50 Hz; 15 V/cm	30 min; 90 °C	Water	(Saberian et al., 2017)

Pulsed electric field (PEF)	Grape pomace	Polyphenols Anthocyanins	40 kV; 0.5 Hz; 13.3 kV/cm; 0-564 kJ/kg; – pulses (10 µs)	n.a.; 22 °C	Water	(Barba et al., 2015)
	Blueberries	Polyphenols Anthocyanins	1-5 kV; 10 Hz; 1-10 kJ/kg; – pulses (20 µs)	n.a.; 20-23 °C	–	(Bobinaitė et al., 2015)
	Tomato	Carotenoids	3.8 kV; 0.33 Hz; 600 pulses (350 µs of total pulses)	n.a.; 40-45 °C	–	(Bot et al., 2018)
	Norway spruce Bark	Polyphenols	40 kV; 0.5 Hz; 20 kV/cm; 200 pulses (10 µs)	10 min; 20 °C	Water + 0.01M NaOH	(Bouras et al., 2016)
	Grape seeds	Polyphenols	40 kV; 0.33 Hz; 20 kV/cm; 400 pulses (10 µs)	n.a.; 50 °C	50% ethanol	(Boussetta et al., 2012a)
	Stevia	Steviol glycosides Polyphenols Flavonoids Chlorophylls Carotenoids	40 kV; 0.5 Hz; 20 kV/cm; 178 kJ/kg; 200 pulses (10 µs)	n.a.; 50 °C	50% ethanol	(Carbonell-Capella et al., 2017)
		Polyphenols Protein Chlorophyll Carotenoid	13.3 kV/cm; 0.5 Hz; 300 pulses (10 µs)	n.a; 20 °C	Water	(Barba et al., 2015)
	Spearmints	Polyphenols	20 mV; 100 kHz; 3 kV/cm; 99 pulses (10 µs)	n.a.	Mannitol solution (followed by 80% ethanol extraction)	(Fincan, 2015)
	Potato peels	Steroidal alkaloids	10 Hz; 0.75 kV/cm; 200 pulses (3 µs)	n.a.; 13-16 °C	Methanol	(Hossain et al., 2015)

Red prickly pear	Colorants	40 kV; 0.5 Hz; 20 kV/cm; 50 pulses (10 μs)	n.a.; 20 °C	Water (followed by 1h water extraction)	(Koubaa et al., 2016)
Microalgae *Chlorella vulgaris*	Proteins	20 kV/cm; – pulses (2 μs)	n.a.; 20 °C	Water	('t Lam et al., 2017)
Apple pomace	Polyphenols	1 Hz; 3 kV/cm; 3.0 kJ/kg; 500 μs of total pulses	n.a.; 25 °C	Water	(Lohani and Muthukumarappan, 2016)
Sorghum flour	Polyphenols	1 Hz; 2 kV/cm; 6.96 kJ/kg; 875 μs of total pulses	n.a.; 25 °C	Water	(Lohani and Muthukumarappan, 2016)
Red beet	Betanine	1 Hz; 7 kV/cm; 2.5 kJ/kg; 5 pulses (2 μs)	n.a.; 30 °C	McIlvaine buffer	(López et al., 2009)
		1 Hz; 6 kV/cm; 50 pulses (3 μs)	n.a.	n.a.	(Luengo et al., 2016)
Orange peels	Polyphenols	1 Hz; 7 kV/cm; 0.06-3.77 kJ/kg; 20 pulses (3 μs)	n.a.	n.a.	(Luengo et al., 2013)
Papaya peels	Polyphenols Proteins Carbohydrates	40 kV; 13.3 kV/cm; 400 pulses (10 μs)	n.a.; 35°C	n.a.; 35°C	(Parniakov et al., 2014)
Papaya seeds	Polyphenols Carbohydrates Isothiocyanates	40 kV; 13.3 kV/cm; 300 pulses (8.3 μs)	n.a.	n.a.	(Parniakov et al., 2015)
Microalgae *Nannochloropsis*	Polyphenols Proteins Carotenoids Carbohydrates	40 kV; 20 kV/cm; 400 pulses (10 μs)	n.a.; 20-30 °C	Water	(Grimi et al., 2014)

Rosé wines	Anthocyanins	30 kV; 5 kV/cm; 122 Hz; 3.67 kJ/kg; 50 pulses (3 μs)	n.a.; 4 °C	n.a.	(Puértolas et al., 2011)
Purple-fleshed potato	Anthocyanins	30 kV; 3.4 kV/cm; 1 Hz; 8.92 kJ/kg; 35 pulses (3 μs)	n.a.	Water and ethanol	(Puértolas et al., 2013)
Red wine	Polyphenols	10 kV; 200 Hz; 5 kV/cm; 3.5 kJ/kg;1 pulses (100 μs)	n.a.	30% ethanol	(Saldaña et al., 2017)
Sesame seeds	Polyphenols Proteins	40 kV; 0.5 Hz; 20 kV/cm; 40 kJ/kg	n.a.; 50 °C	Water	(Sarkis et al., 2015a)
Sesame cake	Polyphenols Proteins Lignans	40 kV; 0.5 Hz; 13.3 kV/cm; 83 kJ/kg; 100 pulses (10 μs)	20 min; 60 °C (40 °C proteins)	10% ethanol (50% for lignans)	(Sarkis et al., 2015b)
Borage	Polyphenols	30 kV; 300 Hz; 5 kV/cm; 6.18 kJ/kg; 50 pulses (3 μs)	n.a.; 40 °C	Acidic water	(Segovia et al., 2015)
Button mushroom	Polysaccharide Polyphenols Proteins	30 kV; 1 Hz; 38.4 kV/cm; 136 pulses (2 μs)	n.a.; 20 °C	Water	(Xue and Farid, 2015)
Bone	Calcium	70 kV/cm; 12 pulses (24 μs)	n.a.; Room temperature	1.25% citric acid	(Yin and He, 2008)
Rapeseed	Polyphenols Proteins	400 V; 0.5 kHz; 5 kV/cm (20 kV/cm for proteins); 200 pulses (10 μs)	20 min; 50 °C (20 °C proteins)	75 % ethanol (water for proteins)	(Yu et al., 2015)
Tea	Polyphenols	1.1 kV/cm; 50 pulses (100 μs)	n.a.	Water	(Zderic and Zondervan, 2016)
Microalgae *Spirulina platensis*	C-Phycocyanin	25 kV/cm; 50 pulses (3 μs)	2.5 h; 40 °C	Water	(Martínez et al., 2017)
		40 kV/cm; –pulses (1 μs)	6 h; Room temperature	Sodium-phosphate buffer	(Jaeschke et al., 2019)

Moderate electric field (MEF)	Microalgae *Heterochlorella luteoviridis*	Carotenoids Lipids	180 V; 60 Hz	10 min; 35 °C	75% ethanol	(Jaeschke et al., 2016)
	Passion fruit peel	Pectin	100 V; 60 Hz	15 min; 45 °C	Acidic water	(De Oliveira et al., 2015)
High voltage electrical discharge (HVED)	Grape seeds	Polyphenols	40 kV; 300 pulses (10 µs)	n.a.; 50 °C	Water	(Liu et al., 2011)
			40 kV; 0.33 Hz; 100 pulses (10 µs)	n.a.; 50 °C	50% ethanol	(Boussetta et al., 2012a)
	Grape pomace	Polyphenols	40 kV; 0.5 Hz; 80 pulses (10 µs)	60 min; 60 °C	Water	(Boussetta et al., 2009)
			40 kV; 80 kJ/kg	30 min; 60 °C	30% ethanol	(Boussetta et al., 2011)
		Polyphenols Anthocyanins	40 kV; 0.5 Hz; 280 kJ/kg	n.a.; 22 °C	Water	(Boussetta et al., 2012b)
	Sugar beet pulp	Pectin	40 kV; 0.5 Hz; 76.2 kJ/kg; 100 pulses (10 µs)	60 min; 90 °C	Water	(Almohammed et al., 2017)
	Flaxseed cake	Polyphenols Lignans	40 kV	60 min; 40 °C	25% ethanol	(Boussetta and Vorobiev, 2014)
	Rapeseed	Lignin	0.5 Hz; 800 kJ/kg	80 min; 200 °C	65% ethanol	(Brahim et al., 2017)
	Olive kernel	Polyphenols Proteins	40 kV; 0.5 Hz; 66 kJ/kg	n.a.; 25°C	50% ethanol	(Roselló-Soto et al., 2015)
	Papaya peel	Polyphenols	40 kV; 35 kJ/kg	272 min; 50 °C	n.a.	(Parniakov et al., 2014)
	Vine shots	Proteins Carbohydrates Polyphenols	40 kV; 0.5 Hz; 609.5 kJ/kg; 100 pulses (10 µs)	n.a.; 50 °C	Water	(Rajha et al., 2015)

	Sesame seeds	Essential oil Polyphenols Proteins	40 kV; 0.5 Hz; 160 kJ/kg	n.a.; 50 °C	Water	(Sarkis et al., 2015b)
	Sesame cake	Polyphenols Proteins Lignans	40 kV; 0.5 Hz; 83 kJ/kg; 100 pulses (10 µs)	20 min; 60 °C (40 °C proteins)	10% ethanol (50% for lignans)	(Sarkis et al., 2015a)
	Pomegranate peel	Polyphenols	20 kV; 1000 Hz; pulses (2 µs)	30 min; 70 °C	Water	(Xi et al., 2017)
	Stevia	Steviol glycosides Polyphenols Flavonoids Chlorophylls Carotenoids	40 kV; 0.5 Hz; 178 kJ/kg; 200 pulses (10 µs)	n.a.; 50 °C	Water	(Carbonell-Capella et al., 2017)
		Polyphenols Protein Chlorophyll Carotenoid	40 kV; 0.5 Hz; 141 kJ/kg; 300 pulses (10 µs)	n.a; 20 °C	Water	(Barba et al., 2015)

2.3 BIOCOMPOUNDS MICROENCAPSULATION PROCESSES

Natural bioactive compounds are chemically unstable and susceptible to oxidative degradation, particularly when exposed to oxygen, light, enzymatic activities, adverse temperature and pH conditions, metal ions and water, which leads to the alteration of their properties (Shishir et al., 2018). The oxidative degradation may deteriorate natural pigments and antioxidant bioactive compounds, leading to the generation of free radicals and subsequently may result in a negative effect on shelf stability, sensory characteristics and consumer acceptability of the final products (Gómez-Mascaraque et al., 2017). Moreover, the direct use of "non-protected/stabilized" biofunctional compounds (e.g. phenolics, pigments, vitamins, essential oils, among others) is very limited in food, chemical and pharmacological formulations due to some of their particular features, *i.e.* low bioavailability, low water solubility, rapid catabolism and excretion and low stability in environmental, processing and gastrointestinal conditions (Gómez-Mascaraque et al., 2017; Shishir et al., 2018; Vulić et al., 2019).

Therefore, in order to preserve the quality and functionality of bioactive compounds, or to enhance their applicability to food, chemical, pharmacological, medical and other biological formulations, encapsulation is considered as a feasible alternative (Ye et al., 2018).

Encapsulation is a process in which active agents are capped or coated by carrier material in order to form particles or capsules (micro or nanometer scale), protecting the active substance from the external medium. The coated materials (active or functional agent) are also designated as core, fill, or internal phase, while the coating materials (carrier material) are known as capsule, shell, wall material, membrane, matrix or external phase (Devi et al., 2017). Encapsulation technology is extensively applied in food (e.g., functional compounds, additives, dyes, flavours), textile, cosmetic, agro-chemical and pharmaceutical (e.g., vaccines) industries to encapsulate bioactive compounds (polyphenols, natural pigments, enzymes, *etc.*) by forming protective barriers against the adverse conditions (Devi et al., 2017). In the finished application, encapsulation enhances bioavailability, controlled release, and targeting precision of bioactive compounds (Shishir et al., 2018). Furthermore, encapsulation contributes to mask undesirable flavors, formation of solid particles, reduction of evaporation or volatiles' loss, enhancement of reactivity barrier for bioactive compounds, and improvement of physical stability, biological activity and shelf life of bioactive compounds (Aguiar et al., 2017; de Souza Simões et al., 2017). Therefore, encapsulation plays an important role to preserve or even enhance the functionality of bioactive compounds.

Moreover, one of the major challenges regarding the use of encapsulation systems for food and medical applications is the replacement of "non-food grade"/toxic materials by bio-based, biodegradable and GRAS alternatives. Other challenges include the design and application of efficient delivery systems for controlled release of bioactive compounds (de Souza Simões et al., 2017).

In encapsulation process, stable macrocapsules (diameters bigger than 5000 μm) micro-capsules (1–5000 μm) and nano-capsules (below 1 μm) are produced (Aguiar et al., 2017; de Souza Simões et al., 2017).

Microencapsulation is not a new process but was first commercially applied in 1954 for carbonless copy paper (Desai and Jin Park, 2005). Microencapsulation techniques are classified into three groups: (i) physical methods, such as spray-drying, lyophilization, supercritical fluid precipitation and solvent evaporation; (ii) physico-chemical methods, including coacervation, liposomes and ionic gelation; (iii) chemical methods, such as interfacial polymerization and molecular inclusion complexation (Ozkan et al., 2019).

2.3.1 Spray-drying encapsulation technique

Spray-drying is one of the oldest encapsulation methods used originally in the 1930's to encapsulate flavours using gum acacia (Shahidi and Han, 1993). Compared to the other methods, spray-drying is the most common method used for microencapsulation thanks to its low process cost (if compared to freeze-drying, the cost of spray-drying method is 30–50 times cheaper (Desai and Jin Park, 2005)), good retention of volatiles, good stability of the final product, large-scale production in continuous mode, controlled particle size, morphology and density of the powder in a single step (Madene et al., 2006). This technique is a process by which a liquid product is atomized and dried by means of a hot gas current (air or more rarely an inert gas as nitrogen) to obtain a powder. Moreover, the spray-drying process is generally divided in three basic steps (Santos et al., 2018):

- *preparation of the dispersion* (formation of a fine and stable emulsion of the core material and wall solution);
- *homogenization of the dispersion* (normally the dispersion must be heated and homogenized);
- *atomization of the mass into the drying chamber* (in this step, the mixture is atomized into a heated air stream supplied to the drying chamber with consequent evaporation of the solvent,

usually water that then leads to the formation of microcapsules. Due to the subsequent reduction in particle size and dispersion of the particles in the drying gas, the surface area of the particles increases exponentially, which helps to dry the feed in seconds. With the small size of droplets and the even distribution of the fluid feed, the moisture removal occurs without disturbing the integrity of the material (Santos et al., 2018)).

The final product can be in the form of granules, powders and agglomerate, and their properties are influenced by the operational parameters. According to Gharsallaoui *et al.* (Gharsallaoui et al., 2007) for efficient microencapsulation it is also necessary to optimize spray-drying conditions, including the feed temperature, the air inlet temperature and the outlet temperature. In fact, the best spray-drying conditions depend on the right compromise between the operating parameters. Despite the numerous advantages of spray drying techniques for food microencapsulation, there are also some limitations of this technology. The main limitation is the relatively limited number of wall materials available in the market, which also have the property of good solubility at high concentration. The main ones are discussed below.

2.3.2 Wall materials utilized in microencapsulation

As reported by Desai and Park (Desai and Jin Park, 2005), an ideal coating should have good rheological properties at high concentration and easy workability during microencapsulation, be able to disperse or to emulsify the active material and stabilize the emulsion produced, and not react chemically with the core materials. In addition, the wall material must seal and hold the active material within its structure and then release completely the solvent or other materials used during the process of microencapsulation. As mentioned before, these materials must be inexpensive, food-grade, biodegradable and GRAS for human health are other mandatory requirements (de Souza Simões et al., 2017). Generally, the microencapsulating materials are biomolecules that derive from various origins (such as plants, marine, animals or microbial), classified into three major categories (Desai and Jin Park, 2005):

- *Proteins*, have an amphiphilic character that offers physicochemical and functional properties required to encapsulate hydrophobic active molecules. The most commonly used proteins for encapsulating food ingredients by spray-drying are whey, pea and soy proteins and gelatin (Gharsallaoui et al., 2007; Sheu and Rosenberg, 1998). However, in presence of high temperatures it is very difficult to predict the effect of spray-drying process on the stability of wall proteins (Gharsallaoui et al., 2007; Shaddel et al., 2018). In all cases, it should be noticed that

there are sometimes certain issues that may limit the use of proteins as encapsulating agents, for example, allergy and precipitation of protein when microcapsules are added to products having pH near their isoelectric point (Gharsallaoui et al., 2007).

- *Lipids*, such as fatty acids, fatty alcohols, waxes, glycerides and phospholipids. Lipids are generally used as secondary coating materials applied to primary microcapsules and in the efficient encapsulation of bioactive lipophilic compounds, forming a powder to improve their moisture barrier properties. Lipids can also be incorporated in an emulsion formulation to form a matrix or film around the bioactive core (H. D. Silva et al., 2020).

- *Carbohydrates,* such as starches, MDs and gums. These materials are good encapsulating agents because they show low viscosities and good solubility, but most of them require more properties for high microencapsulation efficiency. In this context, chemical modifications of carbohydrates are a novel approach to improve encapsulating properties of common wall materials (Gharsallaoui et al., 2007). For example, some modified starches have surface-active properties and are widely used in the process of microencapsulation by spray-drying. Among hydrolyzed starches, MDs have the advantages of being low cost, good flavor and protection against oxidation, but lack appropriate emulsifying properties (Desai and Jin Park, 2005; Pourashouri et al., 2014). Gum Arabic is a natural exudate polysaccharide of *Acacia spp* and is a well-known effective wall material because of its good emulsifying capacity, low viscosity in aqueous solution and good volatile retention. Problems associated with the use of gum arabic in encapsulation are the high cost and limited supply (Kuan et al., 2009).

In research and especially in the food industry, among different wall materials, carbohydrates are the most commonly used if compared to proteins and lipids.

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CHAPTER III

BIOLOGICAL POTENTIAL OF *PINUS PINASTER* BARK EXTRACTS

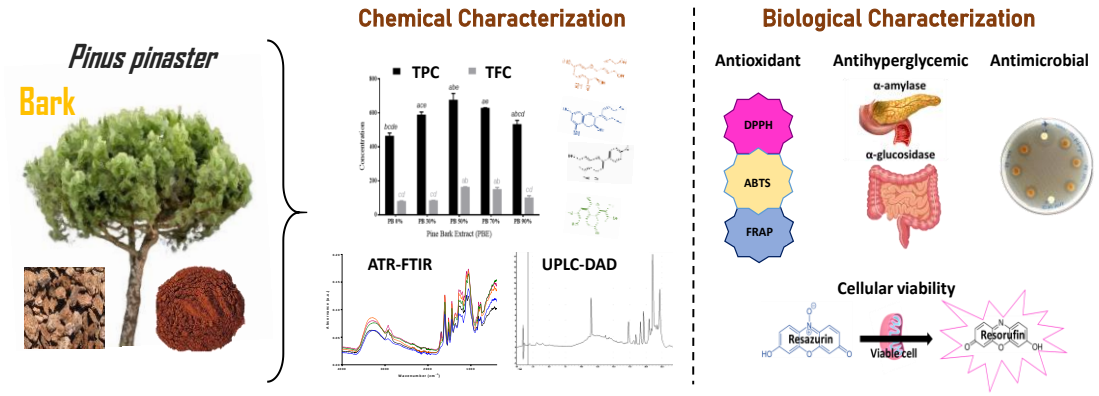
This chapter was based on the following paper:

Pedro Ferreira-Santos; Zlatina Genisheva; Cláudia M Botelho; Joana Santos; Carla Ramos; José Teixeira; Cristina Rocha. **2020.** Unravelling the Biological Potential of *Pinus pinaster* Bark Extracts. *Antioxidants,* 9, 3342020. https://doi:10.3390/antiox9040334

Abstract

Natural compounds from agro-food by-products have fostered interest in food industries. The aim of this study was to unravel potential uses for *Pinus pinaster* bark extracts (PBE). As functional features of this type of extracts are usually attributed to phenolic compounds, the extraction process was studied. Different PBEs were achieved, with high content in phenolic compounds, using different water/ethanol combinations as a solvent. These PBEs were chemically characterized, and their bioactivity and in vitro cell viability were evaluated. Extracts obtained with hydroethanolic solvents had higher content in phenolic and flavonoid compounds. All the PBEs presented high antioxidant, antibacterial and antihyperglycemic activities. Moreover, PBEs have low cytotoxicity and a selective activity against cancer cells as these were negatively affected. These features may allow the extracts to be used in food formulation and processing (as preservatives, antioxidants or bioactive ingredients), but they showed also potential for the pharmaceutical or nutraceutical sectors.

Graphical Abstract



Keywords: *Pinus pinaster* bark; extraction; pine bark extracts; phenolic compounds; flavonoid compounds; antioxidant activity; antihyperglycemic activity; antimicrobial activity; cells' metabolism

3.1 INTRODUCTION

Natural compounds, such as phenolics, flavonoids, proteins, carotenoids, among others, have fostered interest in different industries including paints, fertilizers, surfactants, textiles, rubbers, pharmaceuticals, etc. (Raza et al., 2019). Additionally, in food industry technology, they are used as natural preservatives against oxidation and microorganisms (bacterial and fungal contaminations), and in the development of functional food ingredients (Mark et al., 2019).

The biological activity of plant extracts can be attributed to secondary metabolites such as phenolic acids, flavonoids and other phenolic compounds. The popularity of these extracts is linked to their biological properties, such as antioxidant, anti-inflammatory, antimicrobial, antiviral, antiatherogenic, etc. (Calvo Torras et al., 2005; Ferreira-Santos et al., 2019; Lee et al., 2020; Salehi et al., 2019; Sharma et al., 2016). In particular, the extracts of *Pinus pinaster* (a conifer plant found in some Mediterranean countries and used in afforestation of Africa, New Zealand and Australia), are rich in phenolic acids, flavanols and flavonoids (e.g., cinnamic acid, hydroxybenzoic acid, catechin, quercetin and taxifolin) with a potent AA (Ferreira-Santos et al., 2019; Mármol et al., 2019). These extracts have demonstrated beneficial effects for the treatment of several diseases, such as cardiovascular, metabolic, neurological, etc. (Iravani and Zolfaghari, 2011; Maimoona et al., 2011; Mármol et al., 2019; Rohdewald, 2002). The activities reported for *P. pinaster* extracts make this underexploited by-product (bark) of the wood industry of high interest for the pharmaceutical and food industries. There are only a few studies about the potential bioactivities and toxicity of the extracts from *P. pinaster* bark. However, the existent studies are focused on the commercial product Picnogenol®, an aqueous extract used as an active supplement (Iravani and Zolfaghari, 2011; Rohdewald, 2002).

The extracts from *P. pinaster* bark are predominantly obtained by conventional solvent extraction, Soxhlet, MW, supercritical CO_2 and, more recently, by OH-assisted extraction (Braga et al., 2008; Chupin et al., 2015; Ferreira-Santos et al., 2019; Jerez et al., 2006; Mellouk et al., 2016). However, the variety of extraction conditions (such as type of solvent, solid-liquid ratio, time and temperature) may potentially affect the processes' yield and the phenolic profile of the extracts. To prevent the environmental impact and reduce the biowastes and by-products of the agro-food industry, it is necessary to optimize the recovery of bioactive compounds with high added value and to enable their re-introduction in the market (Chemat et al., 2019; Herrero and Ibañez, 2018; Torres-Valenzuela et al., 2019). In addition, the applied methodology needs to be sustainable and "green" by using alternative and highly clean and nontoxic solvents (Lourenço et al., 2019).

It is known that polar solvents, such as ethanol or aqueous mixtures containing ethanol, are frequently used for the recovery of phenolic compounds from plant tissues (Chemat et al., 2019). In this sense, the aim of this research work was to study the functional potential of phenolic-rich extracts from PB using water and water-ethanol in different ratios as a solvent. The pine bark extracts (PBE) with the highest phenolic compounds' content and highest in vitro AA were chemically characterized, and its bioactivity (antioxidant, antimicrobial and antidiabetic) and in vitro cell viability (in normal and cancer cell lines) were evaluated.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Folin-Ciocalteu reagent, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), porcine pancreatic amylase (EC 3.2.1.1, type VI), *Saccharomyces cerevisiae* α-Glucosidase (EC 3.2.1.20, type I), *p*-nitrophenyl-R-D-glucopyranoside (pNPG), aluminium chloride ($AlCl_3$), acarbose, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution, resazurin sodium salt, dimethyl sulfoxide (DMSO, ≥99.9%) and all standard markers for HPLC were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were analytical grade, and ultra-pure water was used throughout the experiments.

3.2.2 Raw material preparation and characterization

Bark from *P. pinaster* (approximate age 15 years) was collected in Ponte de Lima, Portugal (April 2016). Firstly, the bark was washed with distilled water and dried at 40 °C for 48 h and subsequently milled in a cutting mill (Retsch SM 2000) to a granulometry of 0.1–0.45 mm for general chemical composition and 1–1.6 mm for extraction process.

Chemical summative analyses were determined in accordance to the National Renewable Energy Laboratory (NREL) official protocols, and included ethanol extractives (NREL/TP-510-42619), structural carbohydrates (namely cellulose and hemicellulose), klason and acid soluble lignin (NREL/TP-510-42618) and ash content (NREL/TP-510-42622). The mineral content was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES), after PB

digestion with HNO_3. Fat content was determined according to the official AOAC method (n° 920.39). Total proteins content estimated by using the N×6.25 conversion factor, was performed using a Kjeldahl distillator (Kjeltec 8400 Analyzer, FOSS, Hilleroed, Denmark) by quantification of Nitrogen after PB digestion. PB moisture was determined gravimetrically using a moisture analyzer (MAC 50/1/NH, RADWAG, Radom, Poland). All experiments were performed in triplicate.

3.2.3 Extraction conditions and extracts preparation

In the first part of the work, the procedure to study the influence of the extraction parameters of phenolic compounds present in PB was carried out using 100 mL cylindrical reactors duly protected from light in thermostatized water bath with shaking (170 rpm). The volume of extraction for all experiments was 40 mL. Experiments were performed using response surface methodogy (RSM) (2^3 central composite design (CCD)) for TSE with water or hydroethanolic solvent (30%–90% (v/v)). The levels of independent variables were selected based on the results obtained from our preliminary experiments (data not shown) and data from the literature (Jerez et al., 2006; Mellouk et al., 2016). The five levels of each of the three variables were coded in 18 runs (including four replicates of the center point) and were performed in a random order. Independent variables for extraction were time (min, x_1), temperature (°C, x_2) and solid: liquid ratio (g/mL, x_3). Dependent variables (Y_1 and Y_2) were total phenolic content (TPC, mg GAE/g PB) and ferric reducing antioxidant power (FRAP, mmol Fe^{2+}/g PB), respectively. Coded and actual values of the independent variables together with data of dependent variables are given in **Table 1**. Data were correlated following the polynomial Equation 1.

$$Y_i = \beta_{0i} + \beta_{1i}x_1 + \beta_{2i}x_2 + \beta_{3i}x_3 + \beta_{11i}x_1^2 + \beta_{22i}x_2^2 + \beta_{33i}x_3^2 + \beta_{12i}x_1x_2 + \beta_{13i}x_1x_3 + \beta_{23i}x_2x_3 \quad (1)$$

where, Y_i correspond to the dependent variables; x_1, x_2 and x_3 value of independent variables; β_{0i}, β_{1i}, β_{2i}, β_{3i}, β_{11i}, β_{22i}, β_{33i}, β_{12i}, β_{13i} *and* β_{23i} are regression coefficients calculated from experimental data by multiple regression using the least-squares method.

The experimental data were fitted to the proposed model using Statistica software (Statistica 8.0). The statistical analysis was performed using ANOVA, which established the model significance, the significance for each polynomial coefficient, and the determination coefficient R^2.

For the best conditions selected in the previous stage, new experimental assays were carried out in order to characterize and evaluate the bioactive potential and cell viability of extracts. The obtained extracts were dried by freeze drying and keep at 4 °C for further analysis.

Table 1. Experimental runs using coded levels of time (min, x_1), temperature (°C, x_2) and solid: liquid ratio (g/mL, x_3) according to the 2^3 full factorial central composite design and data of total phenolic content (TPC) and reducing antioxidant activity (FRAP) of extracts obtained under those conditions for the tested experimental model (EtOH 0%, EtOH 30%, EtOH 50%, EtOH 70% and EtOH 90%).

Runs	Time (min)	Temperature (°C)	Solid-Liquid Ratio (g/mL)	EtOH (*v/v*)									
				0% (H_2O)		30%		50%		70%		90%	
	x_1	x_2	x_3	*TPC*	*FRAP*	*TPC*	*FRAP*	*TPC*	*FRAP*	*TPC*	*FRAP*	*TPC*	*FRAP*
1	35 (−1)	38 (−1)	0.05 (−1)	18.70	0.13	20.81	0.29	68.56	0.42	60.94	0.44	59.74	0.34
2	35 (−1)	38 (−1)	0.15 (1)	31.77	0.26	93.44	0.93	99.04	1.15	97.34	1.06	91.54	0.83
3	35 (−1)	82 (1)	0.05 (−1)	20.18	0.17	60.94	0.35	69.04	0.48	71.04	0.50	61.74	0.34
4	35 (−1)	82 (1)	0.15 (1)	37.59	0.33	106.14	0.99	119.44	1.39	126.24	1.15	98.84	0.97
5	115 (1)	38 (−1)	0.05 (−1)	19.86	0.16	58.14	0.32	61.34	0.43	63.44	0.45	60.64	0.37
6	115 (1)	38 (−1)	0.15 (1)	31.76	0.26	86.84	0.94	99.04	1.40	108.54	1.11	99.94	0.61
7	115 (1)	82 (1)	0.05 (−1)	22.68	0.16	75.74	0.41	93.24	0.58	82.04	0.60	62.44	0.43
8	115 (1)	82 (1)	0.15 (1)	48.13	0.35	120.14	1.35	163.64	1.50	136.54	1.44	123.84	1.14
9	8 (−1.682)	60 (0)	0.1 (0)	5.30	0.08	72.24	0.49	77.24	0.61	76.34	0.55	75.64	0.53
10	142 (1.682)	60 (0)	0.1 (0)	30.59	0.25	83.14	0.71	102.54	1.21	100.24	0.96	89.34	0.94
11	75 (0)	23 (−1.682)	0.1 (0)	20.31	0.18	69.24	0.49	79.54	0.66	81.54	0.57	73.64	0.51
12	75 (0)	97 (1.682)	0.1 (0)	35.61	0.29	95.14	1.08	115.94	1.07	124.34	1.43	104.24	0.96
13	75 (0)	60 (0)	0.016 (−1.682)	9.08	0.09	19.38	0.17	59.69	0.21	57.64	0.38	25.35	0.17
14	75 (0)	60 (0)	0.184 (1.682)	36.09	0.33	118.64	1.30	138.84	1.34	133.04	1.40	115.64	1.07
15	75 (0)	60 (0)	0.1 (0)	27.97	0.25	83.64	1.01	92.54	1.09	106.94	0.94	89.14	0.81
16	75 (0)	60 (0)	0.1 (0)	29.24	0.26	94.94	0.79	92.74	0.98	99.24	1.01	85.04	0.78
17	75 (0)	60 (0)	0.1 (0)	28.28	0.25	91.34	1.00	94.94	1.16	95.24	0.95	79.44	0.81
18	75 (0)	60 (0)	0.1 (0)	27.87	0.25	86.74	0.80	94.14	1.15	103.24	1.24	80.24	0.95

TPC, Total Phenolic Content (mg GAE/g PB); FRAP, Ferric Reducing Antioxidant Power (mmol Fe^{2+}/g PB).

3.2.4 Chemical analysis of extracts

3.2.4.1 Total phenolic content (TPC)

The total content of phenolic compounds was measured by the Folin–Ciocalteu method that was based on the colorimetric reduction/oxidation reaction of phenols (Ferreira-Santos et al., 2019; Singleton et al., 1999). Gallic acid was used to perform the standard curve (R^2 = 0.996) and the results were expressed as milligram gallic acid equivalents (GAE) per gram of PB (optimization process) or dry extract (extract characterization).

3.2.4.2 Total flavonoid content (TFC)

The applied method for the determination of total flavonoids content has been previously described by Barros *et al.* (Barros et al., 2010). An aliquot (500 µL) of the PBE solution was mixed with distilled water and $NaNO_2$ solution (5%). After 6 min, $AlCl_3$ solution (10%) was added and allowed to stand further 6 min; thereafter, NaOH solution (4%) was added to the mixture. Then, the mixture was properly mixed and allowed to stand for 15 min, and the absorbance was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (R^2 = 0.997) and the results were expressed as mg of catechin equivalents (CE) per g of extract (mg CE/g extract).

3.2.4.3 Liquid chromatography

Identification and quantification analysis of phenolic presents in PBE were performed as described previously (Ferreira-Santos et al., 2019) using a Shimatzu Nexpera X2 UPLC chromatograph equipped with Diode Array Detector (DAD) (Shimadzu, SPD-M20A, Columbia, Malyland, USA). Separation was performed on a reversed-phase Aquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size; from Waters, Milford, Massachusetts, USA) and a pre-column of the same material at 40 °C. The HPLC grade solvents used were water/formic acid (0.1%) and acetonitrile as eluents and the flow rate was 0.4 mL/min. Phenolic compounds were identified by comparing their UV spectra and retention times with that of corresponding standards. Quantification was carried out using calibration curves for each compound analyzed using concentrations between 250–2.5 mg/L (250, 125, 100, 50, 25, 10, 5, 2.5 mg/L). In all cases, the coefficient of linear correlation was R^2 > 0.99. Compounds were quantified and identified at different wavelengths (209–370 nm).

3.2.4.4 ATR-Fourier transform infrared spectroscopy

Chemical groups and bonding arrangement of constituents present in the PBE dried samples were determined by Fourier Transform Infrared Spectroscopy (FTIR) using an ALPHA II- Bruker spectrometer (Ettlingen, Germany) with a diamond-composite attenuated total reflectance (ATR) cell. The measurements were recorded with a wavenumber range from 4000 to 400 cm^{-1}, with a resolution of 4 cm^{-1} and 24 scans per sample.

3.2.5 Evaluation of *in vitro* bioactivities

3.2.5.1 Antioxidant activity

Three different methods of measuring the AA were used: DPPH, ABTS and FRAP, as previously described by Ferreira-Santos *et al.* (Ferreira-Santos et al., 2019).

Free radical scavenging assay (DPPH assay) consists in the reduction of the DPPH· radical in the presence of hydrogen-donating antioxidant, and in the formation of the non-radical DPPH-H form at the end of the reaction.

Scavenging activity of ABTS radical cation ($ABTS^{\cdot+}$) (ABTS assay) is based on interaction between antioxidant and ABTS radical, that, in the presence antioxidant compounds, the $ABTS^{\cdot+}$ nitrogen atom quenches the hydrogen atom, causing the solution decolorization.

The concentration of the tested PBE and Trolox as a standard compound ranged between 1 and 250 µg/mL. The lyophilized extracts were re-suspended in the respective solvent used in the extraction process, and the Trolox prepared in absolute ethanol. A corresponding control was used for each solvent.

The radical scavenging activity for DPPH and ABTS methods (% inhibition) was calculated as Equation 2.

$$\% \text{ Inhibition} = \frac{\text{Ac}-\text{As}}{\text{Ac}} \times 100 \quad (2)$$

where As represents the sample absorbance and Ac the control sample absorbance. The results were expressed as the sample concentration (µg/mL) required to inhibit 50% of the activity (IC_{50}) calculated from a dose response curve using GraphPad software (San Diego, CA, USA).

FRAP assay consists in the ability of extracts to reduce ferric ions (Fe^{3+} to Fe^{2+}), in the form of TPTZ. FRAP values are expressed as micromoles of ferrous equivalent per g of dry weight (µmol Fe^{2+}/g PBE).

3.2.5.2 Antihyperglycemic activity

Two different methods of measuring the potential antihyperglycemic activity were used: α-amylase and α-glucosidase inhibition assays. These methodologies were previously reported by Irondi and coworkers (Irondi et al., 2017), and used with some minors modifications. The concentration of the tested PB extracts (dissolved in 20% DMSO and 80% water) were between 50–1000 μg/mL for α-amylase assay (1000, 750, 500, 250, 125, 50 μg/mL) and 1–250 μg/mL for α-glucosidase assay (250, 200, 150, 100, 50, 25, 10, 1 μg/mL, prepared by successive dilutions). For the α-amylase inhibition assay, different concentrations of PBE were incubated with α-amylase (0.5 mg/mL) and 1% of starch solution for 15 min at 37 °C. Afterwards, dinitrosalicylic acid color reagent (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH) was added to the reaction and placed 10 min in a boiling water bath. This heating step also allowed to stop the reaction (inactivate the enzyme). Finally, the mixture was diluted 10 times in distilled water. In these conditions, the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid allows to quantify maltose which being detectable at 540 nm. Acarbose was used as a reference control, and the concentration used for a-amylase assay is between 1–200 μg/mL (200, 100, 50, 25, 10, 1 μg/mL). The α-amylase inhibition (%) was calculated using the same equation (Equation 2) used for DPPH assay.

For α-glucosidase inhibition assay, α-glucosidase solution (10 U/mL) was incubated with different concentrations of PBE and pNPG (3 mM). The mixture was then incubated at 37 °C for 15 min, and the reaction was stopped by adding Na_2CO_3 solution (1M). The activity of α-glucosidase was determined by measuring the absorbance of *p*-nitrophenol released at 400 nm. Acarbose was used as a reference control, and the concentration used for α-glucosidase assay is between 2500–15,000 μg/mL (15,000, 12,500, 10,000, 7500, 5000, 2500 μg/mL). The α-glucosidase inhibitory activity was calculated using the same equation (Equation 2) used for DPPH assay.

The results were expressed as the sample concentration (μg/mL) required to inhibit 50% of the activity (IC_{50}) calculated from a dose response curve using GraphPad software.

3.2.5.3 Antimicrobial activity

For this assay, a disk diffusion method was used to determine the diameter of the inhibition zone of tested extracts (50 mg/mL of each PBE´s reconstituted in DMSO (≥99.9%)) and was performed following the protocols established by the Clinical and Laboratory Standards Institute

(Clinical and Laboratory Standards Institute, 2012). The PBE concentration was selected based on the results obtained from preliminary experiments (data not shown) and data from the literature in which extracts rich in polyphenols were used (Gontijo et al., 2019).

Strains of Bacillus cereus ATCC 11778, Clostridium perfringens ATCC 13124, Escherichia coli ATCC 25922, Listeria monocytogenes ATCC 13932, Staphylococcus aureus ATCC 25923, Salmonella enterica serovar Enteritidis ATCC 25928, Aspergillus brasiliensis ATCC 16404, Saccharomyces cerevisiae NCTC 10716 and Candida albicans ATCC 10231 were inoculated in Columbia Agar + 5% Sheep Blood (COS, Biomérieux, France). Active cultures (0.5 McFarland) were spread onto Mueller-Hinton Agar (MHA) (Oxoid, England) for general bacteria and MHA + 0.2% glucose for fungus. Blank disks 6 mm in diameter were placed onto inoculated plates and impregnated with 10 µL of PBE or controls (DMSO and commercial solution of sodium hypochlorite (LX)). In this study, a solution of sodium hypochlorite was used as positive control instead of commercial antibiotics, in order to control the sensitivity of the microbial test and reduce antibiotic use in basic research studies. Afterwards, the plates were incubated for 24 h at 37 °C (for bacteria) and 48 h (for yeasts and fungi). Zones of inhibition were measured in mm with the help of ImageJ software (US National Instiutes of Health, http://rsb.info.nih.gov/ij/).

3.2.5.4 Cell Viability

In vitro cell metabolic activity of the PB extracts was assessed in different cell lines: normal mouse fibroblast (L929-ATCC® CCL -1), human embryonic kidney (HEK293T- ATCC® CRL-11268) and human lung cancer (A549- ATCC® CCL-185); these cell lines were kindly provided by Andreia Gomes (Department of Biology, University of Minho). The metabolic activity of each cell line was evaluated by the resazurin reduction assay (Helm et al., 2017). Cells were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere with 5% CO_2. When the cell culture reached 70%–80% of confluence, the cells were trypsinized and seeded in a 96-well plate at a density of 1×10^5 cells per well. The different cell lines were incubated with supplemented DMEM and PBE in a concentration ranging from 75 to 1000 µg/mL for 24 h. After incubation, cell viability was measured using the resazurin assay (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt). The supernatant was replaced by 200 µL culture media containing resazurin (0.5 mM in Phosphate Buffer Solution (PBS)). After 2 h of incubation at 37 °C, 150 µL of the supernatant were transferred to a new 96-well microplate and the pink fluorescent resultant

product (resorufin) was detected at 560 nm (λ ex) and 590 nm (λ em) using a microplate reader (Cytation 3, BioTek Instruments, Inc., Winooski, Vermont, USA).

The % cell viability was calculated correcting blank values (cell-free medium) and related to untreated controls (0.5% DMSO). IC_{50} values were calculated from a dose response curve using GraphPad software.

3.2.6 Statistical Analysis

All experiments were performed in triplicate and the data are presented as mean ± standard deviation (SD) values. GraphPad Prism® software (version 6.0; San Diego, CA, USA) was used for statistical analyses. The analysis of variance (ANOVA) and Tukey's multiple comparisons test were used to determine statistically different values at a significance level of $p < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Chemical Characterization of PB

The chemical composition of the PB is summarized in **Table 2**. The results show that the majority fractions of the total bark composition are lignin, representing 41.6% (being 41.0% klason and 0.6% acid soluble lignin). The monomeric composition of polysaccharides, which correspond to an average of approximately 30% of pine bark, shows a predominance of the cellulose fraction (17.4% of glucose content). In the hemicellulose fraction, xylans are most representative (10.9%), being arabinan and mannan groups present in 1.4% of total monosaccharides.

In this work, the ethanol soluble extractives represent 13.2% of total PB composition.

The inorganic substances represent 2.6% of total bark composition, and the most relevant minerals, determined by plasma atomic emission spectrometry, are potassium, magnesium, calcium and iron.

Other constituents such a lipid fraction (fat) and protein content represent 2.5% and 1.6% of total composition of PB, respectively.

Table 2. Chemical composition of *Pinus pinaster* bark, expressed as percentage of dry raw material weight (composition by 100 g).

Composition (%)	
Cellulose[a]	17.39 ± 0.37
Hemicellulose	12.31 ± 0.20
Xylose	10.92 ± 0.19
Arabinose + manose	1.39 ± 0.01
Acetyl group	n.d.
Lignin	41.65 ± 0.24
Klason	41.05 ± 0.24
Acid soluble	0.60 ± 0.00
Fat	2.54 ± 0.26
Protein	1.64 ± 0.03
Ash	0.87 ± 0.00
Moisture	8.15 ± 0.02
Ethanol extractives	13.20 ± 0.31
Inorganic substances	2.56 ± 0.33
Macro minerals *(Na, K, Ca, Mg, Fe)*	2.54 ± 0.33
Micro minerals *(Zn, Mn, Cu)*	0.02 ± 0.00

[a]estimated from the glucan content; n.d.: not detected.

3.3.2 Solid–Liquid Extraction

3.3.2.1 Influence of Variables on the TPC and Antioxidant Activity

Assays were performed using water and ethanol (30%, 50%, 70% and 90% (*v/ v)*) as solvents, in order to select the best extraction conditions for each solvent. In this way, five different extracts were generated with different polarities and features presumably representative of the PB potential. These extracts were used in the subsequent analyzes. The solvents used (water and ethanol) are considered natural, environmentally friendly (green solvents), nontoxic and food grade (Chemat et al., 2019; Prat et al., 2015). This is very important, as the extracts obtained will be studied as possible food and nutraceutical applications for human or animal consumption.The influence of independent variables, such as time, temperature and solid-liquid ratio for different ethanol concentration on the TPC and antioxidant activity (FRAP), was evaluated by the models shown in **Table 1** and in **Figure 1** (Contour line plots for FRAP variable) and **Figure 2** (Pareto chart).

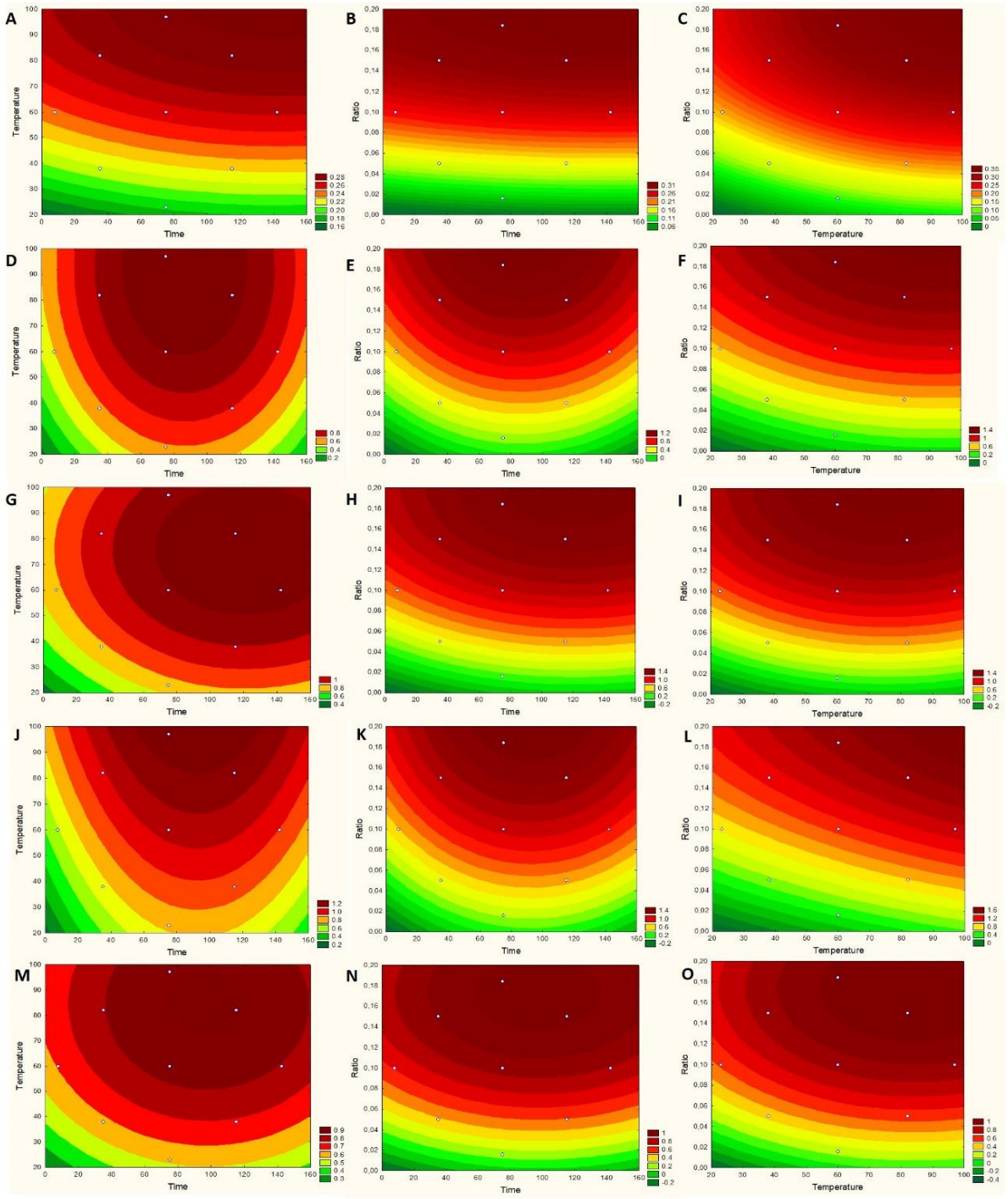


Figure 1. Contour line plots representing the antioxidant activity (FRAP assay) under different conditions of extraction (time, temperature, liquid-solid ratio) of the tested experimental models. EtOH 0% (A,B,C), EtOH 30% (D,E,F), EtOH 50% (G,H,I), EtOH 70% (J,K,L), EtOH 90% (M,N,O).

The significance of each coefficient was also determined using *F-value* and the corresponding *p*-value (**Table 3**). Statistical analysis for each solvent (water (EtOH 0%) or water-ethanol mixtures) is represented in **Figure 1** and **Table 3**.

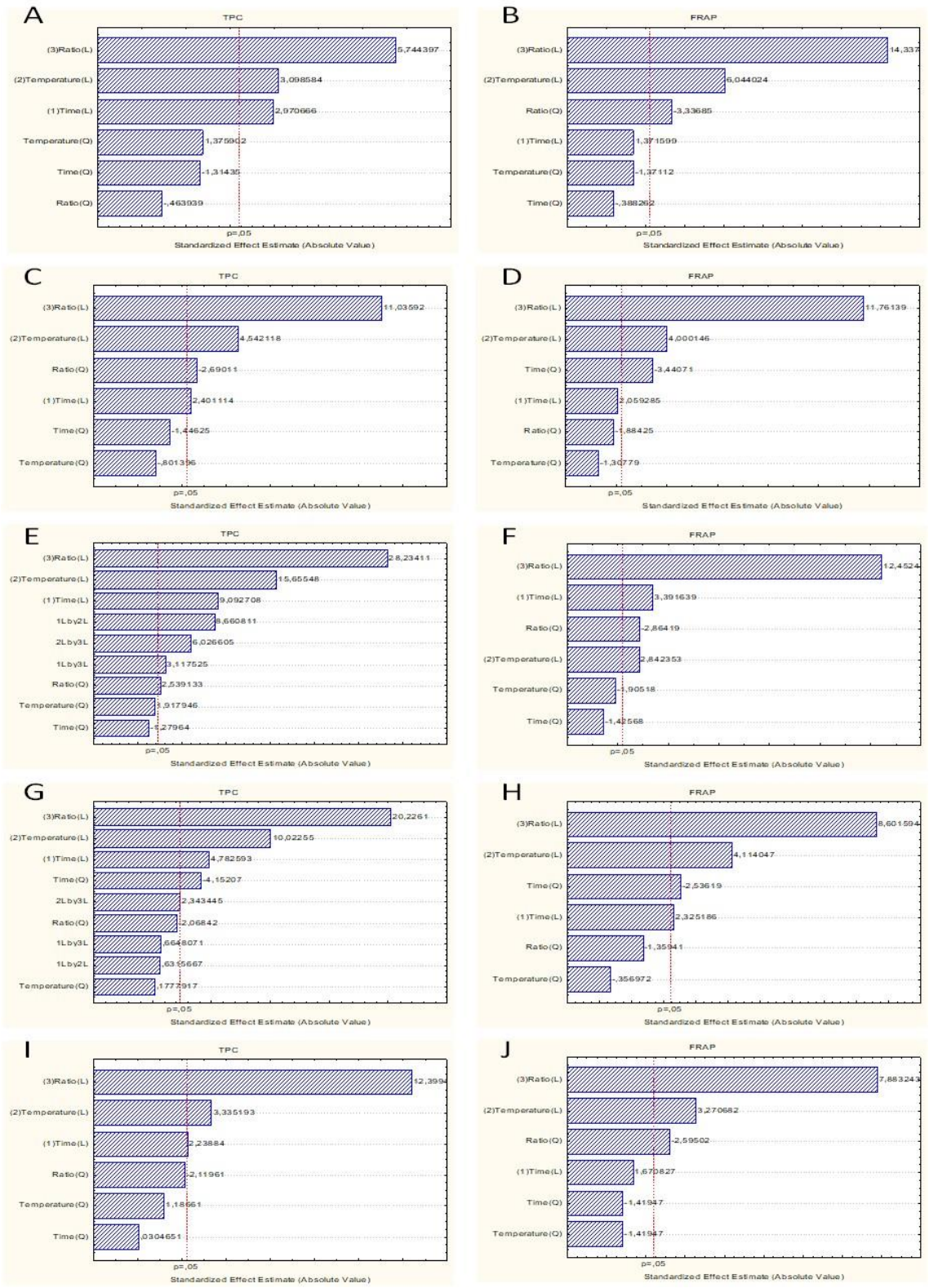


Figure 2. Pareto chart for the effects of time (x1), temperature (x2), liquid-solid ratio (x3), and possible interactions, on the total phenolic content (TPC) (**A**,**C**,**E**,**G**,**I**) and FRAP antioxidant activity (**B**,**D**,**F**,**H**,**J**) of the tested experimental models. EtOH 0% (**A**,**B**), EtOH 30% (**C**,**D**), EtOH 50% (**E**,**F**), EtOH 70% (**G**,**H**), EtOH 90% (**I**,**J**). L and Q correspond to the effects at linear and quadratic levels, respectively.

The experimental variables were correlated following Equation 1 (quadratic model). The proposed mathematical models describing the extraction time (x_1), temperature (x_2) and solid-liquid ratio (x_3) as function, and using normalized values of regression coefficient for TPC and FRAP, are presented in the **Table 4**. In addition, the validation experiments performed under the predicted conditions derived from the experimental design demonstrated that the experimental values were close to the predicted values (data not shown), confirming the validity and adequacy of the proposed mathematical models.

The conditions of test number 8 of all experimental models, which extraction conditions of 115 min, 82 °C and a solid-liquid ratio of 0.15 g/mL (6 g /40 mL), showed a higher TPC (48.1 mg GAE/g PB for EtOH 0%, 120.1 mg GAE/g PB for EtOH 30%, 163.6 mg GAE/g PB for EtOH 50%, 136.5 mg GAE/g PB for EtOH 70%, 123.8 mg GAE/g PB for EtOH 90%, respectively) compared to the other conditions tested. In addition, the AA evaluated by the FRAP method was also in agreement with the TPC of the obtained extracts. Taking into account these results to obtain extracts with high content of bioactive polyphenols, the conditions mentioned above were selected in order to evaluate the chemical profile, potential bioactivity and cytotoxicity of the extracts.

Table 3. Factors and interaction effects of time (x_1), temperature (x_2) and liquid-solid ratio (x_3) on total phenolic content (TPC) and reducing antioxidant activity (FRAP) of the tested experimental model (EtOH 0%, EtOH 30%, EtOH 50%, EtOH 70% and EtOH 90%).

Model	Response	Factor	SS	df	MS	*F-test*	*p-value*
EtOH 0% (H_2O)	TPC	(1)Time (L)	255.635	1	255.635	8.825	0.013
		Time (Q)	50.042	1	50.042	1.728	0.215
		(2)Temperature (L)	278.124	1	278.125	9.601	0.010
		Temperature (Q)	54.838	1	54.839	1.893	0.196
		(3)Ratio (L)	955.876	1	955.876	32.999	<0.001
		Ratio (Q)	6.235	1	6.235	0.215	0.652
		Error	318.644	11	28.968		
		Total SS	1947.954	17			
	FRAP	(1)Time (L)	0.001	1	0.001	1.881	0.198
		Time (Q)	0.001	1	0.000	0.151	0.705
		(2)Temperature (L)	0.014	1	0.013	36.530	<0.001
		Temperature (Q)	0.001	1	0.001	1.880	0.198
		(3)Ratio (L)	0.076	1	0.076	205.571	<0.001
		Ratio (Q)	0.004	1	0.004	11.135	0.006
		Error	0.004	11	0.001		
		Total SS	0.098	17			
EtOH 30%	TPC	(1)Time (L)	443.91	1	443.910	5.765	0.035
		Time (Q)	161.05	1	161.049	2.092	0.176
		(2)Temperature (L)	1588.50	1	1588.498	20.631	0.001
		Temperature (Q)	49.45	1	49.450	0.642	0.440
		(3)Ratio (L)	9377.50	1	9377.512	121.792	<0.001
		Ratio (Q)	557.20	1	557.199	7.237	0.021
		Error	846.96	11	76.996		
		Total SS	12877.44	17			
	FRAP	(1)Time (L)	0.050	1	0.050	4.241	0.064
		Time (Q)	0.141	1	0.141	11.839	0.006
		(2)Temperature (L)	0.190	1	0.190	16.001	0.002

		Temperature (Q)	0.020	1	0.020	1.710	0.218
		(3)Ratio (L)	1.645	1	1.645	138.330	<0.001
		Ratio (Q)	0.042	1	0.0422	3.550	0.086
		Error	0.131	11	0.0122		
		Total SS	2.177	17			
EtOH 50%	TPC	(1)Time (L)	787.87	1	787.867	82.677	<0.001
		Time (Q)	15.60	1	15.604	1.638	0.237
		(2)Temperature (L)	2335.60	1	2335.602	245.094	<0.001
		Temperature (Q)	35.05	1	35.054	3.679	0.091
		(3)Ratio (L)	7596.51	1	7596.514	797.165	<0.001
		Ratio (Q)	61.44	1	61.438	6.447	0.035
		1L by 2L	714.80	1	714.798	75.010	<0.001
		1L by 3L	92.62	1	92.616	9.719	0.014
		2L by 3L	346.11	1	346.108	36.320	<0.001
		Error	76.24	8	9.529		
		Total SS	12073.57	17			
	FRAP	(1)Time (L)	0.160188	1	0.160	11.503	0.006
		Time (Q)	0.028	1	0.028	2.033	0.181
		(2)Temperature (L)	0.113	1	0.113	8.079	0.016
		Temperature (Q)	0.051	1	0.051	3.630	0.083
		(3)Ratio (L)	2.159	1	2.159	155.062	<0.001
		Ratio (Q)	0.114	1	0.114	8.207	0.015
		Error	0.153	11	0.014		
		Total SS	2.730	17			
EtOH 70%		(1)Time (L)	414.02	1	414.024	22.873	0.001
		Time (Q)	312.05	1	312.053	17.240	0.003
		(2)Temperature (L)	1818.26	1	1818.258	100.452	<0.001
		Temperature (Q)	0.57	1	0.572	0.032	0.863
		(3)Ratio (L)	7404.97	1	7404.968	409.095	<0.001
		Ratio (Q)	77.44	1	77.442	4.278	0.073
		1L by 2L	7.22	1	7.220	0.399	0.545

		1L by 3L	8.00	1	8.000	0.442	0.524
		2L by 3L	99.40	1	99.405	5.492	0.047
		Error	144.81	8	18.101		
		Total SS	10266.68	17			
	FRAP	(1)Time (L)	0.113	1	0.113	5.406	0.040
		Time (Q)	0.134	1	0.134	6.432	0.027
		(2)Temperature (L)	0.352	1	0.352	16.925	0.002
		Temperature (Q)	0.003	1	0.003	0.127	0.728
		(3)Ratio (L)	1.540	1	1.540	73.987	<0.001
		Ratio (Q)	0.038	1	0.038	1.848	0.201
		Error	0.229	11	0.021		
		Total SS	2.384	17			
EtOH 90%	TPC	(1)Time (L)	246.668	1	246.668	5.012	0.047
		Time (Q)	0.046	1	0.046	0.001	0.976
		(2)Temperature (L)	547.405	1	547.405	11.124	0.007
		Temperature (Q)	69.292	1	69.292	1.408	0.260
		(3)Ratio (L)	7566.128	1	7566.128	153.748	<0.001
		Ratio (Q)	221.094	1	221.094	4.493	0.058
		Error	541.326	11	49.211		
		Total SS	9261.411	17			
	FRAP	(1)Time (L)	0.042	1	0.042	2.792	0.123
		Time (Q)	0.030	1	0.030	2.015	0.183
		(2)Temperature (L)	0.162	1	0.162	10.698	0.007
		Temperature (Q)	0.030	1	0.030	2.015	0.183
		(3)Ratio (L)	0.940	1	0.940	62.146	<0.001
		Ratio (Q)	0.102	1	0.102	6.734	0.025
		Error	0.166	11	0.015		
		Total SS	1.434	17			

L and Q correspond to the effects at linear and quadratic levels, respectively. SS: sum of squares; MS: mean square; df: degrees of freedom.

Table 4. Quadratic models describing the responses variation of total phenolic compounds (TPC) and antioxidant activity (FRAP) in function of independent variables of the tested experimental model (EtOH 0%, EtOH 30%, EtOH 50%, EtOH 70% and EtOH 90%) and their correspondent R^2 coefficients.

Response	Model	Mathematical models [c]	R^2
TPC [a]	EtOH 0%	$27.35 + 4.33x_1 + 4.51x_2 + 8.37x_3$	0.84
	EtOH 30%	$89.06 + 5.70x_1 + 10.78x_2 + 26.20x_3 - 6.64x_3^2$	0.93
	EtOH 50%	$93.55 + 7.60x_1 + 13.08x_2 + 23.58x_3 + 2.20x_3^2 + 9.45x_1x_2 + 3.40x_1x_3 + 6.58x_2x_3$	0.99
	EtOH 70%	$101.26 + 5.51x_1 + 11.54x_2 + 23.29x_3 - 0.10x_1^2 + 3.53x_2x_3$	0.99
	EtOH 90%	$83.37 + 4.25x_1 + 6.33x_2 + 23.54x_3$	0.94
FRAP [b]	EtOH 0%	$0.26 + 0.03x_2 + 0.07x_3 - 0.02x_3^2$	0.96
	EtOH 30%	$0.90 + 0.12x_2 + 0.35x_3 - 0.11x_1^2$	0.94
	EtOH 50%	$1.09 + 0.11x_1 + 0.09x_2 + 0.40x_3 - 0.10x_3^2$	0.94
	EtOH 70%	$1.04 + 0.09\,x_1 + 0.16x_2 + 0.34x_3 - 0.10x_1^2$	0.90
	EtOH 90%	$0.84 + 0.11x_2 + 0.26x_3 - 0.09x_3^2$	0.88

[a] Total Phenolic Content (mg GAE/g PB); [b] Ferric Reducing Antioxidant Power (mmol Fe^{2+}/g PB); [c] x_1: time; x_2: temperature; x_3: solid-liquid ratio. The equations are expressed in terms of coded values (-1, 0, +1).

3.3.3 Phenolic and flavonoid contents of PBE

The spectrophotometric determination of TPC and TFC of aqueous and hydroethanolic extracts from *P. pinaster* bark are presented in **Figure 3**. Moreover, the individual phenolic compounds were identified and quantified using liquid chromatography, and the results are presented in **Table 5**.

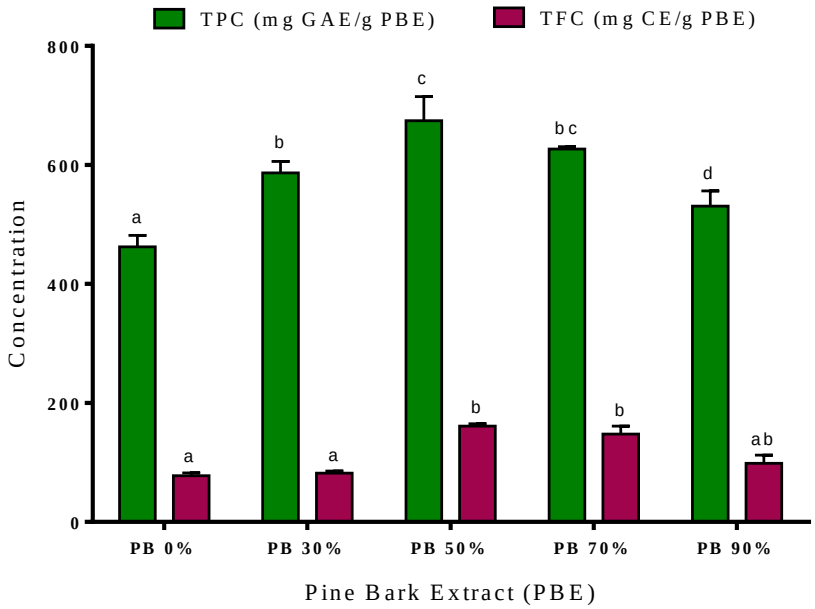


Figure 3. Total phenolic content (TPC) and total flavonoid content (TFC) of aqueous and hydroethanolic extracts from *Pinus pinaster* bark. Values are expressed as mean ± SD of 3–4 experiments; GAE: gallic acid equivalents; CE: catechin equivalents. Different letters show significant differences ($p < 0.05$) between groups for the same experiment.

The use of specific solvents is responsible for the selectivity of the compounds in the extract, and the dissolution of intracellular compounds of the raw material, i.e., plants or by-products (Aryal et al., 2019). Important metabolites with antioxidant properties of natural resources, such as phenolic compounds, are more soluble in polar solvents, due to the presence of a hydroxyl group (Aryal et al., 2019; Rafińska et al., 2019). Therefore, in this work, water and ethanol were used as effective environmentally friendly polar solvents (Chemat et al., 2019).

Our results show that the PB extracts have a high content of phenolic compounds. The phenolic content was determined by the Folin–Ciocalteu method, ranging from 460 to 675 mg of GAE/g PBE. The extracts obtained using 50% and 70% ethanol have higher concentration of TPC (674.5 ± 23 and 626.8 ± 2 mg GAE/g, respectively) when compared to aqueous, 30% and 90% hydroalcoholic extracts (462.5 ± 11, 586.5 ± 11 and 530.8 ± 15 mg GAE/g, respectively). Even so, water extracts present a very significant number of phenolic compounds showing high bioactive or functional potential, and water should be considered as a promising solvent due to its greener character.

The extracts' flavonoid content ranged from 77 to 161 mg CE/g PBE depending on the solvent used in the extraction process. The highest content was observed in the 50% and 70% ethanol extracts (161 ± 3 mg CE/g and 148 ± 9 mg CE/g, respectively), while the extracts obtained with

water, 30% and 90% of ethanol showed the lowest TFC (77.5 ± 4 mg CE/g, 81.9 ± 3 mg CE/g and 98.6 ± 9 mg CE/g, respectively) ($p < 0.05$).

These results are in accordance with those described previously by our research group, where it was observed that *P. pinaster* bark extracts are very rich in phenolic compounds (Ferreira-Santos et al., 2019b). Moreover, Chupin *et al.* (Chupin et al., 2015) use *P. pinaster* bark and reported values from 236 to 306 mg GAE/g extract, depending on the extraction conditions, but the maximum was achieved with 80% ethanol. In another study (Chupin et al., 2013), the values of TPC are between 22–62 mg GAE/g bark, results that are in agreement with those obtained in our study (30.75, 54.0, 68.2, 65.1, 57.9 mg GAE/g bark for H_2O, EtOH 30%, EtOH 50%, EtOH 70%, EtOH 90%, respectively). Also, extracts from other species of pine (Bocalandro et al., 2012; Royer et al., 2013; Sharma et al., 2016) (*Pinus roxburghii*, *P. wallichiana, P. radiata*, *P. gerardiana, P. mariana* and *P. banksania*) showed high content in TPC and TFC. For example, Sharma *et al.* (Sharma et al., 2016) obtained hydroethanolic extracts (90% ethanol) from bark of three different pine species *(Pinus roxburghii, P. wallichiana* and *P. gerardiana)*, and the extracts showed TPC concentrations between 222 to 249 mg GAE/g extract and high TFC content (477–597 mg rutin equivalents/g extract). The study by Royer *et al.* (Royer et al., 2013) demonstrated that aqueous and hydroethanolic extracts (95% ethanol) of Canadian pine species have TPC values between 27 to 346 mg GAE/g extract and 6–39 mg quercetin equivalents/g extract for TFC, values below those obtained in our work for *P. pinaster* bark.

The ratios of 50:50 and 30:70 (*v/v*) water-ethanol were the ones presenting the highest TPC and TFC content. If the ethanol concentration used on the extractive process is higher (90% *v/v*), the extracts contain lower amounts of these secondary metabolites. Other research works also reported higher phenolic compounds extraction when intermediate ethanol concentrations are used as solvents for other extraction matrices (Jesus et al., 2019; Jiménez-Moreno et al., 2019). Jiménez-Moreno and co-workers (Jiménez-Moreno et al., 2019) studied different extraction conditions to obtain grape stem extracts rich in polyphenols, and their results showed that using 50% ethanol the extraction is maximized and the extracts show greater AA. Recently, Tanase *et al.* (Tanase et al., 2019) gathered bibliographic information on the extraction of phenolic compounds from the bark of woody plants and their potential biological activity, showing that several solvents are used to obtain phenolic compounds. In this review, the authors report that the mixture of water with ethanol has been widely used to maximize the extraction of these compounds derived from lignocellulosic matrices, such as the *Pinus* species. This may have to do with the ability of the

solvent to penetrate the cell wall, allowing the phenolic compounds to escape (Jiménez-Moreno et al., 2019).

Chromatographic analysis of extracts was performed to identify and quantify the individual compounds and to evaluate the selectivity of each solvent used in the extraction process over the chemical profile (phenolic composition) of the extract (see **Table 5**).

Table 5. Phenolic compounds identification and quantification in aqueous and hydroethanolic extracts from *Pinus pinaster* bark.

Phenolic Compound (mg/L)	Extracts				
	PB 0%	PB 30%	PB 50%	PB 70%	PB 90%
Hydroxycinnamic acids					
caffeic acid	4.2 ± 0.0 [a]	11.5 ± 0.0 [b]	13.8 ± 0.6 [b]	12.0 ± 0.0 [b]	12.0 ± 0.0 [b]
ferulic acid	9.7 ± 0.7 [a]	23.2 ± 2.4 [b]	24.5 ± 0.1 [b]	21.2 ± 0.1 [b]	21.3 ± 0.2 [b]
cinnamic acid	5.4 ± 0.6 [a]	29.5 ± 0.3 [b]	38.1 ± 1.0 [c]	53.4 ± 2.5 [d]	47.4 ± 0.2 [e]
chlorogenic acid	5.8 ± 1.0 [a]	11.0 ± 0.3 [b]	15.7 ± 0.1 [c]	15.5 ± 0.5 [c]	17.2 ± 0.8 [d]
p-cumaric acid	n.q.	n.q.	n.q.	n.q.	n.q.
Hydroxybenzoic acids					
vanillic acid	3.0 ± 1.0 [a]	8.0 ± 1.0 [b]	9.5 ± 0.5 [c]	10.0 ± 0.0 [c]	10.5 ± 0.5 [c]
gallic acid	n.q.	n.q.	n.q.	n.q.	n.q.
3,4 dihydroxybenzoic acid	29.7 ± 1.3 [a]	35.5 ± 1.0 [b]	36.0 ± 0.3 [b]	47.4 ± 5.1 [c]	64.1 ± 1.7 [d]
ellagic acid	53.6 ± 2.0 [a]	67.2 ± 0.4 [b]	120.6 ± 5.1 [c]	122.6 ± 2.1 [c]	124.4 ± 16.1 [c]
Flavan-3-ols					
catechin	n.d. [a]	105.0 ± 1.0 [b]	133.5 ± 0.9 [c]	135.5 ± 1.0 [c]	133.0 ± 2.5 [c]
gallocatechin	149.3 ± 9.0 [a]	140.3 ± 7.0 [a]	n.d. [b]	n.d. [b]	n.d. [b]
epicatechin	n.q.	n.q.	n.q.	n.q.	n.q.
Flavonoids					
naringenin	n.d. [a]	128.0 ± 19.2 [b]	170.5 ± 18.7 [c]	249.5 ± 11.0 [d]	239.6 ± 4.3 [d]
hesperidin	n.q.	n.q.	n.q.	n.q.	n.q.
quercetin	n.d. [a]	n.d. [a]	10.1 ± 0.1 [b]	8.5 ± 0.5 [b]	10.9 ± 2.5 [b]
apigenin	n.d. [a]	1.9 ± 0.3 [b]	6.2 ± 0.1 [c]	12.4 ± 1.2 [d]	5.1 ± 0.0 [c]
taxifolin	73.1 ± 11.6 [a]	166.4 ± 33.9 [b]	422.9 ± 8.9 [c]	463.2 ± 6.4 [d]	463.9 ± 4.4 [d]
Stilben					
resveratrol	3.8 ± 0.1 [a]	10.9 ± 0.5 [b]	13.5 ± 0.0 [c]	18.9 ± 0.3 [d]	17.5 ± 0.0 [d]
Total	**337.6**	**738.5**	**1014.9**	**1171.1**	**1163.9**

Values of phenolic compounds are expressed as concentration (mg/L) mean ± SD of 3 experiments. n.d.: not detected; n.q.: not quantified. Different letters (a–e) show significant differences ($p < 0.05$) for the same compound.

A total of 18 polyphenol compounds belonging to the groups of acids, flavan-3-ol, flavonoids and stilbens were identified according to their corresponding standards. The extraction with hydroethanolic mixtures resulted in the highest extraction of almost all phenolic compounds, except for the gallocatechin, which was only detected in aqueous and 30% ethanol extracts (149.3 and 140.3 mg/L, respectively).

All extracts contain high concentrations of taxifolin and ellagic acid, which increase as the ethanol concentration increases in the extraction process. Taxifolin was found in concentrations ranging from 73.1 mg/L to 463.9 mg/L (representing 21%–42% of the total phenolic composition) and ellagic acid concentration was 53.6–124.4 mg/L (9%–16% of the total phenolic composition). Narigenin and catechin have only been identified and quantified in hydroethanolic extracts, representing an average of 20% and 13% of the extract composition, respectively. Taxifolin was also one of the main constituents of pine seed extracts from *P. sibirica* (Lantto et al., 2009). Catechin and taxifolin were found in extracts of pine barks from five different species *P. pinea*, *P. sylvestris*, *P. nigra*, *P. parviflora*, and *P. ponderosa* harvested from different locations (Yesil-Celiktas et al., 2009). The authors concluded that higher concentrations of catechin and taxifolin are due to climatic stress conditions like less rainfall, higher temperatures and longer duration of sun exposure. However, taxifolin was not found in *P. nigra*, and *P. sylvestris* needle extracts harvested in Macedonian flora (Karapandzova et al., 2015). Quercetin has been identified in extracts obtained with the highest concentration of ethanol (i.e., 50% to 90% (*v/v*)) having concentration values of 8.5 to 10.9 mg/L. Ferulic acid was found in extracts of spruce bark in higher concentrations (between 23.9 and 28.6 mg/g) than presented here (Talmaciu et al., 2016). Epicatechin, *p*-coumaric acid, gallic acid and hesperidin were identified in all samples, but could not be quantified because they coeluted. Epicatechin, *p*-coumaric acid were found to be the main constituents in the extracts of pine needles (Yen et al., 2008).

Though the phenolic composition of pine extracts may depend on variables like pine species, used solvent, environmentally conditions and geographical location, the detected and determined individual phenolics of PBE are in agreement with data from our previous studies (Ferreira-Santos et al., 2019) and the literature results (Chupin et al., 2015).

Polyphenols are very diverse compounds, with different polarities and chemical characteristics. Their extraction depends on several factors, such as solvent to matrix ratio, type of matrix, extraction method, extraction time, extraction temperature, extraction solvent, among others. The solvents with different polarities proportioned extracts with different amounts or types of

constituents (Sasidharan et al., 2011). More polar solvents are expected to extract more polar molecules (Sasidharan et al., 2011). For example, the extraction of hydrophilic compounds uses polar solvents such as methanol or ethanol, and for extracts, more lipophilic compounds, dichoromethane or mixtures with methanol are used. This affinity for the solvent will determine also the solubility of the different compounds, and may limit the maximum possible extractable amount (Cuevas-Valenzuela et al., 2015). In this context, the results obtained are within the expected. Flavonoids have very poor solubility in water. Even though they may have oxygen atoms for hydrogen bonding with water in side chains, they have a relatively large and complex structure with a high number of carbon atoms which gives them the strong apolar character (Ko et al., 2014). Very poorly soluble in water compounds, such as cathechin, naringenin, taxifolin, quercetin or resveratrol, are almost not detected when water is used as solvent. However, their solubility can be strongly increased in the presence of ethanol, which reflected in the much higher amounts extracted particularly above 50% in ethanol content. However, this increase was not visible for higher amounts of ethanol, in particular when comparing the results from 70% and 90% ethanol. This may indicate that there is a balance to be made between the affinity of the solute towards the solvent and the ability of the solvent to enter in the matrix structure. As referred to above, this last one seems to decrease for very high amounts of ethanol. On the other hand, smaller phenolics such as 3,4-dihydroxybenzoic acid, caffeic acid or ferulic acid have higher (though still poor) solubility in water, with the differences between using just water or hydroethanolic mixtures as a solvent being not so sharp.

This detailed analysis of the chemical composition of PBE obtained with non-toxic solvents allows us to state that these natural extracts are rich in phenolic acids, flavan-3-ols, flavonoids and stilbene compounds, that could be used as high added value products, i.e., a viable alternative for food and nutraceuticals industry applications.

3.3.4 ATR-FTIR spectra analysis

The ATR-FTIR technique was used to obtain information regarding the major functional groups present in the plant extract samples. The spectra of the PB compounds extracted with different solvents are recorded in the 400–4000 cm^{-1} region and are presented in **Figure 4**.

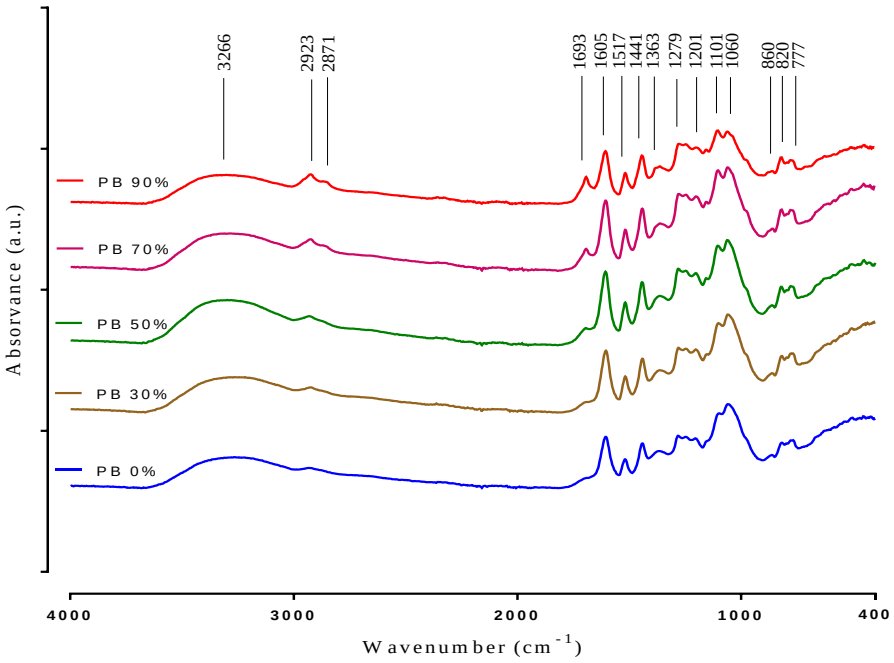


Figure 4. ATR-FTIR spectra of aqueous and hydroethanolic extracts from *Pinus pinaster* bark.

The IR spectra interpretation was performed according to Chupin *et al.* (Chupin et al., 2015), Coates (Coates, 2006) and Ricci *et al.* (Ricci et al., 2015), and clearly shows differences between the PBE´s corroborating the results obtained in the other chemical determinations (*i.e.*, TPC, TFC and UPLC-DAD). The very broad band observed at 3600–3000 cm^{-1} is assigned to the hydroxyl compounds, O–H stretching vibration in phenolic and aliphatic structures. Other characteristic vibration that is observed in this region is the C–H stretch of a terminal alkyne. It exhibits a relatively narrow absorption at 3300 cm^{-1}. Small peaks at 2923 and 2871 cm^{-1} originate from C–H stretch vibration in methyl groups. These peaks are clearly more intense in the extracts when obtained with the highest ethanol concentrations, and could be attributed to hydrophobic phenolic compounds extracted with this conditions. Nevertheless, these peaks may also be related with a higher amount of lipids that may be co-extracted in these conditions, as they seem to increase up to the 90% ethanol solvent (while most phenolics decrease from 70% to 90%).

Absorption in the region 1850–1650 cm^{-1} usually indicates the presence of a C=O group (carbonyl compound). On the other hand, the band identified at 1693 cm^{-1} is attributed to carbonyl stretch and it is characteristic of hydrolysable tannins. It is obvious that this peak is increasing with the increase of ethanol. Compounds that have C=O are better extracted with ethanol. The peaks at 1605 cm^{-1}, 1517 cm^{-1} correspond to aromatic skeleton vibrations and to –CH deformation at 1440 cm^{-1}. Additionally, the peak at 1517 cm^{-1} indicates the presence of non-gallate procyanidins. These

structures identified by the presence of the previous peaks indicate the presence of phenolic compounds in the PBE samples. Therefore, in the spectra relative to the PB extracts 30%, 50% and 70% are presented with a higher intensity than in the spectra of the samples PB 0% and PB 90%, according to the results of TPC and TFC. The band at 1363 cm^{-1} is attributed to phenolic stretch vibration of –OH and aliphatic –CH deformation in methyl groups. The bands located at 1201 cm^{-1} and 1060 cm^{-1} represents the asymmetric and symmetric stretching vibrations of –CO and aromatic –CH bending in plane bending vibrations detected at 1101 cm^{-1} demonstrating aromatic ring deformations and interactions with rings substituents, due to phenols and flavonoids structures. The peaks intensity differences of PBEs at 1101 cm^{-1} and 1060 cm^{-1} can be due to an opening of the cyclic ether structure of phenols. Peaks between 860 to 777 cm^{-1} show the stretching and bending vibrations of –CH from aromatic rings, related to phenolic compounds. This is also supported by the peaks in the range 1600–1500 cm^{-1}, and the locations of the bands are often indicative of the nature of the substitution of the aromatic ring. In simple structures, it is possible to differentiate mono- and di- (ortho, meta and para) substitution. For example, the peak 860 cm^{-1} indicates for para substitution. That is in accordance with the identification of the individual phenolic compounds, as all samples had *p*-coumaric acid.

3.3.5 Antioxidant activity of PBE

The antioxidant capacity of natural molecules and extracts is one of the most studied biological activities, being referenced as a mechanism to prevent the oxidative stress and several diseases. Moreover, the AA of the plant extracts depends on the composition and structure of the biocompounds, such as a phenolic acids and flavonoids and their ability to neutralize ROS and other free radicals, i.e., chelators and free radical scavengers activities (Carocho and Ferreira, 2013).

In the food context, the emergence of new antioxidant sources has been the target of recent research and applications in food protection and nutritional enrichment, promoting the consumption of bioactive compounds.

In this sense, different methods were used for the determination of AA, allowing the analysis of different mechanisms of extracts action. The FRAP method is based on the reduction of an iron complex (Fe^{3+} to Fe^{2+}), and DPPH and ABTS are the most widely used method for determining the free radical scavenging capacity (Fan et al., 2020; Lee et al., 2020).

The results of DPPH, ABTS and FRAP for the PBE are represented in **Table 6**. Our data shows that the extracts obtained with 50% ethanol (PB 50%) have the highest radical scavenging activity (IC_{50} value of 49.74 μg/mL for DPPH and 59.41 ± 2.1 μg/mL for ABTS) ($p < 0.05$). The other extracts also have a high inhibition percentage, obtaining an increase on the IC_{50} values for PB 70%, PB 30%, PB 0% and PB 90%, respectively. This is in accordance with previous statement that ethanol have an important role in the extraction of compounds with higher antioxidant activities (Braga et al., 2008).

The FRAP assay also proved the potent reducing power of PBE, and the results are in agreement with those obtained in the DPPH and ABTS methods. The PB 50% extract shows the greatest reducing power (138.5 mmol Fe^{2+}/g PBE), followed by extracts PB 70% (122.9 mmol Fe^{2+}/g PBE), PB 30% (112.4 mmol Fe^{2+}/g PBE), PB 0% (101.9 mmol Fe^{2+}/g PBE) and lastly the extract PB 90% (101.3 mmol Fe^{2+}/g PBE).

These results show the correlation of the total phenolic and flavonoids content of the bark extracts with the AA measured by the DPPH, ABTS and FRAP assays, demonstrating the contribution of these extracted biocompounds to the AA.

Recently, Gascón and coworkers (Gascón et al., 2018) proved the high AA of phenolic extracts from *P. pinaster* bark using in vitro methods, *i.e.* ABTS, and in the control of cellular ROS in Caco-2 cells. Also, our group reported that extracts obtained from this by-product using different extraction methods have a large antioxidant capacity, determined by the DPPH, FRAP and ABTS methods (Ferreira-Santos et al., 2019). Moreover, the total phenolic compounds of the PBE were highly correlated with their antioxidant power (Ferreira-Santos et al., 2019). Other authors also reported the potential AA of *P. pinaster* extracts. Yesil and co-authors (Yesil-Celiktas et al., 2009) also found a high correlation between the AA of PBE and its TPC, as well as its total amount of constituents. Moreover, the author demonstrated that the DPPH values of PBE of the same species harvested from different locations did not show considerable differences in comparison to the total phenol values. In another study, the authors managed to connect the increment in the AA of extracts with the increment in catechin + epicatechin contents, which were the only phenolic compounds measured (Braga et al., 2008).

Table 6. Antioxidant (DPPH, ABTS and FRAP) and antihyperglycemic (α-amylase and α-glucosidase) and antimicrobial activities of aqueous and hydroethanolic extracts from *Pinus pinaster* bark.

Antioxidant Activity						
Extract	**PB 0%**	**PB 30%**	**PB 50%**	**PB 70%**	**PB 90%**	**Trolox**
DPPH IC_{50} (μg/mL)	99.96 ± 0.1 [a]	73.11 ± 0.0 [b]	49.74 ± 0.1 [c]	55.04 ± 0.1 [c]	100.1 ± 0.1 [a]	10.81 ± 0.1 [d]
ABTS IC_{50} (μg/mL)	106.61 ± 8.0 [a]	89.18 ± 0.9 [b]	59.41 ± 2.1 [c]	65.57 ± 5.0 [c]	112.1 ± 9.5 [a]	23.15 ± 4.0 [d]
FRAP (mmol Fe^{2+}/g PBE)	101.9 ± 0.3 [a]	112.4 ± 1.4 [b]	138.5 ± 4.0 [c]	122.9 ± 4.6 [d]	101.3 ± 1.2 [a]	136.1 ± 1.0 [c]
Antihyperglycemic Activity						
Extract	**PB 0%**	**PB 30%**	**PB 50%**	**PB 70%**	**PB 90%**	**Acarbose**
α-Amylase IC_{50} (μg/mL)	531.5 ± 5.4 [a]	536.4 ± 7.1 [a]	546.3 ± 2.9 [a]	254.2 ± 9.2 [b]	300.3 ± 3.9 [b]	35.42 ± 1.0 [c]
α-Glucosidase IC_{50} (μg/mL)	166.2 ± 1.1 [a]	132.8 ± 10.8 [b]	122.7 ± 11.3 [b]	138.4 ± 7.4 [b]	162.8 ± 3.7 [a]	11000 ± 1.0 [c]
Antimicrobial Activity (expressed in zone of inhibition, mm)						
Extract	**PB 0%**	**PB 30%**	**PB 50%**	**PB 70%**	**PB 90%**	**LX**
A. brasiliensis	n.d.	n.d.	n.d.	n.d.	n.d.	12.5 ± 1.2
S. cerevisiae	n.d.	n.d.	n.d.	n.d.	n.d.	39.6 ± 4.2
C. albicans	n.d.	n.d.	n.d.	n.d.	n.d.	24.8 ± 0.0
C. perfringens	12.7 ± 0.2	13 ± 0.7	13.0 ± 0.2	13.0 ± 0.2	12.7 ± 0.4	18.5 ± 0.8
B. cereus	9.6 ± 0.2	10.2 ± 0.0	9.9 ± 0.1	10.8 ± 0.1	10.3 ± 0.2	12.5 ± 2.6
S. aureus	9.4 ± 0.2	10.1 ± 0.1	9.7 ± 0.2	10.1 ± 0.3	10.2 ± 0.1	12.5 ± 0.5
L. monocytogenes	7.4 ± 0.0	7.8 ± 0.0	7.9 ± 0.6	8.7 ± 0.7	8.3 ± 0.6	9.8 ± 0.3
E. coli	n.d.	n.d.	n.d.	n.d.	n.d.	11.4 ± 0.8
Salmonella Enteritidis	n.d.	n.d.	n.d.	n.d.	n.d.	12.3 ± 1.2

Values are expressed as mean ± SD of 3–4 experiments. LX: commercial solution of sodium hypochlorite; n.d.: not detected. Different letters show significant differences ($p < 0.05$) between groups for the same experiment.

3.3.6 Antihyperglycemic activity

In recent years, compounds of natural origin, such as phenolic compounds, have been extensively studied for their high biological activity. Among the most prominent activities of phenolic compounds are the activity of free radicals' inhibition, antibacterial, anticancer, etc., and more recently the activity of inhibiting digestive enzymes, such as α-amylase and α-glycosidase, involved in the pathological state of hyperglycemia associated with diabetes mellitus. Inhibition of these enzymes blocks carbohydrate hydrolysis and decreases glucose absorption in the body (Chen et al., 2018; Irondi et al., 2017; Maqsood et al., 2019; Sharma et al., 2016).

In this sense, PBEs were studied to find out if they had inhibitory activity of these enzymes, and the results are presented in **Table 6**. The obtained results showed that all of the extracts, regardless of the solvent used in the extraction, have α-amylase (IC_{50} values between 254.2 ± 9.2 to 576.4 ± 2.9 μg/mL) and α-glycosidase (IC_{50} values from 122.74 ± 11.3 to 166.22 ± 1.1 μg/mL) inhibitory activity. It is possible to observe that the extracts obtained with highest concentrations of ethanol (70% and 90% *v/v*) have a higher α-amylase inhibition ($p < 0.05$), presenting lower IC_{50} values. On the other hand, the hydroethanolic extracts obtained with 30%, 50% and 70% ethanol (PB 30%, PB 50% and PB 70%, respectively) showed the greatest inhibition in α-glucosidase activity.

These results indicate that PB extracts have a potent inhibition of digestive enzymes, and may be considered when seeking for alternatives in the prevention or treatment of diabetes.

Both methods used to evaluate the antihyperglycemic activity of the extracts were validated using a commercial inhibitor, acarbose. In the α-amylase assay, acarbose has an IC_{50} of 35.42 ± 1.0 μg/mL, noting that all extracts have less activity than the commercial inhibitor. When activity is evaluated using α-glucosidase, acarbose has IC_{50} values of 11.00 ± 1.0 mg/mL, much higher than those obtained in PB extracts. The authors Irondi (Irondi et al., 2017), Bezerra (Bezerra et al., 2019) and their collaborators, also reported a decrease in the α-amylase and α-glucosidase inhibitory effects using doses of acarbose similar to those presented here.

Schäfer and Högger (Schäfer and Högger, 2007), and Liu and coworkers (Liu et al., 2004) conducted clinical studies on diabetes patients using a commercial aqueous PBE (Picnogenol®, Horphag Research, Geneva, Switzerland), and reported a potent antidiabetic activity of the procyanidin-rich extract. In another work, authors studied the effect of PBE (70% EtOH) on diabetic mice and observed an inhibition effect against salivary α-amylase and yeast α-glucosidase, decreasing carbohydrate absorption (Kim et al., 2005).

These results show that aqueous and hydroethanolic extracts from *P. pinaster* bark have the potential to reduce glucose absorption, and can be used as a food supplement with antidiabetic properties.

3.3.7 Antimicrobial activity

It is known that the aqueous and ethanolic plant extracts may exhibit antimicrobial activity. In this sense, the screening of antibacterial and antifungal activities of PBEs were assessed by the agar well diffusion plate method, by estimating the diameter of zone of inhibition against Gram-positive (*S. aureus, C. perfringens*, *L. monocytogenes and B. cereus*) and Gram-negative (*E. coli*, *Salmonella* Enteritidis) bacteria, two yeasts (*C. albicans* and *S. cerevisiae*) and a fungi, *A. brasiliensis*. These microorganisms were selected due to the well-known causes of these pathogens to foodborne diseases, except *C. albicans* (Deng et al., 2015). The diameter of zone of inhibition by all extracts is presented in **Table 6**.

Generally, Gram-negative bacteria are more resistant than Gram-positive bacteria. Therefore, it was important to evaluate the antimicrobial properties of PBEs obtained under different conditions and the in presence of different of bacteria, yeast and fungi strains.

The results of PBEs demonstrate a potent antibacterial activity against Gram-positive bacteria. At the concentration of 50 mg/mL, the highest inhibition zones were achieved against *C. perfringens*, and the most resistant bacteria was *L. monocytogenes*. These extracts did not show activity for Gram-negative bacteria, nor antifungal activity against *C. albicans*, *S. cerevisiae* and *A. brasiliensis*. It is apparent that the extracts obtained by hydroethanolic extraction have a higher antibacterial activity than the aqueous extracts. This may be due to the higher contents of phenol and flavonoids in these extracts (**Figure 3** and **Table 5**).

While plants serve as rich, natural and safer sources of antimicrobials, the rapid incidences of increased resistance to available antibiotics worldwide have turned the attention of researchers and the pharmaceutical industries to plants in search of viable alternatives.

There are different studies that demonstrates the antimicrobial activity of the phenolic compounds (Daglia, 2012), more specifically, plant bark extracts used as a natural preservatives. In a previous study, different polyphenolic bark extracts from Canadian forest species and a commercial product from PB were tested against two non-pathogenic bacteria strains (*E. coli* and *L. ivanovii*). Their results show that these extracts were more active for Gram-positive bacteria (Royer et al., 2013). These results are consistent with the data obtained in our study.

Natural extracts, such as PBEs, rich in antioxidant bioactive compounds like phenolics (phenolic acids and flavonoids) can be a viable alternative to the serious problem of microbial resistance to antibiotics. In this sense, PBEs are a good option to be considered as a complement to bio-preservation for the food industry, particularly in foods where Gram-positive bacteria are important and common contaminants.

3.3.8 Cell viability

As demonstrated, the PBE has enormous potential to be used for human consumption and as a nutraceutical component. Therefore, the next step is to verify in which concentrations the extract is safe to be used. For that three different cell lines, one normal mouse cell line (L929) and two human cell lines (normal—HEK293T—and derived from cancer tissue—A549) were used. Several authors have used similar cell lines to evaluate the potential toxicity of other plant extracts and isolated products (Delazar et al., 2019; Mao et al., 2017). Different concentrations of active extracts were placed in contact with the cells and their ability to metabolize resazurin into resorufin in the presence of PBE was used as a measurement of cells metabolic activity. A decreased of resazurin conversion indicates impairment of cellular metabolism (Helm et al., 2017), being an indication of the PBE toxicity.

The cells were exposed to different concentrations of each extract for 24 h (0 to 1000 μg/mL). The three cells lines demonstrated a dose-dependent effect. However, the behavior of non-tumor cells was different from the behavior of tumor cells. The presence of low concentrations of all extracts (75 and 125 μg/mL) stimulated their metabolic activity, while, for the same concentration, the tumor cells had a decrease on their metabolic activity. Though the variability of results is high (which is common in these type of tests), this effect is statistically different and this result is in accordance with the literature, where it is described that naturally available extracts of different sources selectively inhibit abnormal cell proliferation without interrupting normally functioning cells (Abraham et al., 2017). Therefore, this finding is the ideal condition for a potential anti-cancer effect, as it would only affect the cancer cells and not the healthy cells. However, this is still a very preliminary indicator (useful in a first potential screening) and further tests at different levels (in vitro, ex vivo and in vivo) are needed to validate (or not).

When analyzing the IC_{50}, it is clear that the action mechanism of the compounds present in the PBE extracts is different for the three cells lines. As it can be seen on **Figure 5** the IC_{50} decreases with the increase of ethanol on the extraction solvent for the mouse cells line (L929) and for the

human cancer cell line (A549), on the hand, there is no clear relationship between the IC_{50} and the solvent extraction composition for the human normal cells line (HEK 293T). As it is known the solvent extraction composition influences the extracted compounds. The highest IC_{50} for the non-tumor cells was observed for the PBE extracted using 30% of ethanol (PB 30%), while for the tumor cell the highest is for aqueous (PB 0%), followed by PB 30%. Interestingly the extract PB 30% does not have the highest content in TPC or TFC, which indicates that the total amount of these compounds is not the main reason the cellular mechanisms observed. Even when comparing with the DPPH, ABTS and FRAP, there is no clear evidence. The extracts PB 0% and PB 90% present similar anti-oxidative activity. Moreover, these extracts showed the lowest anti-oxidative activity comparatively to the rest of the extracts. Therefore, it is not clear which mechanism was responsible for the observed cell behavior. Moreover, the antioxidant effect of PB extracts on the oxidative status of these cancer cell lines should be studied in the future.

When analyzing the composition of the extracts, it is possible to observe that gallocatecchin is only present in PB 0% and PB 30% and quercetin is absent (or undetected), only being detected on the PB 50%, 70% and 90% that induced the highest cytotoxicity (lower IC_{50}), except for the non-tumoral cell line HEK293T. It has been described that gallocathecin can inhibit the tumor cell line HCT-116 growth up to 57%, and that quercetin can also induce selective growth inhibition and apoptosis in hepatic tumor cells, but not in normal cells (Du et al., 2012). Also, the quercetin showed the strongest dose-dependent anti-proliferative activities to colon cancer cells (HT-29) and liver cancer cells (HepG2) (Fan et al., 2020).

Taking in consideration the results obtained, it is hypothesised that the selectivity of the PBE obtained are related to a synergetic effect of several molecules, in particular to gallocathecin and quercetin.

Recently, Gascón and coworkers (Gascón et al., 2018) described the antiproliferative, apoptotic and redox system controlling effects of bark extracts from three pine species, including *Pinus pinaster*, on Caco-2 cells. Touriño and collaborators (Touriño et al., 2005) reported that *P. pinaster* bark extracts have a high AA and can control proliferation in a human melanoma cell line. Additionally, Mao *et al.* (Mao et al., 2017) demonstrated that extracts from the *Pinus massoniana* bark inhibit migration of the lung cancer A549 cell line.

These results demonstrate that the aqueous and hydroethanolic extracts of *P. pinaster* bark at the tested concentrations present low cytotoxicity, and may have the potential to inhibit the tumor cell growth to some extent, though this has to be further validated.

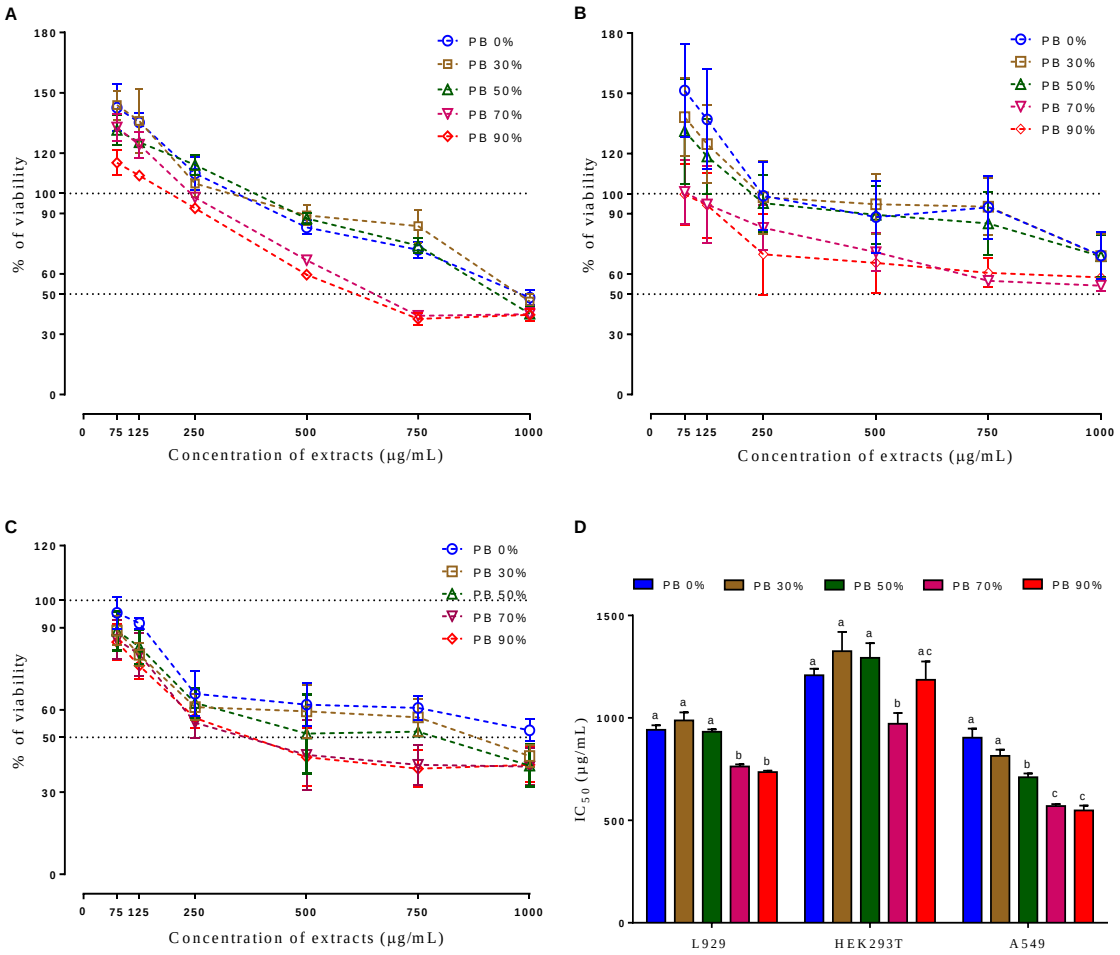


Figure 5. Cellular viability (%) of aqueous and hydroethanolic extracts from *Pinus pinaster* bark against normal mouse fibroblast (L929) (**A**), human embryonic kidney (HEK293T) (**B**), human lung cancer (A549) (**C**) cell lines, and the respective IC_{50} values (**D**). Values are expressed as mean ± SD of 4-5 experiments. Different letters show significant differences ($p < 0.05$) between groups for the same experiment.

3.4 CONCLUSIONS

The present study revealed a wide range of phytochemicals in *P. pinaster* extracts, belonging to different chemical groups: phenolic acids, flavonoids, flavonols and stilbens. Extracts showed different phenolic profiles, depending on the solvent used. However, all demonstrated high potential antioxidant, antidiabetic and antimicrobial activities. The extracts from intermediate ethanol concentrations (50% and 70%) showed the highest bioactivities. These features demonstrate the potential of these extracts to be used in food formulation and processing, either with a technologic function (such as preservative or antioxidant) or as a bioactive ingredient. Moreover, the PBEs have low cytotoxicity, but most importantly, they act selectively on cancer cells, as these are negatively affected and the non-tumor cells are not. These results unravel the potential use of PBE in the medical or nutraceutical sectors.

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CHAPTER IV

MODERATE ELECTRIC FIELDS AS A POTENTIAL TOOL FOR SUSTAINABLE RECOVERY OF PHENOLIC COMPOUNDS FROM *PINUS PINASTER* BARK

This chapter was based on the following paper:

Pedro Ferreira-Santos; Zlatina Genisheva; Ricardo N. Pereira; José A. Teixeira; Cristina M.R. Rocha. **2019.** Moderate electric fields as a potential tool for sustainable recovery of phenolic compounds from *Pinus pinaster* bark. *ACS Sustainable Chem. Eng.*, 7(9), 8816–8826. https://doi.org/10.1021/acssuschemeng.9b00780.

Abstract

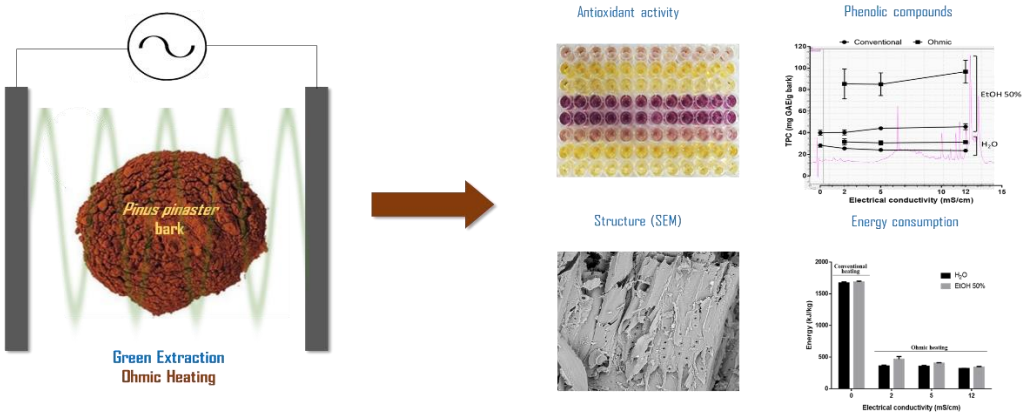
Pinus pinaster (pine) bark is a widely available wood industry by-product but an underexploited source of phenolic compounds with a strong antioxidant activity.

The aim of this study was to evaluate the effectiveness of ohmic heating (OH, a recognized eco-friendly, electric fields-based processing technology) for the sustainable recovery of phenolic compounds from *Pinus pinaster* bark.

Results of OH evidenced increased extraction of antioxidant phenolic compounds with no degradation of pine bark extracts for all tested conditions. Moreover, the enhancement in phenolic content in OH-assisted extraction was particularly evident when using a hydroethanolic solvent with 50 % ethanol, reaching improvements higher than 100 %, when comparing to the conventional approach. This fact may indicate preferential extraction, presumably due to the action of electric field effects acting in synergy with heat and solvents. Furthermore, additional changes in the morphological structure of bark cells were observed upon OH-assisted extraction, suggesting membrane electroporation effects. A significant reduction in energy consumption when compared with the conventional process was observed, leading to an energetically more sustainable approach.

OH technology holds the potential to be a "green" alternative, with higher extraction yields, reduced energy consumption and offering the possibility of tuning the selectivity towards phenolic compounds.

Graphical Abstract



Keywords: *Pinus pinaster* bark; phenolic compounds; conventional thermal extraction; ohmic heating; cells permeability; energy consumption; sustainable extraction

4.1 INTRODUCTION

Pinus pinaster (maritime pine) is considered a plant of the conifer family native to North-Western Africa and some European countries, growing and adapting strongly in the forests of France, Spain and Portugal (Seabra et al., 2012). The Portuguese *P. pinaster* forest reached its highest area in 1995 (978×10^3 ha) and has been reduced since that time, being 714×10^3 ha in 2010. However, this species represents an important part of the total forest (about of 23 %), being the third most important species after eucalyptus (812 × 103 ha, 26 %) and cork oak (737 × 103 ha, 23 %) (ICNF, 2013).

PB is a by-product of the wood industry (sawmills, wood panels, pulp and paper, carpentry, packaging and furniture) representing 10 to 20% of the total pine trunk composition. This waste is usually used for energy production or simply discarded, being only a very low percentage used for high value and industrial applications (pharmaceutical and supplement nutrition; polymers formation and bio-based materials; biopesticides; etc.) (Miranda et al., 2012). Thus, knowing that this lignocellulosic waste has several profitable characteristics, such as low price, long-term stability and important phytochemical constituents, which together make the use of this residue highly attractive for investigations that aiming to improve the value of such material(Fradinho et al., 2002). The transformation of this wastes in high added value products are important for the economy of countries where this species grows abundantly.

Presently, the exclusive commercial product of *P. pinaster* bark (Pycnogenol®), obtained by the application of conventional heating and the use of water as solvent, is the result of the use of this material to generate products of high industrial interest (Rohdewald, 2002). The extracts of PB are highly rich in numerous phenolic compounds such as catechin, taxifolin, procyanidins and phenolic acids. These extracts have been shown to exhibit different modes of action, including antiradical, antioxidant, anti-inflammatory and antiviral properties, and some applications of such extracts are described in the literature (Iravani and Zolfaghari, 2011; Jerez et al., 2007b, 2007a; Maimoona et al., 2011; Rohdewald, 2002).

Traditional extraction techniques (such as maceration and Soxhlet) used to recover polyphenolic compounds from different plant-based matrices are time-consuming and often require high solvent and energy consumptions, thus generating large amounts of waste (Aspé and Fernández, 2011; Rocha et al., 2018). In recent years, greater consciousness of environmental issues has allowed a growing movement towards the use of so-called "green" technologies. An example of this paradigm shift is related to the growing demand for cleaner extraction techniques, such as microwave-

assisted extraction, high pressure extraction and ultrasound-assisted extraction, for the recovery of bioactive compounds (Arshadi et al., 2016; Ghitescu et al., 2015; Mellouk et al., 2016). These new green processes are meant to be more environmentally friendly with shorter extraction times, lesser consumption of organic solvents and energy, while maintaining a high quality of extracts (Chemat et al., 2015; Cvjetko Bubalo et al., 2018; Herrero and Ibañez, 2018; Mellouk et al., 2016). Recently, electrotechnologies such a PEF, HVED and OH have been also applied to the extraction of molecules of interest from plant materials (Pereira et al., 2016a; Rocha et al., 2018). OH is a promising technology, as a process in which heating occurs by the transformation of internal energy from electrical to thermal (Loypimai et al., 2015; Rocha et al., 2018), leading to almost instantaneously and uniform heating. OH can further induce not only thermal but also the electro-permeabilization of the cell membranes and this phenomenon was assumed to be relevant in the experiments conducted on extraction of essential oils and bioactive molecules from different bioresources (Aamir and Jittanit, 2017; Loypimai et al., 2015; Pereira et al., 2016a; Rocha et al., 2018).

In this context, this study was designed to evaluate the aptness of OH-assisted extraction combined with water or water/ethanol mixture as solvent to obtain extracts with high content of phenolic compounds from *P. pinaster* bark. Moreover, studies were made in order to access possible chemical differences of the extracts, as well as morphological changes of the bark cells. Results of OH-assisted extraction were also compared with a conventional heating performed under identical conditions. Effects of electrical conductivity and EF were also addressed to a better understanding of the non-thermal effects of the OH in the extraction process.

The final aim was to achieve a feasible, low-cost and low-environmental impact extraction process. As far as we know, this is the first time that OH-assisted extraction is used to extract phenols from lignocellulosic residues, and its effects on the process yield, selectivity and energetic efficiency are evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Raw material

PB from *P. pinaster* was collected in the North region of Portugal (Ponte de Lima, Viana do Castelo, Portugal) in April 2016. The trees had an approximate age of 15 years. Firstly, the bark was washed with distilled water several times to remove dirt, lichens, and resin. The bark was dried at 40 °C for 48 hours and subsequently milled in a cutting mill (Retsch Mill MM 2000, Retsch,

Haan, Germany) to a granulometry of 1-1.6 mm. Finally, the ground PB was kept in a sealed bags and stored in a dry and dark place until further analysis.

4.2.2 OH equipment

Extractions, were performed in a cylindrical glass reactor, double-walled water-jacketed (3 mm of internal diameter and 100 mm height) with two inox electrodes. The design of reactor was described elsewhere (Pereira et al., 2010). The distance between electrodes was kept constant (3.2 cm). The power source working with a sinusoidal wave at 25 kHz (Agilent 33220A, 1 Hz-25 MHz and 1-10 V; Penang, Malaysia) allowed changing the voltage. Temperature was recorded with a type-K thermocouple (temperature precision of ±1 °C; Omega Engineering, Inc., Stamford, CT, USA), located in the geometric center of the extractor's volume. The thermocouple was connected to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA) and Lab View 7 Express software (National Instruments, NI Data logger) was used to extract the data. A portable oscilloscope (ScopeMeter® 125/S, Fluke, WA, USA) was used to measure electrical frequency, voltage and current intensity during OH treatments.

The reactor was covered properly to avoid the loss of solvent and the direct incidence of light.

4.2.3 Conventional and OH heating extraction conditions

For extractions, 1.5 g of dried PB was mixed with 15 ml of solvent (water or ethanol 50 %) in a reactor (described above) with a magnetic stirrer (size of 0.5 cm) at 150 rpm, introduced inside the reactor vessel to homogenize the solution and improve heat transfer during the heating cycle. For the conventional thermal treatment (0 V/cm), a thermostatic circulator water system (F25-ED, Julabo, Seelbach, Germany) was used to get the same heating rates in all types of treatments. Sodium chloride (NaCl) was selected as a conducting medium due to higher electrical conductivity and prepared to ensure a homogeneous current flow and poured to the processing chamber in a solid-liquid ratio (Nair et al., 2014). Experiments were carried out using different concentrations of NaCl in the extraction medium (i.e from 0 to 0.5 M) that allowed to maintain the values of electrical conductivity comparable between the different types of solvent used in the extraction (water and ethanol), as well as to change for each solvent the EF intensity applied (see **Table 1**). The extractions were made in triplicate and kept at 83 ± 1 °C for 30 minutes (conditions chosen using data shown in chapter III).

4.2.4 Total extractives

Total extractives were determinate in accordance to NREL procedure (Sluiter et al., 2008). For the exhaustive extraction were used 2 g of PB in 100 mL of ethanol 96 % (v/v) during 21 h at 100 °C in an automatic Soxhlet extraction system (Soxtec™ 8000, FOSS, Denmark).

4.2.5 Conductivity and pH

The pH of the solutions and extracts was measured using a pH meter (HANNA Instruments Inc., HI2210, USA), and the conductivity was measured using a conductivity/TDS/Salinity Meter (HANNA Instruments Inc., edge®, HI2003, USA) at 21 ± 1 °C.

4.2.6 Extraction yield

The solvent efficiency in extracting target compounds from a dry material can be measured using the extraction yield. Yield calculation (presented in %) was made using Equation1, considering cumulative mass of extract :

$$\text{Yield} = \frac{\text{extracted solids (g)}}{\text{initial dry material (g)}} \times 100 \qquad (1)$$

4.2.7 Total phenolic compounds determination

The concentration of total phenolic compounds (TPC) was measured using the Folin–Ciocalteu method, which is based on the colorimetric reduction/oxidation reaction of phenols (Singleton et al., 1999). For all analyses, 5 µL of extract (water or ethanol 50 % for control) was mixed with 60 µL Folin–Ciocalteu reagent, 15 µL of Na_2CO_3 (75 g/L). The prepared solution was kept at 60 °C for 5 min. Absorbance was measured at 700 nm by an UV/vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). A calibration curve was prepared using a standard solution of gallic acid. Final values were expressed as milligram gallic acid equivalents (GAE) per gram of dry material (mg GAE/g PB).

4.2.8 Determination of antioxidant activity

Three different methods of measuring the AA was used: DPPH, ABTS and FRAP. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH assay) of extracts from PB was determined as described by Ballesteros *et al.* (Ballesteros et al., 2015). with some modifications.

Four different dilutions of each sample were prepared considering that the percent inhibition had to be between 20% and 80%. The control solution consisted in using solvent of extraction instead of the sample. A calibration curve was prepared with a standard solution of Trolox. The radical scavenging activity was calculated by using the Equation 2.

$$\% \text{ Inhibition} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100 \quad (2)$$

where Ac and As are the absorbance of the control solution and the absorbance of the sample solution, respectively (measured at 515 nm).

DDPH percent inhibition data were plotted as a function of antioxidant concentration to obtain DPPH inhibition concentration at 50% (IC_{50}). The IC_{50} values were expressed as micromoles of Trolox equivalent (TE) per g of dry material (µmol TE/g PB).

The radical cation decolorization (ABTS assay) of extracts was determined as described by Ballesteros *et al.* (Ballesteros et al., 2015) using the ABTS reagent (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt). Each sample was diluted as previously mentioned for the DPPH assay, and the absorvance was measured at 734 nm. Solvent of extraction was used as control solution instead of the sample. A standard solution of Trolox was used to build the calibration curve. The same equation employed in the DPPH radical scavenging was used to calculate the percent inhibition of ABTS radical cation. The IC50 values were expressed as micromoles of TE per gram of dry material (µmol TE/g PB).

FRAP assay was based in the method described by Benzie and Strain(Benzie and Strain, 1996) and modified by Meneses *et al.* (Meneses et al., 2013). The absorbance is determined at 593 nm. An aqueous solution of ferrous sulphate was used to build the calibration curve. FRAP values are expressed as micromoles of ferrous equivalent per g of dry weight material (µmol Fe^{2+}/g PB).

4.2.9 Identification and quantification of phenolic compounds by UPLC-DAD

Samples was analysed by Shimatzu Nexpera X2 UPLC chromatograph equipped with DAD (Shimadzu, SPD-M20A). Separation was performed on a reversed-phase Aquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size; from Waters) and a pre-column of the same material at 40 °C. The flow rate was 0.4 mL/min. HPLC grade solvents water/formic acid 0.1% (A) and acetonitrile (B) were used. The elution gradient for solvent B was as follows: from 0.0 to 5.5 min eluent B at 5%, from 5.5 to 17 min linearly increasing from 5 to 60%, from 17.0 to 18.5 min a linearly increasing from 60 to 100%; lastly, the column is equilibrated at 5% from 18.5 to

30.0 min. A comparison between the UV spectra (at different wavelengths) and the retention times of each standard was used to identify and quantify the phenolic compounds. All analyses were made in triplicate.

4.2.10 Morphological characterization of bark

The PB, treated and untreated form, were characterized using a desktop scanning electron microscope (SEM) (SEM-Phenom ProX, Phenom-World BV, Netherlands), applying an acceleration voltage of 15 kV, at 550 fold magnifications. All SEM pictures were acquired using the ProSuite software (Phenom-World BV, Netherlands).

The samples were added to aluminum pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were imaged without coating. The aluminum pin stub was then placed inside a Phenom Charge Reduction Sample Holder (CHR).

4.2.11 Determination of energy consumption

The electrical energy consumption of the conventional and OH treatments was calculated taking into account the data of voltage (V) and current intensity (A) applied during the treatment time and expressed in kW.h.

4.2.12 Statistical analysis

The extraction and analyses were performed in triplicate and the data are presented as mean ± SD values. GraphPad Prism® software (version 6.0; GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analyses. The ANOVA and the least significant difference test were used to determine statistically different values at a significance level of $p < 0.05$. Principal component analysis (PCA) was used on phenolic compounds to discriminate between extracts. PCA was made using the software XLStat-Pro (Addinsoft, Paris, France, 2011).

4.3 RESULTS AND DISCUSSION

4.3.1 pH and electrical conductivity

All results on pH and electrical conductivity obtained for the solvent before extraction and the extracts of *P. Pinaster* bark after complete extraction using different conditions are summarized in **Table 1**. The results presented show that the electrical conductivity of the solvent is modified using a different concentration of NaCl, as intended, while pH does not show much variation. After

the aqueous and ethanolic extraction the medium containing the extractives show an increase of electrical conductivity ($p < 0.05$), especially in hydroethanolic extracts when higher concentrations of NaCl (0.18 M and 0.50 M) were used. This increase of electrical conductivity noticed after extraction of the compounds from *P. Pinaster* bark can be explained by the leakage of intracellular and extracellular minerals and other conductive compounds from the material to the extraction medium (Rohdewald, 2002). This fact was also observed when electrical technologies (such as PEF´s) were applied to different sources, such as foods, plants, etc (Vorobiev and Lebovka, 2010). The pH of extractives is lower than that of the solvent used for their extraction, and it was observed a tendency of pH decrease when increasing conductivity of the medium confirming the extraction of compounds of acid origin, such as phenolic acids from *P. pinaster* bark. These results may be explained by an ion exchange reaction between the phenolic compounds and Na^+. Sodium cation is exchanged against a H^+ of carboxyl group. This reaction forms a water molecule and phenolic ion, which is more soluble in polar solvents (such as water and ethanol) than the initial form of the polyphenol (Bouras et al., 2016). No significant differences were detected between the conventional and the OH-assisted extractions in terms of final conductivities and pH.

Table 1. Electrical conductivity and pH of solvents and extractives.

	Concentration NaCl (M)	Solvent		Extactives			
				Conventional		Ohmic	
		Conductivity (mS/cm)	pH	Conductivity (mS/cm)	pH	Conductivity (mS/cm)	pH
H_2O	0.00	0.02 ± 0.00	5.79 ± 0.13	0.32 ± 0.01	4.51 ± 0.04	–	–
	0.02	2.04 ± 0.15	5.63 ± 0.12	2.29 ± 0.04	4.22 ± 0.05	2.33 ± 0.00	4.20 ± 0.01
	0.05	4.95 ± 0.00	5.61 ± 0.05	5.50 ± 0.01	4.03 ± 0.01	5.62 ± 0.00	4.03 ± 0.02
	0.15	12.05 ± 0.08	5.68 ± 0.00	12.65 ± 0.13	3.86 ± 0.00	12.53 ± 0.23	3.81 ± 0.01
EtOH 50%	0.00	0.00 ± 0.00	6.10 ± 0.14	0.08 ± 0.01	4.76 ± 0.02	–	–
	0.04	2.01 ± 0.00	5.76 ± 0.38	2.32 ± 0.02	4.18 ± 0.05	2.44 ± 0.09	4.06 ± 0.03
	0.18	4.94 ± 0.06	5.57 ± 0.24	5.98 ± 0.011	3.98 ± 0.01	5.99 ± 0.04	3.93 ± 0.01
	0.50	12.16 ± 0.01	5.75 ± 0.07	14.47 ± 0.04	3.77 ± 0.03	14.42 ± 0.44	3.68 ± 0.04

The solvent (with addition of different concentration of NaCl) before extraction and of the extracts obtained from *Pinus pinaster* bark after conventional or OH-assisted extraction methods using water (H_2O) or ethanol 50% (v/v) (EtOH 50%) as solvents. Values are expressed as mean ± SD of 3 experiments.

4.3.2 Extraction yields

The influence of solvent and electrical conductivity on the extraction yield for the OH-assisted and conventional extraction techniques is shown in **Figure 1**. The total amount of extractible material, and thus the maximum possible yield value of extractive compounds, was determined after exhaustive Soxhlet extraction (ethanol 96% (v/v), 24h at 83 °C), representing 13.2% of total PB composition.

For this by-product, the results clearly indicate that the extraction yields were significantly increased ($P < 0.05$) when the extraction is performed with ethanol 50% (v/v) compared to the aqueous extraction for both methods of extraction (conventional and OH), which was expected. It is known that most phenolic compounds have higher affinity towards ethanol than water. When extraction is performed with mixtures of organic solvents (such as ethanol or methanol) and water the process is usually more cost-effective in recovering these compounds. The results achieved for the conventional heating treatment with no salt are similar to those referred in literature. For instance, Fradinho and co-workers (Fradinho et al., 2002) report that the extractives yield of *P. pinaster* bark was of 10.3% for ethanol extractives and 3.2% for water extractives obtained by Soxhlet extraction. In another study using super-critical CO_2 extraction or high pressure CO_2 and ethanol mixtures (ethanol content ranging from 30% to 70% (v/v)), the extraction yield was of approximately 4.0 %, results much lower than that obtained in this work for ethanolic extracts (Seabra et al., 2012). A recent study using microwave assisted extraction and different conditions for extraction of PB compounds reports that extraction yields of approximately 9.24% (Chupin et al., 2015). Besides the extraction methods, these differences are probably also due to a number of other factors, such as PB age, growing conditions or extraction conditions. Nevertheless, none of the applied reported unconventional methods (high pressure CO_2 or microwave), though efficient, seems to have significantly improved extraction yields.

Our results also indicate that the OH-assisted extraction provides a yield very closed to the maximum possible yield using moderate extraction conditions (ethanol 50% (v/v), 30 min at 83 °C), being able to extract almost 100% of the extractable material. When comparing OH-assisted extraction with conventional heating, it can be observed that in both studied solvents the extraction yield was significantly higher ($p < 0.05$) in the OH extraction independently of the tested electrical conductivity. The maximum increase of extraction yield was 17% in aqueous extraction and 30% in hydroethanolic extraction when compared to conventional heating. This increase in the extraction yield may be related with the cell wall integrity, which could be weaken by the simultaneous effect

of internal temperature increase (Joule effect) and permeation due to electric fields, resulting in a higher contact of the solvent and sample (Moreira et al., 2017).

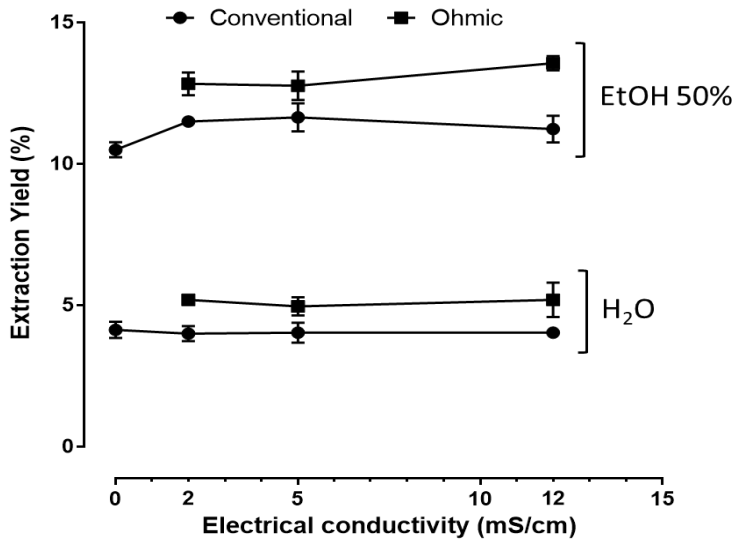


Figure 1. Extraction yields (%), influence of solvent and electrical conductivity using conventional heating and OH-assisted extraction methods in *Pinus pinaster* bark. Values are expressed as mean ± SD of 3 experiments.

4.3.3 Total phenolic content

The TPC from *P. pinaster* bark at different conditions of extraction are presented in **Figure 2**. The trends observed are similar to those obtained for the extraction yield. The highest amounts of total phenols are achieved using 50% ethanolic solution, presenting significantly higher amounts ($p < 0.05$) than with water for both techniques studied and without significant influence of electrical conductivity. It is proved that an increase in the concentration of ethanol accelerated the mass transfer between solvent medium and material, and improved the solubility of the polar phenolic compounds. The findings obtained from our study are in agreement with previous studies, which reported that adding water to the alcohol shows synergistic effect, increasing in the extraction yield of phenolic compounds from plant material (Seabra et al., 2012). This phenomenon can be explained by the action and properties of the solvents used in the process. Water causes structural changes in the sample, acting as a swelling agent, facilitating the process of transferring solvent into the bark and compounds from the bark to the extraction medium. In addition, the solvent inside the bark causes the disruption of bonds between the solutes and the sample (Bouras et al., 2015; Ghitescu et al., 2015). Furthermore, the high dielectric constant of water is a very important

parameter in the extraction process particularly in specific extraction technologies such a microwave, US and also OH (Moreira et al., 2017).

A recent investigation using microwave technology and ethanol 80% (v/v) as solvent yielded 28.3 mg GAE/g bark of phenolic compounds (Chupin et al., 2015). This result is comparable to the phenolic content obtained in the present work by the extraction with water for 30 min at 83 °C, and are in agreement with the results obtained for the total extraction yield.

In the case of TPC, our results demonstrate that OH is markedly more efficient in extracting phenolic compounds when compared to conventional heating. This is more obvious for hydroethanolic extracts where OH allowed to almost double the TPC ($p < 0.001$) in comparison with the TPC achieved with the conventional thermal extraction (89.00 mg GAE/g bark and 42.55 mg GAE/g bark, respectively). This increase in the extraction of phenolics from PB using OH technology is also observed using water as solvent (31.21 mg GAE/g bark), compared to the conventional extraction technique, 25.38 mg GAE/g bark ($P < 0.05$), though the differences are not so pronounced. This improvement of extraction with OH treatment is usually ascribed to an electropermeabilization mechanism which facilitates intracellular compounds extraction (Aamir and Jittanit, 2017; Lebovka et al., 2005; Loypimai et al., 2015; Rocha et al., 2018). However, differences in the solvent nature seem to have a major role in the OH-based extraction efficiency. In fact, though the improvement in extraction yield caused by OH is proportionally similar when water or 50% ethanol are used, differences in TPC are much higher for hydroethanolic extracts, showing that the OH process is much more selective towards phenolic compounds when the mixture water/ethanol is used. This different extraction behavior may be due to changes in the solvent properties (e.g. in the dielectric constant) caused by the presence of the electric field. In fact, the EF maybe causing a screening effect of the available charges, leading to higher affinity between the solvent and phenolic compounds and thus increasing their preferential extraction.

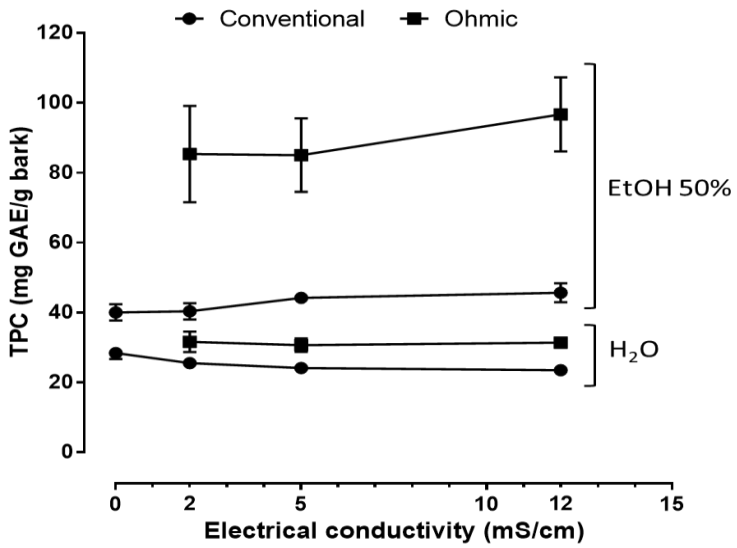


Figure 2. Total phenolic content (TPC) from *Pinus pinaster* bark. Influence of solvent and electrical conductivity using conventional heating and OH-assisted extraction methods. Values are expressed as mean ± SD of 3 experiments; GAE: gallic acid equivalents.

4.3.4 Antioxidant activity

The AA of all extracts were evaluated and results are shown in **Table 2**.

Antioxidants are known to be beneficial for the human health by diminishing the oxidative stress of the body. In general, the bioactive properties (as antioxidant) of the natural resources, in our case of pine bark, may differ from plant to plant due to a number of environmental factors such as the level of maturity, the growing conditions (location, soil status, climate, agricultural practices) and also the conditions of storage of post-harvest material (temperature, time, humidity, presence of contaminants, etc.) (Hoon et al., 2015).

The different methods used for determination of AA allows to evaluate different mechanisms of action of extracts. The FRAP method is based on the reduction of a Fe^{3+} complex to Fe^{2+}, confirming the presence of reducing antioxidants. DPPH is the simplest and most widely used method for determining the free radical scavenging capacity. The ABTS assay is based on interaction between the antioxidant and ABTS cation radical ($ABTS^{\cdot+}$), that, in the presence of hydrogen donating antioxidant, the $ABTS^{\cdot+}$ nitrogen atom quenches the hydrogen atom, causing the solution decolorization. This is a stable method and applicable to hydrophilic and lipophilic antioxidant compounds (Alam et al., 2013; Barros et al., 2010).

In current work, and in all the methods of antioxidant activities studied, the results of AA were always higher in the hydroethanolic extracts comparing with aqueous extracts ($p < 0.05$), as

expected both from literature and from the yield and TPC results. Moreover, antioxidant activities of extracts made with OH method, independently of the solvent used were always higher than the extracts obtained by conventional extraction. Extracts made with the same solvent and method, but with different electrical conductivity had similar antioxidant values. Albeit this may be a critical parameter in OH processes, as it will dictate the rate of heating, it has no relevant influence on the final extraction yields, nor does it alter the quality of the extracts, at least in the case of PBE in the experimental range tested. Extractives made with ethanol 50% (v/v) using OH had the highest antioxidant activities, and showed antioxidant activities that in some cases were almost double of those of extracts obtained with the conventional method ($p < 0.01$). For example: for extracts made with 50 % ethanol and electrical conductivity of 5 mS/cm the AA of the conventional and OH methods using FRAP were 1248.78 µmol Fe^{2+}/g PB and 2158.59 µmol Fe^{2+}/g PB, respectively; for DPPH method the measured activity was 165.09 µmol TE/g PB and 237.27 µmol TE/g PB, respectively; and for the ABTS method the antioxidant values were 394.28 µmol TE/g PB and 807.73 µmol TE/g PB, respectively. As mentioned previously the increase of the AA is more visible when OH and hydroethanolic solvents are used. Increased activity determined by DPPH was more pronounced in the aqueous extracts (up to 72%), compared with FRAP and ABTS methods (up to 24% and 30%, respectively). The increased activities in the hydroethanolic extracts measured with the different methods were as follows: up to 43% for DPPH, up to 73% for FRAP and up to 123% for ABTS. According to the results in the present work, significant correlations can be found between TPC and antioxidant activities of the obtained extracts. This is of a great importance for the industry, since the extracts of these by-products are finding increasing applications as bioactive substances for example in food, chemical and pharmaceutical formulations.

This claim is supported by other researchers who previously demonstrated that extracts of the *Pinus pinaster* bark have a high AA (Braga et al., 2008; Chupin et al., 2015; Maimoona et al., 2011; Mellouk et al., 2016; Seabra et al., 2012).

Table 2. Antioxidant activity of the extracts obtained from *Pinus pinaster* bark measured by different methods (FRAP, DPPH and ABTS).

	Electrical Conductivity (mS/cm)	FRAP (µmol Fe^{2+}/g PB)		DPPH (µmol TE/g PB)		ABTS (µmol TE/g PB)	
		Conventional	Ohmic	Conventional	Ohmic	Conventional	Ohmic
H_2O	0	531.52 ± 12.94	–	163.84 ± 1.32	–	118.41 ± 2.43	–
	2	484.76 ± 9.19	603.19 ± 61.34	119.48 ± 7.12	199.14 ± 6.54	120.01 ± 11.66	155.88 ± 28.10
	5	497.54 ± 7.75	611.70 ±25.97	144.11 ± 21.20	196.40 ± 8.25	119.65 ± 5.25	136.86 ± 4.73
	12	473.93 ± 8.74	559.57 ± 14.86	113.29 ± 2.15	195.00 ± 16.50	110.60 ± 5.31	139.90 ± 11.45
EtOH 50%	0	1178.78 ± 70.38	–	195.14 ± 41.53	–	425.21 ± 4.69	–
	2	1320.07 ± 20.57	2011.56 ± 191.36	175.17 ± 7.69	214.94 ± 15.80	444.44 ± 45.79	918.49 ± 36.13
	5	1248.78 ± 124.55	2158.59 ± 236.95	165.09 ± 7.22	237.27 ± 38.99	394.28 ± 23.58	807.73 ± 115.40
	12	1286.55 ± 96.17	2047.48 ± 279.18	165.07 ± 6.77	235.45 ± 26.55	443.40 ± 20.40	989.75 ± 94.49

Values of phenolic compounds are expressed as mean ± SD of 3 experiments. The extracts were obtained at different electrical conductivities using two extraction methods (conventional and ohmic heating) and with two solvents: water (H2O) or ethanol 50% (EtOH 50%).

4.3.5 Identification and quantification of phenolic compounds

In total, 18 phenolic compounds were tested in the extracts of *P. pinaster* bark by UPLC (**Table 3**). The study of the individual phenolic compounds was meant to understand the action of the extraction technology and type of solvent over the chemical profile of the extracts. As far as we know there is little work done on the evaluation of extracts of *P. pinaster* by liquid chromatography. In this context the results presented here are also useful in terms of conventional extraction, bringing more information on the phenolic profile of extracts of *P. pinaster*.

In terms of the effect of the salt concentration (and thus, the ionic strength and electrical conductivity) and as previously observed, there are no relevant differences in the concentration of phenolic compounds between extracts made by the same method and using the same solvent. The compounds with the highest concentrations in all samples were ellagic acid and taxifolin. Ellagic acid was found in concentrations between 179.6 mg/L - 402.2 mg/L that accounts between 24.0% and 49.7% of the total phenolic compounds. Taxifolin was found in concentrations between 93.4 mg/L - 470.2 mg/L, values that accounted between 15.5% and 35.1% of the total phenolic compounds. Extracts from *P. pinaster* obtained with boiling water had 10.2 mg/L of catechin and 12.8 mg/L of taxifolin (Gascón et al., 2018). These concentrations are three to ten times lower than the ones obtained in the present work for aqueous extracts.

Epicatechin and *p*-coumaric acid were identified in all samples. However, it was not possible to quantify these compounds, as they co-eluted. Vanilic acid was only found in extracts made with conventional method and using hydroethanolic solution as solvent. The compounds apigenin, resveratrol and rosmarinic acid were found only in extracts made with 50% ethanol. This can be explained by the nature of these phenols, once they are poorly soluble/extracted in water in normal conditions (such as pressure). In terms of the effect of moderate electric fields, extracts made with OH and hydroethanolic solvent had the highest total concentrations of phenolic compounds (between 1430.1 mg/L and 1565.1 mg/L). This result is in accordance with the results obtained for the yields, TPC and antioxidant activities of the obtained extracts. Antioxidant activities of extracts increased in the way the total concentration of phenols increased. Extracts made with conventional and OH with water are similar in terms of concentration of individual phenol compounds, while extracts made with conventional and OH with 50% ethanol, were different from the ones made with water, as well as different from each other. Some individual compounds (such as caffeic acid, gallic acid, *o*-coumaric acid, ferrulic acid, narginin, apigenin, rosmarinic acid, taxifolin and quercetin) had concentrations in the extracts made with OH and hydroethanolic solvent

which were almost twice as higher as in the correspondent extracts obtained through conventional heating. This is well notorious for quercetin and taxifolin (dihydroquercetin). Both flavonoids have been described as good radical scavengers, and have demonstrated a wide range of benefits, such as anticarcinogenic, antioxidant and antidiabetic activities, among many others, increasing the potential of application in food, chemical and pharmacological areas (Akinmoladun et al., 2018; Baranowska et al., 2018; Guo et al., 2015; Iravani and Zolfaghari, 2011; Patil and Masand, 2019; Sun et al., 2014). Thus, the conditions and technologies studied in this work may be advantageous for the recovery and reuse of this type of molecules and may be interesting at the industrial level. Furthermore, the results also indicate that the process can be tuned for increased performance in terms of selective extraction of bioactive phenolic compounds.

Table 3. Phenolic compounds identification and quantification from *Pinus pinaster* bark extracts by UPLC-DAD.

Extraction method	**Conventional**								**Ohmic**					
Solvent	H_2O				EtOH 50%				H_2O			EtOH 50%		
EC (mS/cm)	0	2	5	12	0	2	5	12	2	5	12	2	5	12
Catechin	28.3 ± 14.4	35.7 ± 9.5	31.6 ± 5.5	33.2 ± 9.1	97.0 ± 4.9	101.3 ± 4.9	100.6 ± 39.6	116.3 ± 5.5	37.9 ± 12.0	40.2 ± 7.9	34.6 ± 12.8	67.3 ± 14.0	106.3 ± 28.4	117.0 ± 8.0
Epicatechin	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Gallocatechin	11.4 ± 2.4	11.5 ± 6.5	15.0 ± 6.2	7.0 ± 0.9	15.3 ± 2.9	19.6 ± 5.3	17.1 ± 1.4	16.9 ± 4.5	13.1 ± 6.3	12.1 ± 2.8	9.5 ± 1.8	17.8 ± 4.0	17.1 ± 3.2	16.8 ± 4.9
Taxifolin	103.4 ± 16.8	114.0 ± 16.0	117.4 ± 6.4	93.5 ± 12.3	253.5 ± 28.4	320.1 ± 30.7	360.3 ± 39.7	349.7 ± 22.5	109.9 ± 11.7	116.4 ± 5.4	121.1 ± 6.2	448.5 ± 25.7	470.2 ± 26.9	447.7 ± 32.5
Quercetin	29.8 ± 2.2	28.1 ± 0.9	29.0 ± 1.5	27.6 ± 0.6	49.2 ± 0.4	49.7 ± 0.4	49.2 ± 2.5	50.7 ± 2.8	32.0 ± 5.7	34.9 ± 6.0	28.5 ± 3.0	104.2 ± 2.2	104.3 ± 2.5	105.5 ± 2.7
3,4 hydroxybenzoic acid	10.6 ± 1.1	10.0 ± 1.2	10.3 ± 0.7	8.0 ± 1.0	10.4 ± 2.1	9.5 ± 0.3	9.7 ± 1.5	11.2 ± 1.3	10.7 ± 0.6	11.1 ± 0.5	10.2 ± 0.9	14.0 ± 3.9	13.8 ± 3.6	17.3 ± 2.4
Gallic acid	n.d.	n.d.	n.d.	n.d.	2.6 ± 0.4	0.3 ± 0.0	2.2 ± 1.4	2.6 ± 0.1	n.d.	n.d.	n.d.	4.7 ± 0.6	4.1 ± 0.7	3.6 ± 0.7
Vanilic acid	n.d.	n.d.	n.d.	n.d.	5.8 ± 0.1	5.7 ± 0.4	6.3 ± 1.4	6.2 ± 0.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeic acid	6.2 ± 0.1	6.8 ± 0.4	6.8 ± 0.4	6.4 ± 0.1	10.9 ± 0.2	11.9 ± 0.4	11.4 ± 0.4	11.2 ± 0.4	6.4 ± 0.2	6.6 ± 0.4	6.4 ± 0.1	21.4 ± 0.4	20.8 ± 0.7	20.6 ± 1.1
p-coumaric acid	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
o -coumaric acid	33.5 ± 6.4	29.5 ± 1.4	31.4 ± 1.1	29.7 ± 1.9	25.0 ± 6.6	26.0 ± 6.6	44.6 ± 9.7	34.8 ± 8.4	30.5 ± 0.9	30.9 ± 3.2	30.5 ± 4.3	63.2 ± 33.4	63.1 ± 18.5	47.5 ± 25.3
Ferrulic acid	14.9 ± 0.3	15.0 ± 0.5	14.9 ± 0.1	14.5 ± 0.4	27.0 ± 1.5	29.4 ± 0.8	27.5 ± 0,9	26.8 ± 0.1	15.3 ± 0.4	14.9 ± 0.1	14.8 ± 0.3	48.3 ± 0.6	46.7 ± 0.5	47.2 ± 0.8
Rosmaniric acid	n.d.	n.d.	n.d.	n.d.	37.9 ± 2.7	39.0 ± 4.0	39.3 ± 2.2	37.1 ± 4.1	n.d.	n.d.	n.d.	75.4 ± 2.9	74.0 ± 6.1	72.5 ± 4.0
Ellagic acid	332.3 ± 157.3	189.9 ± 49.0	324.4 ± 94.7	123.7 ± 33.4	263.0 ± 55.4	234.1 ± 31.1	231.1 ± 31.7	317.3 ± 18.1	179.6 ± 9.4	259.5 ± 105.3	342.2 ± 61.2	263.4 ± 63.0	384.9 ± 33.6	402.2 ± 51.4
Narginin	97.8 ± 14.1	89.0 ± 3.0	93.1 ± 2.4	89.4 ± 4.1	67.1 ± 6.2	73.2 ± 7.6	78.9 ± 11.7	82.5 ± 16.3	91.2 ± 2.0	92.0 ± 7.1	91.1 ± 9.5	208.0 ± 73.3	207.6 ± 40.7	173.4 ± 55.5
Hisperidin	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Apigenin	n.d.	n.d.	n.d.	n.d.	28.3 ± 0.6	28.6 ± 0.2	28.4 ± 0.7	27.8 ± 0.2	n.d.	n.d.	n.d.	54.2 ± 0.1	54.0 ± 0.1	53.9 ± 0.1
Resveratrol	n.d.	n.d.	n.d.	n.d.	23.0 ± 5.6	24.5 ± 8.8	19.6 ± 0.6	19.5 ± 0.4	n.d.	n.d.	n.d.	39.7 ± 0.5	39.4 ± 1.2	40.0 ± 0.4
Total	**668.4**	**529.5**	**673.6**	**432.9**	**916.1**	**972.9**	**1026.1**	**1110.5**	**526.6**	**618.6**	**688.9**	**1430.1**	**1606.3**	**1565.1**

Values of phenolic compounds are expressed as concentration (mg/L) mean ± SD of 3 experiments. The extracts were obtained at different electrical conductivities (EC), using two extraction methods (conventional and ohmic heating) and with two solvents: water (H2O) or ethanol 50% (v/v) (EtOH 50%).
n.d.: not detected; n.q.: not quantified.

PCA was performed on the phenolic compounds to visualize the differentiation of extracts on the basis of the type of solvent and the type of extraction methods used (**Figure 3**).

The first two principal components (PC1 and PC2) accounted for 81.33% of the total variance, 60.46% and 20.87%, respectively. PCA analysis demonstrated good discrimination between samples. The PCA shows distribution of samples according to the type of solvent used for extraction, ethanol or water. All extracts made with water were similar (C_{H2O} and OH_{H2O}). Hydroethanolic extracts made with conventional method (C_{EtOH}) and with OH (OH_{EtOH}) were distinguished between each other, as well as between the extracts made with water. Conventional extraction in hydroethanol is responsible for the variance in PC2, while OH extraction in ethanol for the variance in PC2. This may give an indication of a synergistic effect when OH and ethanol are combined in the extraction process. All phenolic compounds were distributed along at the positive loading of the PC1 and positive and negative loading of PC2. By the PCA distribution it is possible to conclude that OH with ethanol favors the extraction of compounds such as quercetin, narginin and ellagic acid, but in particular the acids 3,4 hydroxybenzoic and *o*-coumaric.

It also should be taken into consideration that distribution in positive and negative loading of PC2 can be related to the different levels of electrical conductivity and EF intensity; for example, OH extraction in ethanol 50% (v/v) of catechin seems to be at favored at 12 mS/cm, while gallic acid is better extracted at 2 mS/cm. But further studies will be necessary to evaluate EF effects on the mechanism of extraction or even degradation of individual phenolic compounds.

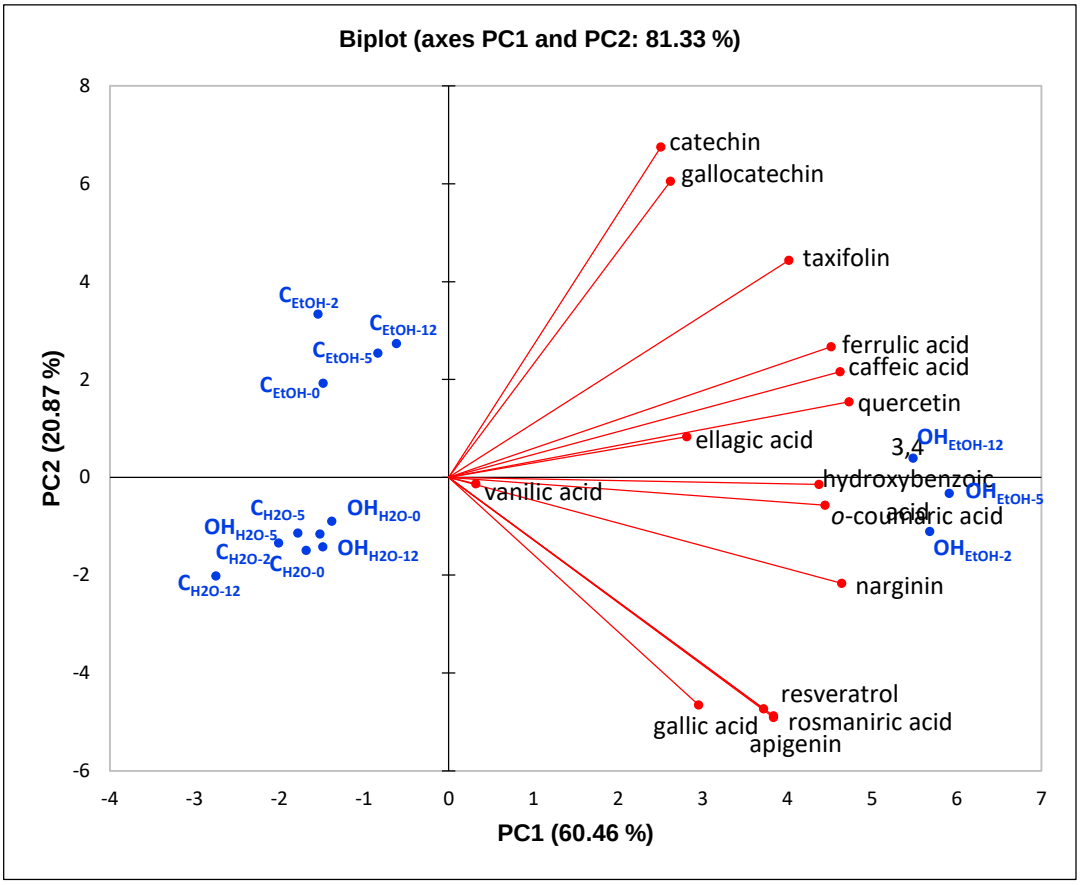


Figure 3. Principle Component Analysis (PCA) on the obtained extracts from *Pinus pinaster* bark. C_{EtOH} – conventional heating with ethanol 50% (v/v) at different conductivities 0, 2, 5 and 12 mS/cm. C_{H2O} – conventional heating with water at different conductivities 0, 2, 5 and 12 mS/cm. OH_{EtOH} – ohmic heating with ethanol 50% (v/v) at different conductivities 2, 5 and 12 mS/cm. OH_{H2O} – ohmic heating with water at different conductivities 2, 5 and 12 mS/cm.

4.3.6 SEM analysis of bark morphology

The changes in the structure of the untreated, conventional heating and OH treated samples of *P. pinaster* bark were imaged by the scanning electron microscope (SEM) (**Figure 4**).

The solvent and extraction method clearly influences the morphology of the PB cells. A visible appearance of pores in the cell walls can be observed in **Figure 4C** and **4E**, which can facilitate extraction by contributing to an increase in the cell membrane's permeability, thus confirming the previous results (increase in extraction yield and quantity of total and individual phenols in extracts obtained by 50% ethanol). In addition, results also demonstrate that action of OH in hydroethanolic solution (**Figure 4E**) contributes to more significant changes in the morphological structure of the *P. pinaster* bark cells, also in accordance with the results described above. In this sense, together with thermal and solvent-driven morphological changes typical from solid-liquid solvent extraction, the eventual existence of an additional effect of electroporation should not be overlooked.

Furthermore, different solvent behavior in the presence of OH indicates that the solvent's solvation and/or extraction properties are altered by the presence of an EF and that this electric-driven effect is different from solvent to solvent.

The bark, leaves and flowers are usually the plant parts richest in bioactive molecules and a rich source of phenolic compounds. These have several beneficial functions, such as protecting the plant from other agents (fungi, parasites, etc.) and promoting plant growth. Several studies have actually shown that bioactive compounds are present in the cellular skeleton of different species, located in different morphological parts of the tissues, both extracellular and intracellular (Koch and Kleist, 2001; Koch and Schmitt, 2013). Moreover, many phenolic compounds are present inside the cell (in the lumens of vessels) and other parenchyma cells and fibers (Koch and Schmitt, 2013; Li et al., 2012). In these cases, the extraction processes usually have to be more aggressive to cause rupture of the cell wall. Therefore the use of temperature and organic solvents becomes necessary.

The results confirm what was expected. When OH is applied, an electroporation effect is expected to create pores in the membrane, helping extracting the compounds from the intracellular medium of the plant cell´s (El Darra et al., 2013; Pereira et al., 2016a; Rocha et al., 2018). In this case, the electrical effect can enhance the cellular permeabilization. Comparing the OH methodology with techniques where high frequencies were applied (such MW) for extraction of intracelular bioactive compounds, the permeabilization of cells are more effective in case of OH (Aspé and Fernández, 2011).

Furthermore, the OH efficiency depends strongly on the dielectric constant of the solvent combination. In our case, the addition of water to the extraction medium leads to an increase in the polarity of the ethanol (or of the organic solvents in general), promoting a greater diffusion of the electric energy inside the sample, simultaneously provoking an increase in temperature. The combination of these factors (electrical and thermal) leads to cellular permeabilization and an easier release of phenolic compounds (Bouras et al., 2016; Lebovka et al., 2005). The application of electric fields in the extraction process of vegetal material usually promotes an increase in the permeability of the cellular wall. This phenomenon occurs because the cell membrane had a specific dielectric strength that has been exceeded by the EF exerted by the OH process, causing an increase in the electroporation mechanism (Lebovka et al., 2005; Nair et al., 2014; Vorobiev and Lebovka, 2010). The electric energy applied between the electrodes in OH was responsible for the breakdown of the PB cell membranes, which enabled the extraction in a much easier way when

compared to the conventional extraction. Despite these promising results, more fundamental studies will be necessary to confirm extraction pathways in this kind of material under OH conditions, which can combine enhanced thermal permeabilization but also electroporation effects, or even combination of both.

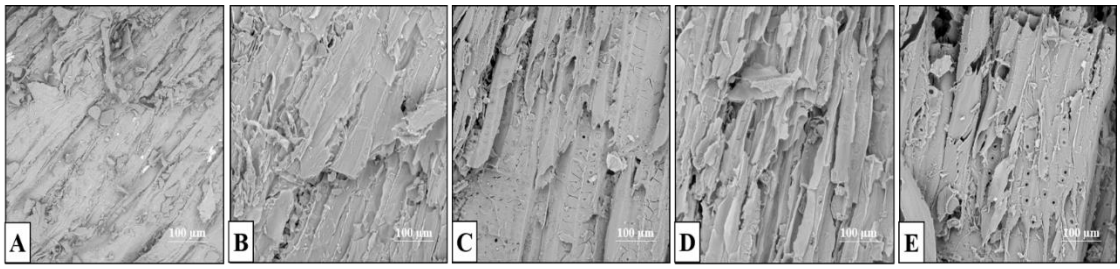


Figure 4. Micrographs by scanning electron microscopy of untreated (**A**), conventional heating (**B**,**C**) and ohmic heating (**D**, **E**) treatements of *Pinus pinaster* bark. **B** and **D** represent the bark after water extraction; **C** and **E** represent the bark after ethanol 50% (v/v) extraction. The material analyzed comes from the residue subjected to extraction with a conductivity of 5 mS/cm, except sample **A**.

4.3.7 Determination of energy consumption

Figure 5 shows total energy consumption of conventional and OH-assisted extraction for the two solvents applied in this work. For this study, the EF strength applied to maintain the temperature of 83 °C during the extraction process was 0 V/cm in the case of conventional heating, and 5 V/cm, 10 V/cm and 15 V/cm for conductivities of 12, 5 and 2 mS/cm, respectively, when applied OH technology. In the case of conventional thermal extraction, the conductivity did not affect the energy consumption, and for calculate the energy supplied during the process, the time and intensity at which the heating source was operated (which was the same for both solvents) was taken into account. As expected, conventional heating resulted in the highest energy consumption (approximately 0.5 kW.h). Despite OH being a thermal process, levels of energy input were significantly lower ($p < 0.001$) when compared with conventional heating for both solvents using the same thermal profile (i.e. heating rate, temperature and treatment time). In case of hydroethanolic extraction the energy consumption of OH decreased with increasing electrical conductivity of extraction medium (and respective decrease of EF applied).

In other studies, the microwave heating thecnology showed relatively low efficiency in comparisson to OH (this study), whereas the magnetron source of MW is typically 67% efficient. If one includes transmission losses, the efficiency becomes closer to 50%. By contrast, OH is over 90% efficient (Mellouk et al., 2016; Pereira and Vicente, 2010).

At an industrial scale, the conventional thermal extraction process uses more energy efficient methodologies, not having as sharp expenditures as those observed in laboratory scale. Nevertheless, OH technology is a process with the potential to reduce energy consumption and associated costs even at a larger scale, wherefore, less environment impact (Pereira and Vicente, 2010).

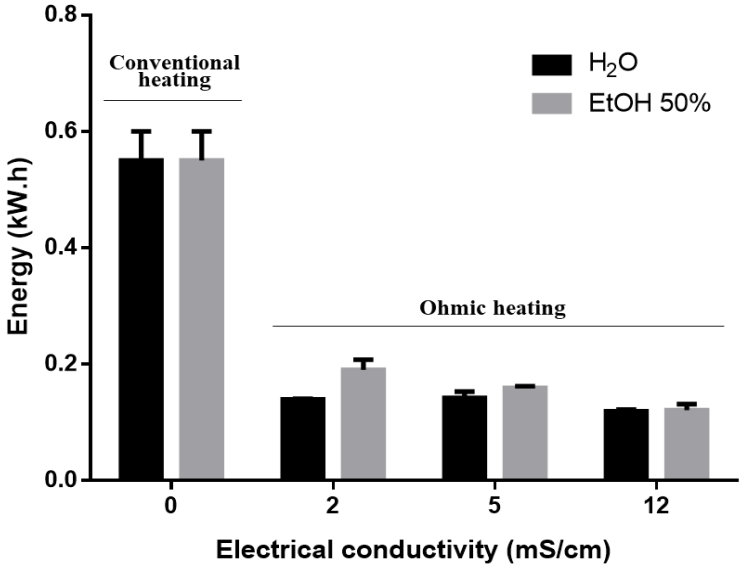


Figure 5. Total energy consumption of conventional heating and OH-assisted extraction methods for water and hydroethanolic (EtOH 50%) extraction at different electrical conductivities. Values are expressed as mean ± SD of 3 experiments.

4.4 CONCLUSIONS

OH-assisted extraction caused a significant increase in total phenolic compounds concentration in *Pinus pinaster* bark extracts (ranging from 17 to 100% increase in comparison with conventional heating) with significant saves in energy consumption. The use of ethanol in combination with OH altered the process selectivity, further boosting the extraction yield of phenolic compounds and increasing the antioxidant activities. A more comprehensive understanding about the influence of OH electrical variables (such as EF intensity, electrical frequency, type of waveform or even current density) on the morphological structure of the *P. pinaster* bark cells, solvents properties and extracts profile may bring new insights on development of extraction protocols aiming at selective extraction.

In short, OH technology holds the potential to be a "green" technique for the extraction of bioactive compounds, namely phenolic compounds from pine bark, with significant improvements in yields

and phenolic content and reduced energy consumption. Moreover, the use of electric fields have a green impact on the environment, as it uses alternative solvents and helps for the wastes reduction. Furthermore, the results also indicate that the process can be tuned for increased performance in terms of selective extraction of bioactive phenolic compounds.

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CHAPTER V

ENCAPSULATED PINE BARK POLYPHENOLIC EXTRACT DURING GASTROINTESTINAL DIGESTION: BIOACCESSIBILITY, BIOACTIVITY AND OXIDATIVE STRESS PREVENTION

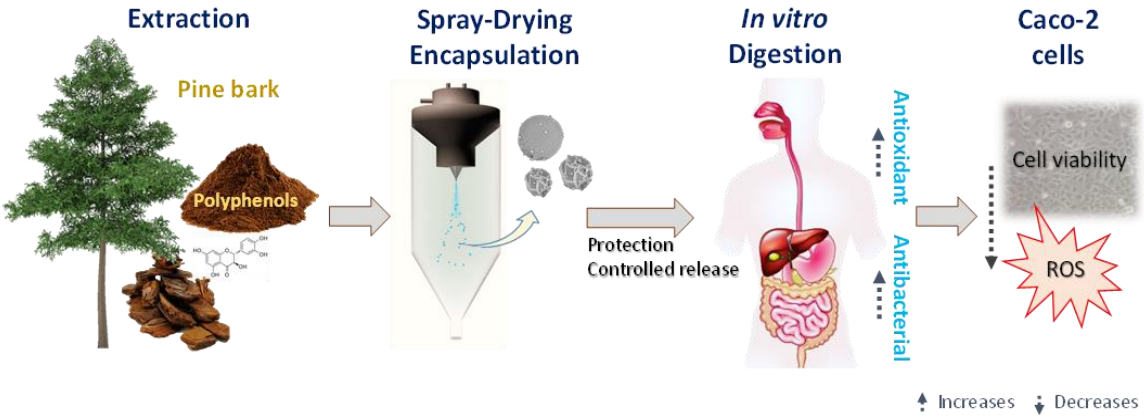
This chapter was based on the following submitted paper:

Pedro Ferreira-Santos; Raquel Ibarz; Jean-Michel Fernandes; Ana Cristina Pinheiro; Cláudia Botelho; Cristina M. R. Rocha; José António Teixeira; Olga Martín-Belloso. Encapsulated pine bark polyphenolic extract during gastrointestinal digestion: Bioaccessibility, bioactivity and oxidative stress prevention. *Foods* (submitted)

Abstract

Polyphenolic extracts from pine bark have reported different biological actions and promising beneficial effects on human health. However, its susceptibility to environmental stresses requires the development of efficient protection mechanisms to allow effective delivering of functionality. The aim of this work was to encapsulate pine bark extract rich phenolic compounds by spray-drying using maltodextrin, and understand the influence of encapsulation on the antioxidant and antimicrobial activity and bioaccessibility of phenolic compounds during gastrointestinal digestion. The optimized process conditions allowed good encapsulation efficiency of antioxidant phenolic compounds. The microencapsulation was effective in protecting those compounds during gastrointestinal conditions, controlling their delivery and enhancing its health benefits, decreasing the production of reactive oxygen species implicated in the process of oxidative stress associated with some pathologies. Finally, this encapsulation system was able to protect these extracts against acidic matrices, making the system suitable for the nutritional enrichment of fermented foods or fruit-based beverages, providing them antimicrobial protection, because the encapsulated extract was effective against *Listeria innocua*. Overall, the designed system allowed protecting and appropriately delivering the active compounds, and may find potential application as a natural preservative and/or antioxidant in food formulations or as bioactive ingredient with controlled delivery in pharmaceuticals or nutraceuticals.

Graphical Abstract



Keywords: *Pinus pinaster*; Polyphenols; Maltodextrin encapsulation; Spray-drying; Antioxidant activity; Antibacterial activity; Gastrointestinal digestion; Oxidative stress

5.1 INTRODUCTION

Pinus pinaster L. bark, residue from the lumber industry, is highly rich in phenolic compounds, mainly including phenolic acids, flavonoids and flavonols (Ferreira-Santos et al., 2019). It has been reported that PBE have health beneficial effects, including anti-inflammatory, antiviral, antitumor, antibacterial, antioxidant and there for can be used as nutraceuticals (Mármol et al., 2019; Tümen et al., 2018). In this sense, the PBE may become highly attractive for the food and pharmaceutical industries, as a potential functional ingredient. Several studies, demonstrated that *P. pinaster* bark (Pycnogenol®), reduces hyperpigmentation and improves the skin barrier function and extracellular matrix homeostasis (Grether-Beck et al., 2016), and shown beneficial effects in several diseases from asthma, lupus erythematosus and cardiovascular diseases (Rohdewald, 2002).

Phenolic compounds are very important molecules that not only act as antioxidants (donors of electrons that neutralize ROS and other free radicals) but also display several functions related to cell differentiation, deactivation of pro-carcinogens, maintenance and reparation of DNA, and other important actions (Jiménez-Moreno et al., 2019; Mrduljaš et al., 2017). Among the phenolic compounds, flavonoids, phenolic acids, stilbenes and tannins, especially proanthocyanidins, are particularly important (Gascón et al., 2018; Mármol et al., 2019). Depending on their structure, phenolics may inhibit the growth and proliferation of certain cancer cells, and the effects are thought to be either direct, due to their electron and proton donor capacity, or indirect due to their ability to alter the activities of key enzymes in cellular response (Gascón et al., 2018).

Furthermore, it is important to take in consideration that, the bioactive compounds of the PBE may suffer significant changes when exposed to adverse environmental (light, oxygen, temperature) and gastrointestinal (GI) conditions (de Vos et al., 2010).

The *in vitro* GI models have been used to simulate the physiological conditions of the gastrointestinal human tract and use a constant proportion of enzymes and salt concentrations, pH and digestion time for each digestive phase to faithfully re-create real-life conditions (Brodkorb et al., 2019). Static *in vitro* digestion models are now very well described and broadly used, once they present numerous advantages over *in vivo* and dynamic GI models (Brodkorb et al., 2019). Moreover, it allows to reach important conclusions about the bioavailability and bioaccessibility of several matrices, such as food, bioactive compounds, supplements and isolated molecules (González et al., 2019; Wang et al., 2019).

In this context, the phenolic compounds protection is of upmost importance. So, encapsulation has been the preferred method to protect bioactive compounds from oxidative processes. Additionally, the encapsulation of these moieties as a another advantage, it allows a controlled released of the molecules from the capsules during the digestion (Gharsallaoui et al., 2007).

Spray-drying is the most common and cheapest technique to produce microcapsules when compared to other encapsulation methods (Desobry et al., 1997). To obtain high encapsulation efficiency (EE) and microcapsule mechanical stability, an optimization step is required taking into account the physicochemical properties of the core and wall materials, the spray-drying operation conditions (feed, air inlet and air outlet temperatures) and the desired functional properties of the final microcapsules (Gharsallaoui et al., 2007). Wall materials are particularly important in spray-drying. They should have sufficient solubility in the feeding liquid, film forming and emulsifying ability, as well as low viscosity at high concentrations. MD are one of the main hydrolyzable carbohydrate-based wall materials used as encapsulating agents, having low cost and easily availability (Desai and Jin Park, 2005). MD forms a coating film, which minimizes oxygen contact with the encapsulated materials and preserves them from other external agents, allowing a release under controlled conditions (Pourashouri et al., 2014).

There are numerous research works on encapsulation of phenolic compounds derived from plant extracts by different methods (spray-drying, electrospinning, *etc.*) using several coating agents (Arepally and Goswami, 2019; Ballesteros et al., 2017; de Vos et al., 2010; Tsali and Goula, 2018); however no study was found on the encapsulation of phenolic PBE, promoting the protection and stability of bioactive compounds, and on the evaluation of their bioactivity and bioaccessibility.

The objective of the current study was to develop an effective protective system for PBE, rich in phenolic compounds, by optimizing the spray-drying encapsulation process using MD as coating material. Additionally, it was proposed to determine the encapsulation influence on the bioaccessibility and bioactivity in terms of antibacterial and AA of the PBE phenolic compounds. Its effect on colorectal adenocarcinoma cell line (Caco-2) viability and ROS production prior and after gastrointestinal digestion (GID) was also evaluated. The extracts and target bioactivities were chosen considering previous results from our research group (Ferreira-Santos et al., 2020), as PBE demonstrated high antioxidant, antidiabetic and antimicrobial activities, with low toxicity, and potential to be used in food formulation

and processing, either with a technological function (such as preservative or antioxidant) or as a bioactive ingredient in therapeutic formulations.

5.2 MATERIALS AND METHODS

5.2.1 Raw material and chemicals

Pine pinaster bark (approximate age 15 years) was collected in Ponte de Lima, Portugal, in April 2016. The bark was washed, dried and milled to a granulometry of 1-1.6 mm. Maltodextrin with 14-17 dextrose equivalent Folin-Ciocalteu reagent, ABTS, DPPH, TPTZ, Trolox, a-amylase (A1031, CAS 9000-90-2), pepsin (P7012; CAS 9001-75-6), pancreatin (P7545; CAS 8049-47-6), bile salts (B8631; CAS 8008-63-7), DMEM, FBS, penicillin-streptomycin solution, resazurin sodium salt, DMSO (≥99.9%) and all standard markers for HPLC were procured from Sigma Aldrich (St. Louis, MO, USA). DCFDA/H2DCFDA - Cellular ROS Assay Kit (ab113851) was procured from Abcam plc (Cambridge, UK). All other chemicals used were of analytical grade and water was bidistilled.

5.2.2 Pine bark extract preparation

The extraction methodology was previously optimized to maximize the extraction of phenolic compounds (Ferreira-Santos et al., 2020). Briefly, 10 g of PB were mixed with 100 mL of water/ethanol (30:70, v/v), using cylindrical reactors protected from light, thermostatized at 83 °C, for 30 min under shaking (170 rpm). The mixture was centrifuged at 3000 rpm for 5 min and vacuum filtered. The extracts produced in the different batches, were combined and the solvent was evaporated at 40 °C using a rotary evaporator (Heidolph VV2000, Germany). The aqueous phase was lyophilized and extracts were stored at 4 °C until use.

5.2.3 Encapsulation process by spray-drying

5.2.3.1 Experimental design

The lyophilized pine bark extracts (LPBE) was encapsulated with MD as wall material (shell). To select the optimal experimental conditions for the encapsulation process, a central composite design according to a RSM was used. The independent variables were air inlet temperature (*T*, 140-180 °C),

ratio of LPBE:MD (*r*, 1:25-1:35 w/w) and flow rate (*F*, 1-4 mL/min). The effect of the spray-drying conditions onto moisture content (MC, %) and EE for the TPC (%), and for the antioxidant activity (%) by ABTS and FRAP assays, were evaluated. For the process, 1 g of LPBE was mixed with MD following the experimental design conditions shown in Table 1. The mixtures were homogenized at 1600 rpm for 5 min with an Ultra-Turrax T25-Basix mixer (IKA, Staufen, Germany). Spray drying was carried out in a Mini Spray Dryer Büchi model B-191 (Büchi Laboratoriums Technik, Switzerland) using compressed air at 6 bar. All samples were atomized at a constant nozzle rate of 600 L/h and at maximum aspiration (100%).

A second-order polynomial model was proposed for each response, Y_i (Equation 1):

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ij} X_{ij}^2 + \sum_{i=1}^{k-1} \sum_{j>1}^{k} \beta_{ii} X_i X_j \quad (1)$$

where Y_i were the dependent variables; X_i and X_j were the independent variables, β_0 was a constant, β_i, β_{ij}, and β_{ii} were regression coefficients; k is the number of the independent parameters.

5.2.3.2 Optimization by RSM

According to the desirability approach method described by Derringer & Suich (Derringer and Suich, 1980), the optimal encapsulation conditions were determined using as response the MC, and the EE's and AA measured by ABTS and FRAP assays, as well as the model parameters determined in Experimental design section. *T*, *r* and *F* were studied at three different levels (-1, 0, 1). The highest desirability is determined assigning the highest level to the EE's and the lowest level to the MC and choosing as factor settings the studied parameters.

5.2.4 Moisture content

The MC (%) was determined by gravimetry according to the *AOAC* (2002) official methods of analysis (Zhang et al., 2018).

5.2.5 Soluble solids content

The soluble solids (°Brix) content were determined by refractometry (UNE-EN 12143) and expressed as grams of sucrose per 100 g of material.

5.2.6 Encapsulation efficiency for TPC and AA

The EE was calculated according to Equation 2.

$$\text{EE}\ (\%) = \frac{\text{encapsulated (LPBE–MD)}}{\text{non–encapsulated (LPBE)}} \times 100 \quad (2)$$

5.2.7 Structural characterization

LPBE and encapsulated LPBE with MD (LPBE-MD) were added to aluminium pin stubs on a Phenom Charge Reduction Holder (CRH) at 10 kV and a spot size of 3.3. The samples were coated with 20 Angstrom Au and characterized using a desktop Scanning Electron Microscope (SEM) (Phenom ProX, Netherlands). All results were acquired using the ProSuite software. The particle size was measured from different zones of microscopic sections using Image J software (US National Institutes of Health, http://rsb.info.nih.gov/ij/).

For optical microscopy, samples were suspended in glycerol, observed using a microscope BX51 with DP72 digital camera (Olympus, Japan) at a magnification of 100X.

5.2.8 Encapsulated extract analysis

To determine the TPC and the AA for the encapsulates (LPBE-MD powders) obtained by the spray-drying process, the LPBE-MD powders were rehydrated to drive the same content of the soluble solids measured before the encapsulation process according to Ballesteros *et al.* (Ballesteros et al., 2017).

5.2.8.1 Total phenolic content

TPC of the extracts was determined following the method based on the chemical reduction of Folin–Ciocalteu reagent (Ferreira-Santos et al., 2019). Gallic acid (0–500 mg/L) was used for calibration ($R^2 = 0.996$). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g dw).

5.2.8.2 Antioxidant activity

The AA was measured with different assays varying their mechanisms of the antioxidant action:

The ABTS and DPPH were measured spectrophotometrically according to the methods described by Ferreira-Santos *et al.* (Ferreira-Santos et al., 2019). Trolox (0–0.55 mmol/L) was used for calibration (R^2 = 0.995). The results were expressed as millimols of Trolox equivalent per 100 gram of dry weight of extract (mmol Trolox/g dw). FRAP assay was determined using ferric sulphate (II) heptahydrate (0–2 mmol/L) for calibration (R^2 = 0.992) (Tsao et al., 2003). The results were expressed as millimols of ferrous equivalent per 100 gram of dry weight (mmol Fe^{2+}/100 g dw).

5.2.8.3 Antimicrobial activity

Antimicrobial activity was determined using non-pathogenic strains of *Escherichia coli* 1.107 (Gram-negative) and *Listeria innocua* 1.17 (Gram-positive), from the stock cultures at the Food and Technology Department, University of Lleida. *E. coli* or *L. innocua* were inoculated into 40 mL of tryptone soy broth (Biokar Diagnostics, Beauvais, France) and incubated at 37 °C, 120 rpm for 11 and 15 hours, respectively to obtain colonies in the stationary growth phase (10^8-10^9 CFU/mL) (Salvia-Trujillo et al., 2014). To determine the reduction in viable cells over time, for each bacterial culture, in 4.5 mL of sterile Mili-Q water, a 0.5 mL bacterial-aliquot was mixed with LPBE or LPBE-MD achieving a final concentration of 0.00001 g/mL of LPBE. Serial decimal dilutions were carried out and counts of *E. coli* and *L. innocua* were performed by spreading the inoculated solution on McConkey and Palcam (Biokar Diagnostics) agar plates, respectively. Water was used as a control. Surviving microorganisms were counted after incubation of agar plates at 37 °C for 24 h.

5.2.9 *In vitro* gastrointestinal digestion

The GID was performed using the INFOGEST digestion standardized procedure (Brodkorb et al., 2019; Minekus et al., 2014), simulating the digestion in mouth (oral phase), stomach (gastric phase), and small intestine (intestinal phase).

The oral phase consisted in the addition of simulated salivary fluid (SSF) with α-amylase (75 U/mL) to the LPBE (40 mg/mL) or LPBE-MD (ratio of LPBE or LPBE-MD to SSF of 50:50 (w/v)). The mixture (pH 7) was gently stirred for 2 min at 37 °C. The LPBE-MD mixture presented the same initial content of phenolic compounds as the LPBE mixture, which was determined taking into account the EE results.

For the gastric digestion, simulated gastric fluid (SGF) with porcine pepsin (2000 U/mL) (ratio of LPBE or LPBE-MD to SGF of 50:50 (v/v)) was added to the oral phase. The pH was adjusted to 3 with HCl (6 M), and the mixture was incubated at 37 °C in a shaking water bath for 120 min at 60 rpm.

For the intestinal digestion, simulated intestinal fluid (SIF) with pancreatin (based on trypsin activity of 100 U/mL) and bile solution (10 mmol/L) was added to the gastric phase (ratio of SIF to the gastric chyme of 50:50 (v/v)). The pH was adjusted to 7 with NaOH (1 M), and the mixture was incubated for 120 min at 37 °C with stirring at 60 rpm.

After each step of the digestive process, a volume of 1 mL of the mixture was taken, centrifuged (10 min at 2700 g) and filtered (0.45 μm). In all fractions, the TPC and AA were determined as described in previous sections (5.2.8.1. and 5.2.8.2., respectively).

5.2.10 Bioaccessibility of phenolic compounds

The bioaccessibility (%) was determined evaluating the effect of each digestion phase on the phenolic content, and was calculated according to the Equation 3 (González et al., 2019).

$$Bioaccessibility\ (\%) = \left(\frac{A}{B}\right) \times 100 \quad (3)$$

where A is the phenolic content (mg GAE/g dw) quantified at each digestion step, and B is the phenolic content in the extract before GID.

5.2.11 Individual phenolic compounds determination and quantification by UHPLC

The LPBE and LPBE-MD individual phenolic compounds were analysed by UHPLC-DAD as described by Ferreira-Santos *et al.* (Ferreira-Santos et al., 2020) in a Shimatzu Nexpera X2 UPLC chromatograph equipped with DAD. Separation was performed on a C18 column (2.1 mm×100 mm, 1.7 μm particle size; from Waters) at 40 °C, using HPLC-grade water/formic acid (0.1%) and acetonitrile as eluents (0.4 mL/min). Phenolic compounds were identified at different wavelengths by comparing their UV spectra and retention times with that of the corresponding standards. Quantification was carried out using calibration curves for each compound analyzed using concentrations between 250–2.5 mg/mL (250, 125, 100, 50, 25, 10, 5, 2.5 mg/mL). In all cases, the coefficient of linear correlation was $R^2 > 0.99$. Compounds were quantified and identified at different wavelengths (209–370 nm).

5.2.12 Biological assays

5.2.12.1 Cell viability

The human colorectal adenocarcinoma Caco-2 cell line (ATCC® HTB-37™) was kindly provided by Dr. Andreia Gomes (Department of Biology, University of Minho). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were grown in DMEM supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin/streptomycin.

When the cell culture reached 70–80% of confluence (confirmed by microscopic observation), the cells were trypsinized (0.25% trypsin-1 mM EDTA) and seeded in a 96-well plate at a density of 2×10^4 cells per ml. The cell line was incubated with different cell culture medium: i) supplemented DMEM; ii) supplemented DMEM with undigested and iii) supplemented DMEM with digested extracts LPBE, LPBE-MD and encapsulated agent (MD) in concentrations ranging from 75 to 2000 μg/mL for a period of 24 h. After incubation, the metabolic activity of Caco-2 cells (cell viability) was evaluated by the resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) reduction assay (Ferreira-Santos et al., 2020). Briefly, the supernatant was replaced by 200 μL culture media containing resazurin (0.5 mM in PBS). After 2 h of incubation at 37 °C, 150 μL of the supernatant were transferred to a new 96-well microplate and the resultant fluorescent product (resorufin) was detected at 560 nm (λex) and 590 nm (λem) using a microplate reader (Cytation 3, BioTek Instruments, Inc., Winooski, VT, USA).

The % cell viability was calculated correcting blank values (cell-free medium) and related to untreated controls (0.5% DMSO).

5.2.12.2 Measurement of intracellular ROS levels

Caco-2 cells were grown in 96-well plates at a density of 2.5×10^4 cells per ml and were incubated overnight at 37 °C in a humidified atmosphere with 5% CO_2. For treatment, extracts were dissolved on cell culture medium at a concentration of 500 μg/mL. Cells were incubated with the extracts for 8h. Then, cells were incubated for 45 min with 25 μM 2',7'-dichlorofluorescein diacetate (DCFDA) at 37 °C in a humidified atmosphere with 5% CO_2 and protected from light. For tert-butyl hydrogen peroxide (tbHP) ROS induction, the cell culture was then replaced by 100 μM tbHP (dissolved in PBS) except negative control cells, which were incubated with PBS. After 1h incubation protected from light, the fluorescence intensity was measured using a microplate reader (Cytation 3, BioTek

Instruments, Inc., Winooski, VT, USA). Excitation and emission wavelength settings were 485 and 535 nm, respectively. The intensity of fluorescence is considered as a reflection of the total intracellular ROS levels.

5.2.13 Statistical analysis

Design Expert DX 7.01 program (Stat Ease Inc., Minneapolis, MN, USA) was employed for the experimental design, data analysis and model building.

All experiments were performed in triplicate and the results are presented as average ± SD. GraphPad Prism® software (San Diego, CA, USA) was used for statistical analyses. The level of significance was determined by one-way ANOVA followed by Bonferroni's test for multiple comparisons. Significance was accepted at $p < 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 Spray-drying process evaluation

5.3.1.1 Moisture content and encapsulation efficiency

Table 1 summarizes the results obtained from the CCD experimental design to optimize the spray-drying encapsulation process. The ANOVA, the significance of *p*-value, the determination coefficient (R^2) and the adjusted determination coefficient (R^2-*adj*) are shown in **Table 2**. To evaluate the model adequacy, the R^2 and R^2-*adj* values were used.

The effect of the temperature (*T*), the core to wall material ratio (LPBE:MD) (*r*) and the flow rate (*F*) were determining factors for the stability of the powder and the viability of the encapsulated PB phenolic compounds. The encapsulated powder *MC* is one of the most important variables to maintain the encapsulated products quality and stability (Nguyen et al., 2018). The lowest *MC* (2.15%) for the spray drying encapsulation conditions was reached at the highest air inlet temperature (180 °C), the lowest ratio LPBE:MD (1:15) and lowest flow rate (1 mL/min). By the contrary, the highest *MC* (4.89%) was obtained when LPBE was encapsulated at 140 °C, with a 1:35 LPBE:MD ratio and flow rate of 4 mL/min (Table 1). The interactions flow rate-LPBE:MD ratio and temperature-LPBE:MD ratio as well as the temperature quadratic term significantly influenced on the *MC.* Thus, for spray drying encapsulation process, a flow rate and ratio core:shell increases, promote the rise of the final *MC*,

while a temperature increase, reduces the final *MC,* wich is the desired situation in this case. The ANOVA data (Table 2) shows that the response functions obtained from the second-order model fitted correctly the experimental data ($p < 0.01$), where the determination coefficient (R^2) was 0.906, meaning that the model was suitable for predicting the response variables. It is pertinent to point out that adjusted R^2 (0.811) was lower than R^2, suggesting that the model effect was significantly improved by the predictor.

The statistical analysis indicated that the second-order models were adequate for describing with accuracy the encapsulation efficiencies in terms of *TPC* (0.856), AA_{ABTS} (0.934), and AA_{FRAP} (0.933) (**Table 2**). Moreover, the adjusted determination coefficients 0.812 (*TPC*), 0.901 (AA_{ABTS}), and 0.910 (AA_{FRAP}) were lower than the corresponding R^2. The maximal *EE* for *TPC* (79%) was reached when spray drying conditions were 160 °C, 1:25 LPBE:MD ratio, and 1 mL/min. The minimal *TPC* (29% and 30%) were reached at 140 °C, 1:35 LPBE:MD ratio and 180 °C, 1:15 LPBE:MD ratio, respectively, at the same flow rate (1 mL/min). The *TPC* decreased when temperature increased and ratio decreased and vice versa. Also, the interaction temperature- LPBE:MD ratio significantly influenced on the *TPC*. That is, as the ratio decreased, the effect of the temperature became more pronounced on TPC encapsulation efficiency.

For the *AA* measured by *ABTS* and *FRAP* assays, the highest *EE* were 120% and 114%, respectively, obtained at 180 °C, 1:15 LPBE:MD ratio, and 4 mL/min for both assays. The *AA* values suggest that the low LPBE:MD ratio used, together with the high flow rate, were not enough to coat the core, being not allowed to create a shell able to protect the phenolic PB compounds, which were susceptible to polymerization at the air inlet temperature used in the drying chamber. At temperatures higher than 80 °C, the Maillard reaction formed novel polymerized products contributing to the *AA* effect leading to values above 100% (Gullón et al., 2016). The air inlet temperature and LPBE:MD ratio significantly influenced on the *EE* of the *AA* (*ABTS* and *FRAP)* during the encapsulation process. *AA* increased with temperature (from 140 to 180 °C) and LPBE:MD ratio (from 1:15 to 1:35) maintaining the flow rate at 1 mL/min. Furthermore, the interaction of temperature and flow rate had a significant effect on *EE*. For example, when LPBE:MD ratio was set at 1:25 at temperature of 160 °C and flow rate from 1 to 4 mL/min, the *EE* augmented from 91% to 103% for AA_{ABTS}, and from 91% to 96% for AA_{FRAP}. In addition, when LPBE:MD ratio was set at 1:25 at a constant flow rate of 2.5 mL/min and the temperature

ranged from 140 °C to 180 °C, the *EE* rises from 101% to 105%, and from 74% to 112% for AA_{ABTS} and AA_{FRAP}, respectively.

Table 1. Experimental design for the independent variables and corresponding response values (measured and predicted) for spray drying encapsulates from pine bark by-product extracts.

Independent variables				Response values							
				MC (%)		EE (%)					
						AA_{ABTS}		AA_{FRAP}		TPC	
Assay*	T (°C)	r (-)	F (mL/min)	Measured	Predicted	Measured	Predicted	Measured	Predicted	Measured	Predicted
1	160	1:25	4	3.01 ± 0.02	3.04	103 ± 12	87	97 ± 5	90	64 ± 6	73
2	160	1:25	2.5	2.91 ± 0.15	3.12	99 ± 54	93	95 ± 3	90	49 ± 12	62
3	160	1:25	1	2.73 ± 0.06	2.82	91 ± 6	99	91 ± 1	90	79 ± 10	65
4	160	1:25	2.5	3.19 ± 0.05	3.12	52 ± 29	93	97 ± 5	90	58 ± 3	62
5	140	1:35	4	4.89 ± 0.04	4.84	112 ± 9	99	68 ± 2	65	53 ± 1	52
6	160	1:25	2.5	3.47 ± 0.25	3.12	111 ± 13	93	88 ± 2	90	67 ± 3	62
7	180	1:15	1	2.15 ± 0.03	3.44	54 ± 37	107	74 ± 5	82	30 ± 1	30
8	140	1:15	1	3.77 ± 0.13	3.58	58 ± 11	73	56 ± 5	63	35 ± 1	36
9	180	1:25	2.5	3.96 ± 0.18	3.71	105 ± 7	96	112 ± 2	109	33 ± 8	44
10	160	1:25	2.5	3.35 ± 0.16	3.12	94 ± 1	93	91 ± 1	90	68 ± 4	62
11	180	1:35	2.5	2.69 ± 0.05	2.76	97 ± 11	112	113 ± 16	137	55 ± 1	53
12	180	1:35	4	3.99 ± 0.11	4.14	91 ± 11	76	127 ± 1	125	49 ± 2	47
13	140	1:15	4	2.74 ± 0.07	2.64	99 ± 10	84	94 ± 13	76	58 ± 1	59
14	180	1:15	4	3.13 ± 0.02	3.1	120 ± 23	87	114 ± 14	94	34 ± 4	29
15	140	1:25	2.5	3.77 ± 0.26	4.13	100 ± 8	90	74 ± 3	70	56 ± 5	49
16	160	1:25	2.5	2.94 ± 0.12	3.12	95 ± 2	93	82 ± 6	90	70 ± 5	62
17	160	1:15	1	2.29 ± 0.20	2.58	119 ± 23	88	85 ± 4	79	44 ± 3	48
18	140	1:35	1	4.07 ± 0.01	4.06	101 ± 2	104	52 ± 18	77	29 ± 3	34
19	160	1:35	2.5	3.51 ± 0.12	3.34	110 ± 8	98	97 ± 3	101	55 ± 1	56

*Assay order was randomized. *T,* temperature (°C); *r,* ratio lyophilized pine bark extract:Maltodextrin (-); *F,* flow rate (mL/min); *MC,* moisture content (%); *EE,* encapsulation efficiency (%); *TPC,* total phenolic content (%); *AA,* antioxidant activity measured by *ABTS* assay (%) and *FRAP* assay (%).

5.3.1.2 Optimal encapsulation conditions and model validation

In the range of the studied spray-drying encapsulation conditions, an optimization was performed in order to find the combination of the spray drying conditions that gives the lowest MC, and the highest EE in terms of *TPC* and *AA*.

The optimum processing condition was achieved at the drying chamber temperature of 158 °C, with a ratio 1:18 [LPBE (core):MD (shell)], and with a flow rate of 0.815 mL/min, where the lowest moisture content was 2.37% and the highest values for the EE were 67%, 98%, and 99% for *TPC*, AA_{ABTS} and AA_{FRAP}, respectively. The desirability of the spray-drying encapsulation conditions treatment was 0.835, which was taken as an indicator of accuracy between the polynomial model predictions and the experimental data (**Tables 1** and **2**). The correlation coefficients between the measured and predicted values were 0.906, 0.856, 0.934, and 0.933 for *MC*, *TPC*, AA_{ABTS} and AA_{FRAP} models, respectively, indicating that the second-order expressions obtained for each assay (**Table 2**) adequately fitted experimental results.

The LPBE-MD obtained at the optimal encapsulation conditions by spray drying was selected to evaluate their antimicrobial properties, *in vitro* stability and bioaccessibility in comparison to LPBE.

Table 2. Analysis of variance of the first-order polynomial models for spray drying encapsulates from pine bark by-product extracts.

Coefficient	MC	EE		
		AA_{ABTS}	AA_{FRAP}	TPC
Model	3.12**	92.78***	89.73***	62.38**
T	-0.21*	2.76*	-19.53***	2.74*
r	0.38**	5.00***	-3.54*	3.98*
F	0.11	6.30***	11.17***	4.15*
*F * T*	-	-7.75*	-0.22*	-
*F * r*	0.43**	-3.76**	-6.16**	-
*T * r*	-0.29*	6.37**	10.26***	-5.96**
T^2	0.80***	-	-	-15.96**
r^2	-	-	-	-10.3***
F^2	-	-	-	6.25*
R^2	0.906	0.934	0.933	0.856
R^2-*adj*	0.811	0.901	0.910	0.812
Eq.	$MC = 52.7 - 0.63\ T + 0.47\ r + 0.39\ F + 0.03\ F\ r - 2.89\ T\ r + 1.20\ T^2$	$AA_{ABTS} = 276.53 + 1.97\ T + 13.19\ r + 25.92\ F - 0.13\ F\ T - 0.25\ F\ r + 0.06\ T\ r$	$AA_{FRAP} = 54.33 - 0.28\ T - 12.43\ r + 9.45\ F - 3.71\ F\ T - 0.41\ F\ r - 0.10\ T\ r$	$TPC = 985.28 + 12.63\ T + 1.54\ r + 6.98\ F - 0.09\ F\ r - 0.04\ T^2 - 0.42\ r^2 + 0.63\ F^2$

Adjusted determination coefficient (R^2-*adj*) and determination coefficient (R^2) for evaluating model goodness-of-fit and model equations are also reported. *T*, temperature (°C); *r,* ratio lyophilized pine bark extract:Maltodextrin (-); *F,* flow rate (mL/min); *M,* moisture content (%); *EE,* encapsulation efficiency (%); *TPC,* total phenolic content (%); *AA,* antioxidant activity measured by *ABTS* assay (%), and *FRAP* assay (%). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.2 Structural characterization

In **Figure 1** it is possible to observe the non-encapsulated LPBE (a and c), and the MD encapsulated extract (b and d) produced under optimized conditions (inlet temperature of 158 °C; ratio LPBE:MD of 1:18 and flow rate of 4 mL/min). The size of microcapsules was not uniform, varying from 5 to 25 μm. It can be observed that spherical-shape microcapsules with rough surfaces were formed, indicating encapsulation and retention of this phenolic extract. This structure may be due to the water evaporation rates during the spray-drying process, using a temperature of 158 °C. Other works report similar structures and size of MD microcapsules, used to encapsulate other polyphenols extracts by spray-drying (Santiago-adame et al., 2015; Zanoni et al., 2020).

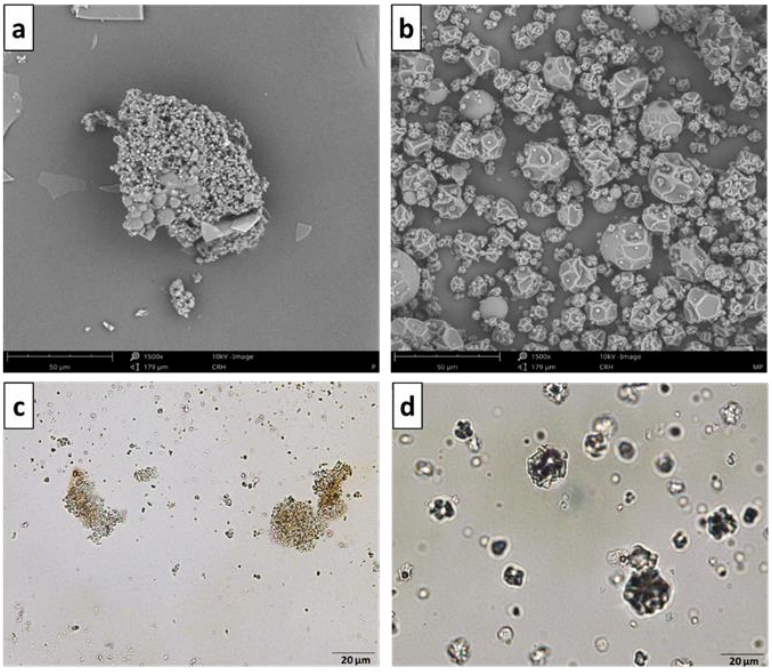


Figure 1. Images of SEM (**a, b**) and optical microscopy (**c, d**) of non-encapsulated LPBE (**a, c**) and encapsulated LPBE-MD (**b, d**) PBE. Scale bar of 50 μm and 20 μm applies to SEM and optical images, respectively.

5.3.3 Antimicrobial activity evaluation

Table 3 shows a significant antibacterial activity of LPBE and LPBE-MD samples against the gram-positive bacteria (*L. innocua*), compared to gram-negative (*E. coli*). Our results are in agreement with Kotzekidou, Giannakidis, & Boulamatsis (Kotzekidou et al., 2008) and Oliveira *et al.* (Oliveira et al., 2013), who suggest that gram-positive microorganisms are usually more sensitive to antimicrobials

such as polyphenols, than gram-negative microorganisms. In this sense, no inhibitory effect against *E.coli* microorganisms was observed (**Table 3**) due to the resistance of gram-negative microbes to antimicrobials, attributed to the strong hydrophilicity of the outer membrane of the two-fold layer structure of the cell envelope of these bacteria, which became a strong barrier (Kozłowska et al., 2015). Moreover, it was observed that for the same concentration (0.00001 g/mL) of PBE the inactivation of *L. innocua* is higher for the encapsulated antimicrobial agent (LPBE-MD) than the non-encapsulated, since after 1 h the microorganisms are already completely inactivated. This behaviour could be attributed to the wall material used for the encapsulation process. Thus, MD modifies the properties of the encapsulated material, affecting their size, geometry, surface morphology, favoring the solubilization of the lypophilic biocompounds (core), facilitating their diffusion through the single membrane of the gram-positive bacteria, enhancing the cellular uptake and promoting the *L.innocua* inactivation (González-Ballesteros et al., 2019; Oliveira et al., 2013).

Table 3. Antimicrobial activity of non-encapsulated (LPBE) and encapsulated (LPBE-MD) PBE against *Escherichia coli* and *Listeria innocua.*

Time	LPBE (0.00001 g/mL PBE)		LPBE-MD (0.00001 g/mL PBE)	
	log *N*		log *N*	
	E. coli	*L. innocua*	*E. coli*	*L. innocua*
Control (0h)	8.974 ± 0.584	7.867 ± 0.313	8.974 ± 0.584	7.927 ± 0.601
1 h	uncountable	2.662 ± 0.027	uncountable	< 1
24 h	uncountable	< 1	uncountable	< 1

Data shown are a mean ± SD of three experiments. LPBE, lyophilized pine bark extrac; PBE, pine bark extract; MD, maltodextrin; *N*, number of microorganisms.

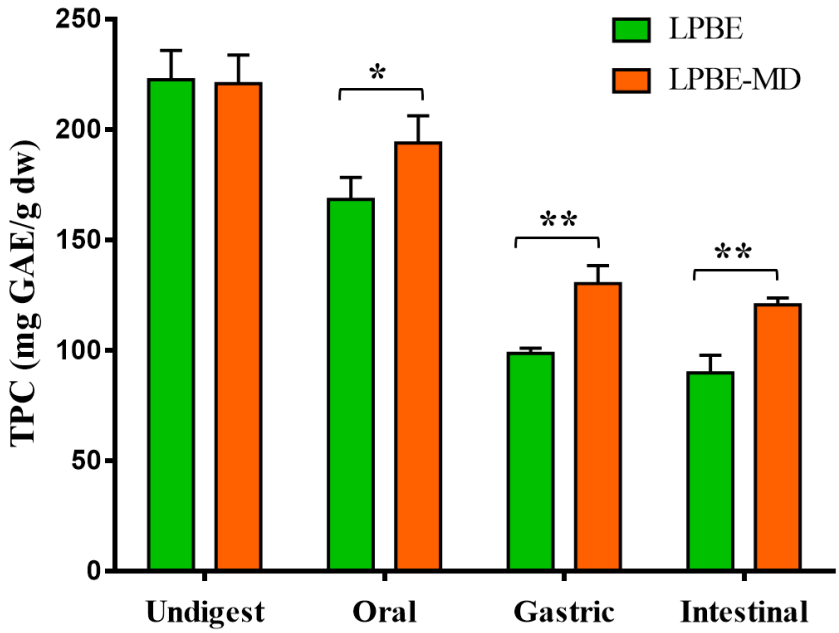
5.3.4 Effect of simulated in vitro GID on pine bark extracts

5.3.4.1 Phenolic compounds determination and bioaccessibility analysis

The impact of GID on TPC and bioaccessibility of LPBE and LPBE-MD is shown in **Figure 2**. The initial phenolic content is the same for LPBE and LPBE-MD with approx. 222 mg GAE/g dry extract. The results of the *in vitro* GID revealed that the TPC strongly decreased after the digestion in comparison to the undigested LPBE. When the extract was encapsulated (LPBE-MD), the degradation of these compounds was slower at the harsh conditions of the GI system (such as enzymes and pH) compared to the non-encapsulated extract (LPBE).

In the oral phase, the content of phenolic compounds decreased, with a significantly higher reduction ($p < 0.05$) for the non-encapsulated extract when compared with the LPBE-MD (168.4 mg GAE/g and 193.9 mg GAE/g, respectively). In the gastric and intestinal phases, the TPC reduced approx. 20% more in LPBE compared to LPBE-MD. This study demonstrates that the phenolic compounds present in the extract have high instability when subjected to gastric conditions, which may be due to the presence of enzymes (such as pepsin) and the acidic pH of this phase. Other researchers also demonstrate that phenolic extracts are highly affected during the digestive process (Vulić et al., 2019; Wang et al., 2019).

In this sense, the MD encapsulation of PBE significantly improved the bioaccessibility of phenolic compounds (**Figure 2**). It can be observed that the encapsulation increased the bioaccessibility of phenolic compounds by approx. 11%, 15% and 14%, when subjected to the digestion conditions of the oral, gastric and intestinal phases, respectively.



	LPBE			LPBE-MD		
Bioaccessibility	**Oral**	**Gastric**	**Intestinal**	**Oral**	**Gastric**	**Intestinal**
(%)	75.7 ± 4	43.2 ± 2	40.4 ± 3	87.1 ± 5	58.5 ± 3	54.3 ± 1

Figure 2. Total phenolic content (TPC, mg GAE/g dw) and bioaccessibility (%) of non-encapsulated (LPBE) and encapsulated (LPBE-MD) PBE before and after gastrointestinal digestion. Values are expressed as mean ± SD of three experiments. * $p < 0.05$, ** $p < 0.01$.

The phenolic profile of LPBE and LPBE-MD before and during GID was tentatively identified and quantified by UHPLC-DAD (**Table 4**). Catechin, epicatechin/*p*-coumaric acid, gallocatechin, narginin, hesperidin and taxifolin were the most abundant phenolic compounds identified in the LPBE, showing values between 125-199 mg/L with the exception of taxifolin having a higher concentration (344 mg/L). Other flavonoids, hydroxybenzoic acids, hydroxycinnamic acids and stilbens (resveratrol) were identified with concentrations between 44 to 94 mg/L, except for apigenin and cinnamic acid, which had lower concentrations. Quercetin and gallic acid were identified in all samples, however, they co-eluted.

The individual phenolic concentrations of the PBE were significantly reduced after *in vitro* GID, in both LPBE and LPBE-MD. However, a more pronounced decrease in concentration was observed in the gastric and intestinal phase of GI digestion.

When the extracts were subjected to the GID, it was observed that, at the end, the composition of the encapsulated extract (LPBE-MD) was similar to the non-encapsulated extract (LPBE). Resveratrol, rosmarinic acid and gallocatechin are the less stable compounds, not being detected at the end of digestion. Other compounds such as taxifolin, 3,4-hydroxybenzoic acid, narginin, o-coumaric acid and cinnamic acid, are also partially destroyed or converted during the intestinal phase, having concentrations below of 53 mg/L, 12 mg/L, 5.2 mg/L, 4.9 mg/L and 2 mg/L, respectively. In addition, ferulic acid, chlorogenic acid and epicatechin/*p*-coumaric acid appear to be the most stable phenolic compounds to digestion, in accordance with results reported previously by Frontela *et al.* (Frontela et al., 2011).

These results are important to unveil the bioavailability of the phenolic compounds present in the PBE, their biological activities and possible applications as nutrients and health promoters.

Table 4. Phenolic compounds identification and quantification of non-encapsulated (LPBE) and encapsulated (LPBE-MD) PBE before and after gastrointestinal digestion by UHPLC-DAD.

	LPBE				LPBE-MD		
Compounds	**Undigested**	**Oral**	**Gastric**	**Intestinal**	**Oral**	**Gastric**	**Intestinal**
catechin	198.2 ± 28.0	153.1 ± 14.9	142.3 ± 13.0	115.3 ± 0.8	128.6 ± 11.2	135.5 ± 13.9	117.4 ± 0.4
vanilic acid	64.5 ± 6.5	55.8 ± 2.3	44.1 ± 2.8	44.3 ± 1.3	59.2 ± 4.0	44.3 ± 2.1	44.1 ± 0.5
gallic acid	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
epicatechin + *p*-coumaric acid	127.7 ± 16.8	105.6 ± 16.7	89.2 ± 10.6	75.5 ± 3.1	121.7 ± 8.3	94.0 ± 9.0	117.7 ± 6.4
o-coumaric acid	57.8 ± 7.8	61.2 ± 30.4	31.3 ± 0.3	7.4 ± 3.4	65.7 ± 11.2	64.5 ± 28.6	4.9 ± 0.1
chlorogenic acid	44.1 ± 4.0	35.6 ± 1.1	30.9 ± 1.8	32.4 ± 0.6	41.5 ± 1.7	33.4 ± 2.0	32.1 ± 0.4
ferulic acid	48.7 ± 9.9	29.6 ± 4.2	35.1 ± 3.5	31.9 ± 1.2	60.2 ± 5.6	54.1 ± 4.7	51.9 ± 0.5
ellagic acid	62.1 ± 12.6	47.9 ± 12.6	34.4 ± 2.1	23.9 ± 3.4	55.8 ± 6.4	57.6 ± 8.6	27.0 ± 5.8
narginin	164.0 ± 23.4	134.5 ± 31.0	97.2 ± 0.9	6.8 ± 2.7	140.3 ± 33.1	117.1 ± 38.7	5.2 ± 0.1
hisperidin	125.9 ± 23.1	84.6 ± 18.9	67.1 ± 10.3	30.0 ± 2.1	115.4 ± 9.8	86.0 ± 9.4	31.1 ± 0.2
apigenin	3.1 ± 0.3	2.3 ± 0.2	2.6 ± 0.1	2.5 ± 0.1	3.1 ± 0.4	3.3 ± 0.3	4.3 ± 1.2
resveratrol	56.8 ± 10.5	48.9 ± 2.9	40.1 ± 0.7	n.d.	61.2 ± 10.9	41.3 ± 0.2	n.d.
cinnamic acid	9.5 ± 3.3	12.4 ± 5.1	5.5 ± 1.4	2.1 ± 0.1	15.6 ± 8.3	20.2 ± 9.6	2.0 ± 0.1
rosmaniric acid	46.4 ± 0.8	34.0 ± 1.6	38.7 ± 1.3	n.d.	49.4 ± 0.5	44.5 ± 1.9	n.d.
gallocatechin	180.5 ± 3.7	188.0 ± 1.3	43.3 ± 1.6	n.d.	161.4 ± 4.6	141.6 ± 16.0	31.7 ± 2.8
taxifolin	344.4 ± 66.8	174.7 ± 73.6	106.1 ± 21.9	23.4 ± 1.0	284.3 ± 10.8	117.3 ± 26.7	53.1 ± 17.9
quercetin	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
3,4 hydroxybenzoic acid	93.0 ± 7.7	76.7 ± 1.1	47.0 ± 3.4	18.4 ± 1.0	75.1 ± 2.6	47.1 ± 7.4	12.5 ± 0.9
Total	**1627**	**1245**	**855**	**414**	**1323**	**1102**	**535**

Values of phenolic compounds are expressed as concentration mean ± SD (mg/L) of three experiments.

n.d., not detected; n.q., not quantified; LPBE, lyophilized pine bark extrac; PBE, pine bark extract; MD, maltodextrin.

5.3.4.2 Antioxidant activity

The influence of GID on the AA of the encapsulated and non-encapsuled extracts is shown in the **Figure 3**. The reduction power (FRAP) of LPBE significantly decreased ($p < 0.01$) during the different stages of GID, and scavenging activity significantly decreased after the gastric and intestinal digestion phases ($p < 0.01$) in comparison to the undigested samples.

When the AA was determined by the FRAP assay (Figure 3a), a significant decrease ($p < 0.001$) was observed in the bioactivity of the LPBE soon in the oral phase compared to the undigested extracts. This reduction was noticeably lower in the LPBE-MD, indicating a high protection level of the microcapsules for reduction AA in oral phase. Also in the gastric phase, the microcapsules showed a protective effect of the phenolic compounds, with significant differences between the LPBE and LPBE-MD extracts ($p < 0.01$). This protective effect was less observed in the intestinal phase.

In agreement with the TPC content, the DPPH results (Figure 3b) demonstrated that, after the digestive process, the encapsulated extract (LPBE-MD) shows a smaller reduction in its AA when compared to the non-encapsulated extract. It can also be observed that, in the oral phase, the encapsulated and non-encapsulated extracts exhibit scavenging activity similar to the undigested extract. These results may lead to state that phenolic compounds that have been degraded do not have relevant AA. The differences are visible in the gastric and intestinal phase, where the LPBE-MD shows 46% and 30% more AA, respectively, compared to the LPBE. These results may be related to the marked decrease in the concentration of certain phenolic compounds with high AA during digestion, such as taxifolin, gallocatechin, resveratrol and rosmarinic acid.

These results demonstrate that the antioxidant capacity of natural extracts is the result of the synergistic effects of the different phenolic compounds among each other and with other components of the matrix or the organism such as proteins, carbohydrates and lipids (Lingua et al., 2019). It has been reported that the AA of phenolic extracts from plant matrices are negatively affected when subjected to the conditions of the gastrointestinal system (Lingua et al., 2019; Wang et al., 2019). In a study, Frontela *et al.* (Frontela et al., 2011) reported that the enrichment of fruit juices with PB phenolic extracts is an expedient strategy to compensate possible phenolic loss through gastrointestinal processing. This study proves that these extracts can be even more efficient if incorporated in an encapsulated form.

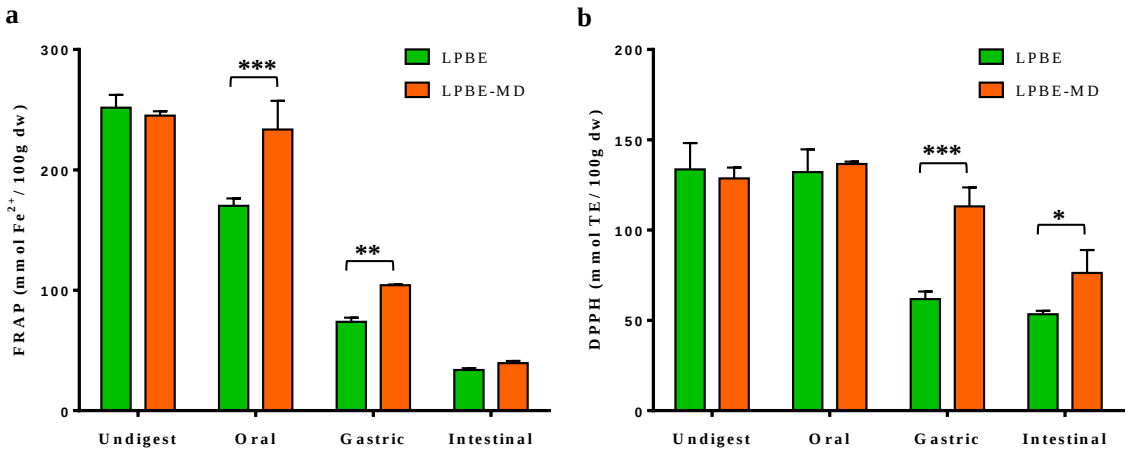


Figure 3. Antioxidant activity by FRAP (**a**) and DPPH (**b**) of non-encapsulated (LPBE) and encapsulated (LPBE-MD) PBE before and after gastrointestinal digestion. Values are expressed as mean ± SD of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.4.3 Cellular viability and antioxidant capacity on intestinal Caco-2 cells

The **Figure 4** represents the cellular viability in the presence of encapsulated (LPBE-MD) and non-encapsulated (LPBE) phenolic extract when subjected (or not) to the conditions of the GID system. Different concentrations of bioactive extracts (0 to 2000 µg/mL) were incubated with the Caco-2 cells for 24h and their ability to metabolize resazurin into resorufin in the presence of extracts was used as a measurement of cells metabolic activity.

Our data shows that MD as wall material was not toxic toward Caco-2 cells, regardless of the concentration tested. On the other hand, the cells exposed with the LPBE non-encapsulated and/or encapsulated with MD, at concentrations higher than 500 µg/mL, show a significant reduction of cell viability in Caco-2 cells. In addition, when the PBE is encapsulated and the cells are exposed to the same concentration, the number of viable cells suffered a greater reduction of viability, compared to the non-encapsulated extract (LPBE).

Furthermore, Caco-2 cells were treated with a bioaccessible fraction of PBE resulting from the simulated GID. The results showed that cell viability decreased significantly when the cells were treated with both digested PBE (LPBE-Dig or LPBE-MD-Dig). This reduction was significantly greater for the encapsulated extracts, despite a smaller reduction compared to undigested extracts.

These results are in agreement with those discussed above, and confirm the protection of phenolic compounds by encapsulation and the promotion of a controlled release, protecting their biological activity and health benefits (*e.g.* anti-proliferative), as well as it can contribute to better sensory properties of encapsulated products.

As it is widely described, cancer development has many steps, from initiation and promotion to progression (Couch, 1996). On this study it was evaluated the influence of bioactive compounds on the latest stage the progression. The literature describes several chemopreventive mechanisms induce by the presence of polyphenols, being one of them the ability to inhibit or reduced the proliferation of cancer cells (Rao et al., 2018; Sharma et al., 2018).

When analyzing the composition in terms of phenolic content of the digested PBE it can observed that the compounds with higher bioavailability for the cells were catechin, taxifolin, ferulic acid and epicatechin/*p*-coumaric acid. Although, it was not possible to isolate the effect of each compound on the anti-proliferation ability of the PBE, it is believed that they may have a synergistic effect based on previous studies found in literature. It is know that the ferrulic acid inhibits osteosarcoma cells by interfering with the cell cycle as well as by inducing a significant increase on cell apoptosis (Wang et al., 2016). Janicke *et al.* described that *p*-coumaric acid has a negative effect on colon cancer progression as it retards cell cycle progression in Caco-2 cells (Janicke et al., 2011). Similarly, taxifolin demonstrated that it can inhibit tumor growth *in vivo*, and it has been reported that this maybe due to cell cycle arrest in G1 (Chen et al., 2017).

Considering these results of low toxicity, LPBE concentrations of 500 µg/mL and their equivalent amount in LPBE-MD were chosen for the following AA studies in Caco-2 cells.

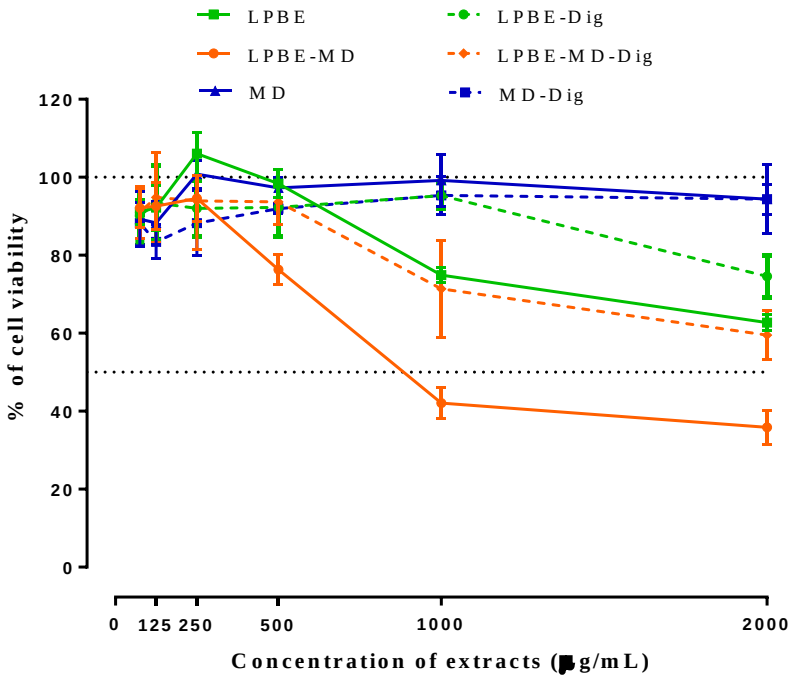


Figure 4. Cellular viability (%) of non-encapsulated (LPBE), encapsulated (LPBE-MD) PBE and maltodextrin (MD) as wall material before and after gastrointestinal digestion against human colorectal adenocarcinoma Caco-2 cells.

Moderate levels of ROS are essential for cellular proliferation, differentiation, and survival. At the same time, and assuming the fundamental role of ROS at the beginning and its influence in a number of diseases, alternative forms have been studied to prevent their increase in the body (Gascón et al., 2018). To overcome cell death induced by oxidative stress, increasing antioxidant defenses has been an interesting therapeutic approach (Victoria Urquiza-Martínez and Fenton Navarro, 2016). Extracts obtained from bioresources (plants, algae, agro-food wastes, etc) have the ability to change cell redox homeostasis, either by increasing or decreasing ROS production. Given previously obtained results of PBE composition (**Figure 2** and **Table 4**) and promising results by FRAP and DPPH antioxidant assays (**Figure 3**), Caco-2 cells were selected to further elucidate the mechanism of extracts action in terms of antioxidant behavior. This cell line was used taking into account the results of cell viability and because they are widely used to understand the influence of nutrients and/or nutraceuticals in the body after ingestion.

The level of ROS produced by Caco-2 cells after 8 h incubation with encapsulated and non-encapsulated LPBE before and after GID is shown in **Figure 5**. In the Figure 5 (a) it is shown the influence of extracts on endogenous oxidative stress in Caco-2 cells. It is known that cancer cells have an altered redox state, expressing higher amounts of ROS that non-cancerous cells. The results showed that the PBE, when encapsulated, presented a significant reduction of endogenous intracellular ROS (about 34%) in the cancer cells used in this work. Figure 5 (b) shows AA of the PBE when Caco-2 cells were subjected to exogenous oxidative stress caused by tbHP, which displayed a greater increase in ROS levels when compared to the control cells (4.8 times more). Moreover, when the cells were incubated with a concentration of 500 μg/mL of extract, the production of ROS induced by tbHP was significantly lower (approx. 30% lower than the control tbHP), confirming its antioxidant capacity. Our results are in agreement with those reported by Gascón *et al.* (Gascón et al., 2018), wherein PBE from three different species (including *Pinus pinaster*) showed AA in Caco-2 cells using concentrations above 20 μg/mL.

As previously evaluated by the *in vitro* antioxidant methods to evaluate iron reduction (FRAP) and scavenger activity (DPPH), it is clear that the digestion process influences the AA of PBE and the MD encapsulation process partially promotes the stabilization of bioactive phenolic compounds, preventing their degradation. This decrease in the AA of the extract after digestion is considered normal, due to the degradation or (bio)tranformation of the phenolic compounds present in the bioactive extract. These data corroborate the results obtained by the intracellular ROS assay in Caco-2 cells in which the digested extracts had less preventive effect of ROS generation than the

undigested extracts, despite the cells treated with the encapsulated extracts (LPBE-MD-Dig) show a lesser increase in intracellular ROS caused by tbHP.

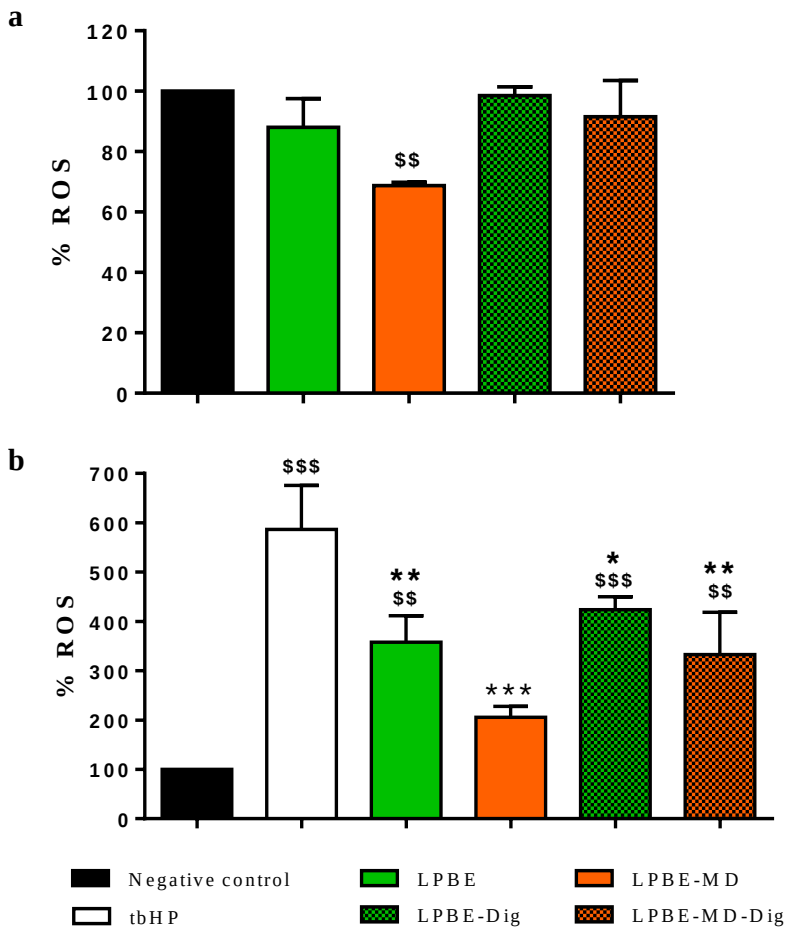


Figure 5. Antioxidant capacity of extracts on Caco-2 cells. (**a**) Measurement of reactive oxygen species levels after 8 h incubation with 500 $\mu g_{extract}$/mL of encapsulated/non-encapsulated (digested and undigested extracts). (**b**) Measurement of reactive oxygen species levels after 8 h incubation with 500 $\mu g_{extract}$/mL of encapsulated/non-encapsulated (digested and undigested extracts) and further tert-butyl Hydrogen Peroxide insult (1h incubation with 100 mM tbHP). Values are expressed as mean ± SD of three experiments. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ versus control cells, and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus tbHP-treated cells.

5.4 CONCLUSIONS

The results obtained in this study demonstrated that PB extracts can be effectively encapsulated in MD through spray-drying, resulting in low moisture content and high encapsulation efficiencies. The structural analysis of the encapsulated extract highlighted the presence of spherical-shape microcapsules containing PB phenolic compounds. The microcapsules obtained under the optimal conditions showed a high efficiency in the encapsulation of bioactive compounds,

preserving the high antioxidant and antimicrobial activity of these extracts. Moreover, the encapsulation demonstrated a protective effect on the phenolic compounds degradation (increased stability and bioaccessibility) during the digestion process, in particular in the oral and gastric phase of the gastrointestinal system. Finally, the MD encapsulation system was able to partially protect the extracts until the intestinal phase, potentiating effective delivery of the PB functionality in this final phase, where the phenolic compounds are absorbed. PBE also provided efficient protection of Caco-2 cells against oxidative stress, and their antioxidative activity was positively affected by encapsulation, increasing the possible therapeutic effects. Furthermore, as the encapsulation process was particularly efficient in protecting the extracts in a highly acidic environment, this system will be suitable for the enrichment of fermented foods or fruit-based beverages, among other foods. Concomitantly, aiming at food safety, the encapsulated extract showed high efficiency in protection against *L.innocua*, making it useful in protecting foods susceptible to this type of contamination. Overall, this system allows increased feasibility of the PBE by protecting and appropriately delivering the active compounds, and may find potential application as a natural preservative and/or antioxidant in food formulations or as bioactive ingredient with controlled delivery in pharmaceuticals or nutraceuticals.

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CHAPTER VI

INFLUENCE OF THERMAL AND ELECTRICAL EFFECTS OF OHMIC HEATING ON C-PHYCOCYANIN PROPERTIES AND BIOCOMPOUNDS RECOVERY FROM *SPIRULINA PLATENSIS*

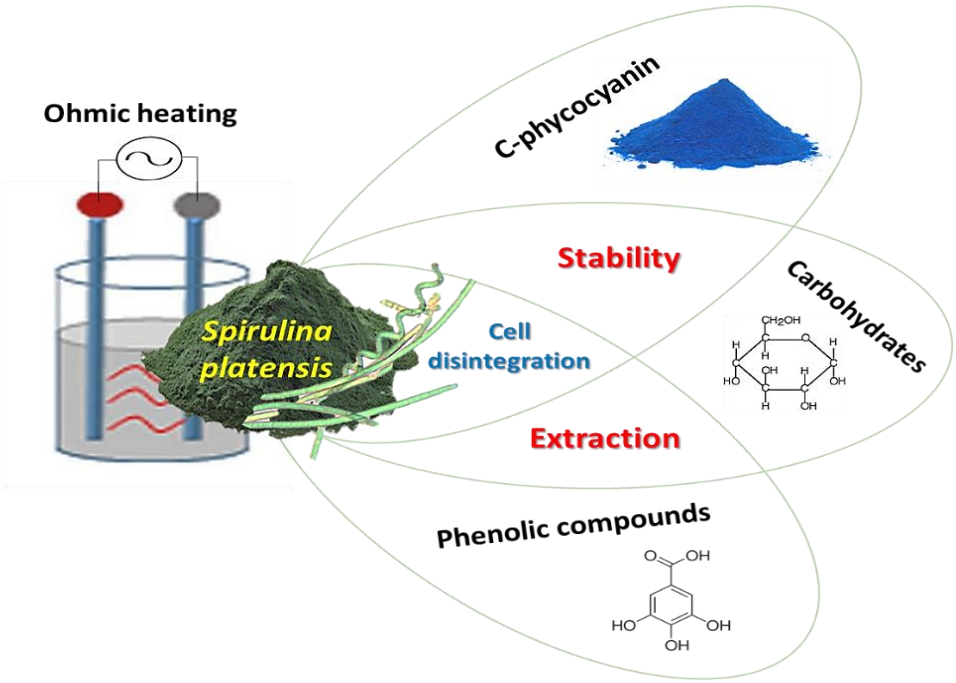
This chapter was based on the following paper:

Pedro Ferreira-Santos; Rafaela Nunes; Filomena De Biasio; Giorgia Spigno; Domenico Gorgoglione; José A.Teixeira; Cristina M. R. Rocha. **2020.** Influence of thermal and electrical effects of ohmic heating on C-phycocyanin properties and biocompounds recovery from *Spirulina platensis*. *LWT-Food Science and Technology*, 128, 109491. https://doi.org/10.1016/j.lwt.2020.109491.

Abstract

Spirulina platensis is interesting for the food industry due to its overall composition and high content in C-phycocyanin. However, the sensitivity of C-phycocyanin makes its extraction a delicate process. The present study focuses on assessing the use of OH in the recovery of C-phycocyanin and other relevant compounds as an alternative method to freeze-thawing or conventional heating. Different ohmic and conventional heating treatments were applied both to purified C-phycocyanin and *Spirulina* powder. Evaluation of fluorescence and circular dichroism showed that moderated electric fields increased C-phycocyanin stability. This was confirmed in the extraction trials which revealed that OH assisted extraction at higher temperatures (44 °C), and shorter times (30 min) allowed significant higher extraction yield of C-phycocyanin (45 mg/g_{dw} *Spirulina*), in comparison with conventional heating and freeze-thawing. OH allowed also up to 80% higher yields in phenolic compounds and carbohydrates.

Graphical Abstract



Keywords: *Arthrospira platensis*; moderate electric fields; C-phycocyanin; biocompounds extraction; cell disruption

6.1 INTRODUCTION

Spirulina (Arthrospira) platensis is a cyanobacterium that is arising attention in various industries and it is commonly used as food supplement due to its particular composition (e.g., proteins, lipids, carbohydrates, vitamins and fibers) and high AA (Andrade et al., 2019; Papadaki et al., 2017; Soni et al., 2017). Regarding *Spirulina* protein content, it is well known that these cyanobacteria have a high content in PC, a high commercial value blue pigment used as a natural colorant. This blue natural colorant has been widely used in nutraceutical, cosmetic and pharmaceutical industries and represents more than 20 % of *Spirulina* dry weight (Batista et al., 2013; Martínez et al., 2017; Sharma et al., 2016). PC is composed of two similar subunits of α-helix and β-sheets and should be easily isolated as a protein complex because it is water-soluble (Kamble et al., 2013; Vernès et al., 2015). On the other hand, though the protein fraction, and in particular C-phycocyanin, is the most explored fraction from *Spirulina platensis*, other compounds may also be interesting and potentially have high added value, such as phenolics and carbohydrates. These compounds may have important bioactive properties, such as antioxidant, antimicrobial, anticancer, prebiotic, antiadherent, etc., which make these compounds interesting for applications in the food, health and pharmaceutical industries (Dai and Mumper, 2010; Ferreira-Santos et al., 2019b; Mena-García et al., 2019).

However, pigments, phenolic compounds, carbohydrates and proteins are presented in cytoplasm or internal organelles which makes difficult their extraction due to the resistance of the cell wall (Chen et al., 2020; Moraes et al., 2011; Zhu et al., 2007). Although various cell disintegration technologies have already been described (e.g., maceration, bead-beating and freeze-thawing), these are time-consuming and often require high solvent and/or energy inputs which lead to an increase in the production costs and limiting their widespread application (Chittapun et al., 2020; Kamble et al., 2013; Kumar et al., 2014). Moreover, the PC extraction without affecting its physico-chemical properties is also a challenge due to its instability to light and heat (low denaturation temperature, i.e. about 45 °C), which makes extraction with traditional methods difficult (Chittapun et al., 2020; Falkeborg et al., 2018).

OH is a promising alternative technology for intracellular compounds' extraction, capable of promoting fast and homogeneous extraction with high energy efficiency. By applying a moderate electrical field through a semi-conductive material that offers resistance to the passage of electric current, heat is generated directly inside the volume of the material causing cell membrane rearrangement, resulting in pores formation (electro-permeabilization), and allowing intracellular

components diffusion (Ferreira-Santos et al., 2019b; Rodrigues et al., 2019). Furthermore, this "green" technology may allow to reduce the ecological impacts caused by the extraction processes, decreasing the water/solvent use, waste generation due to higher extraction yields, energy consumption and/or reducing processing times (Chemat et al., 2017; Rocha et al., 2018). Goettel, Eing, Gusbeth, Straessner, & Frey (2013) and 't Lam et al. (2017) studied other electrical technologies, such as PEF, for extraction of intracellular valuable compounds from microalgae (e.g., *Auxenochlorella protothecoides, Chlorella vulgaris* and *Neochloris oleoabundans*) and revealed that a spontaneous cell disintegration was achieved, with ionic substances, carbohydrates and proteins drained out of the cells.

Based on these premises, the objective of the present work was to find a cell wall disruption and extraction method that would cause the efficient recovery of *Spirulina* protein content without degrading their functional features. It was then essential to study the physicochemical stability of PC when subjected to different environmental conditions of time and temperature (and their combinations) and application of MEF, in order to tune the most efficient extraction method. Furthermore, the impact of the extraction technology in other intracellular compounds content such as phenolic compounds and carbohydrates was also assessed.

6.2 MATERIALS AND METHODS

6.2.1 Raw materials and reagents

Spirulina (Arthrospira) plantesis used in this work was grown, microbiologically controlled and dried at low temperatures in Azienda Agricola Prato della Voja (Bergamo, Italy) and provided by EVRA S.r.l. (Potenza, Italy). Purified PC (lyophilized powder, Sigma P2172, 30-50% protein – commercial product information) isolated from *Spirulina,* Folin-Ciocalteu reagent, sodium chloride, ethanol, sodium carbonate, sulfuric acid, phenol reagent, glucose, chloroform, methanol, nitric acid and hydrogen peroxide were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA).

6.2.2 Experimental plan

In the first part of the study, thermal stability of PC and the influence of EF on protein unfolding was investigated by different treatments. To this purpose, different time – temperature

combinations under both conventional and OH were applied to solutions of purified commercial PC. The protein solutions were analyzed for intrinsic fluorescence and circular dichroism (CD) before and after the treatments.

In the second part of the study, PC and other compounds (phenolics and carbohydrates) were extracted from *Spirulina platensis* powder using conventional heating and OH extraction process with the same time – temperature combinations used on the first part of the study (PC stability). Additionally, freeze-thawing process was applied as reference extraction process. The *Spirulina* powder was characterized for chemical and elemental composition, while the different extracts were analyzed for PC, total phenolic compounds and total carbohydrates content. The *Spirulina* powder before and after the extraction process was visualized by microscopy to observe cell disruption and disintegration.

6.2.3 Ohmic heating and conventional heating equipment

OH was conducted in a double-jacketed glass cylinder containing a stainless-steel electrode at each edge (Pereira et al., 2016a). The distance between electrodes is 2.4 cm and overall volume of the reactor is 20 mL. The temperature was controlled by regulating the voltage output of a function generator (Agilent 33220A, Penang, Malaysia), measured with a type K thermocouple (temperature precision of ± 1 °C; Omega Engineering, Inc., Stamford, CT, USA), located in the geometric center of the extractor's volume and connected to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA) and then amplified on an amplifier system (Peavey CS3000, Meridian, MS, USA). The conventional thermal treatments were conducted using the same apparatus, without the electric influence, and controlling the temperature with a thermo-stabilized bath (F25-ED, Julabo, Seelbach, Germany).

6.2.4 Treatments of c-phycocyanin solutions

A preliminary test (Lowry-TCA) was performed to determine the exact protein concentration in the commercial product. This initial concentration of PC (43% protein powder) was later used to calculate the concentrations of PC solution used in this work.

PC solutions were prepared at an initial concentration of 1 mg/mL using ultra-pure water at room temperature. Then, the solutions were diluted to 0.1 mg/mL with NaCl (0.02 M) to ensure a conductivity of 2 mS/cm. Final pH was checked and adjusted to 6.8 with sodium hydroxide, required for further tests. This pH value was selected considering that the PC is more stable at pH

6.8, taking into account works by other authors (İlter et al., 2018; Sivasankari et al., 2014; Vernès et al., 2015) and previous studies from our group (data not shown). For conventional heating treatment (0 V/cm), protein solutions were maintained in the apparatus previously described at different temperatures (30, 37, 44 and 51 °C) up to 60 min. The solution was maintained protected from the light and stirred, on a magnetic stirrer at constant stirring speed (150 rpm) to ensure homogeneity. For OH, the EF applied was chosen to achieve the same holding temperatures as for conventional heating (approximately 4, 5, 6 and 7 V/cm for 30, 37, 44 and 51 °C, respectively) and simulating exactly the same temperature profile for both processes (including initial transient heating period). These temperatures were used taking into account that the reported denaturation temperature of PC is approximately 44 °C (Falkeborg et al., 2018; Jespersen et al., 2005; Vernès et al., 2015), thus being able to study the influence of electric fields at temperatures lower and higher than the denaturation temperature. Frequency was set at 20 kHz in order to eliminate the electrochemical effects as electrolysis and electrode oxidation (Pataro et al., 2014).

During both ohmic and conventional heating, 1 mL of sample was taken after 15, 30 and 60 min and analyzed for intrinsic fluorescence and CD. All the experiments were performed in triplicate and at room temperature.

6.2.5 Assessment of intrinsic fluorescence

Fluorescence determination was assessed with an Aqualog 800 spectrofluorometer (HORIBA-Jobin Yvon, Inc. Japan) using a 1 cm quartz cell. All solutions were diluted to 5 μg/mL concentration. Samples were excited at 280, 295 and 609 nm and the emissions were recorded over the 280-795 nm range with an integration of 0.1 s and 3 accumulations for each sample with 1.7 nm increment. All determinations were run in triplicate and reported as averages of the triplicate spectra (Selig et al., 2018).

6.2.6 Circular dichroism spectroscopy

CD was used to investigate the PC secondary structure before and after the application of both the conventional heating and the OH. CD spectra were recorded with a spectropolarimeter (JASCO J-1500, Jasco Corp., Japan) at 20 °C under a constant nitrogen gas flow, using a 0.1 cm path length cell. The spectra were recorded between 190 and 260 nm with sampling points every 1 nm and three scans were accumulated and averaged for each sample (de Figueiredo Furtado et al., 2018; Janek et al., 2017). The spectra analysis was performed using CAPITO (CD Analysis and

Plotting Tool) online software, allowing to determine the fractions of secondary structure elements on the protein before and after the thermo-electric treatments (Wiedemann et al., 2013).

6.2.7 Biocompounds extraction from *spirulina*

6.2.7.1 Nutritional characterization of *Spirulina*

Spirulina total protein content was estimated by quantification of total nitrogen after sample acid digestion using a Kjeldahl digestor (Tecator, FOSS, Denmark), applying the nitrogen conversion factor (N × 6.25) (Graziani et al., 2013). Lipid content was evaluated through extraction with a Soxhlet apparatus using chloroform/methanol (2:1 (v/v)) at 150 °C for 12 h (Tibbetts et al., 2015). Ash content was determined gravimetrically through incineration of 5 g of *Spirulina* in a muffle furnace at 575 °C for 24 h. Moisture was determined gravimetrically by drying in an oven at 105 °C for 12-14 h (until constant weight). Total carbohydrates content was estimated by difference using Eq. 1 (Lu et al., 2010). Total dietary fiber content was determined by the AOAC 985.29 gravimetric method using a Megazyme® assay kit. Mineral content was determined using a method by ICP-AES, after *Spirulina* digestion with HNO_3 (65 %) and H_2O_2 (35 %) using a microwave apparatus (Batista et al., 2013).

$$\text{Carbohydrates (\%)} = 100 - (\text{moisture} + \text{ash} + \text{crude protein} + \text{crude lipid}) \quad (1)$$

6.2.7.2 Freeze-thawing extraction method

Spirulina powder was diluted in pure water at a solid/liquid ratio of 1:20 (2.5 g/ 50 mL), stirred at 150 rpm for 30 min and frozen at -20 °C for 4 h. Then, the solution was thawed for 1.5 h at room temperature (Vernès et al., 2015). This freeze-thawing process was repeated four times for each sample. Then, the solution was centrifuged at 847×g for 15 min and the supernatant was recovered and used for further analyses (PC concentration, total phenolic compounds and total carbohydrates content). This method was used as a reference method, as it is one of the most used for cell wall disruption and protein extraction from microalgae and cyanobateria (Sivasankari et al., 2014; Vernès et al., 2015).

6.2.7.3 Conventional heating and Ohmic heating methods

OH and conventional heating treatments were investigated as potential methods for cell disruption and extraction of intracellular compounds such as proteins, carbohydrates and phenolic

compounds. For the experiments, the same equipment as described above (section 6.2.3) was used with the same solid/liquid ratio of 1:20 g/mL as in the freeze-thawing method. This powder concentration ensured an electrical conductivity of 2 ± 0.2 mS/cm at room temperature. Thus, 15 mL of pure water was added to 750 mg of *Spirulina* powder and stirred at 150 rpm for 30 min. The extraction process was performed at 30, 37, 44, 51 °C as reported for PC at section 6.2.4. At different time intervals (30, 60, 90 and 120 min), 1 mL of sample was taken and treated and analyzed as reported for the freeze-thawing method.

6.2.7.4 C-phycocyanin quantification

PC concentration was determined using a UV-Vis spectrophotometer (V-560, Jasco Inc., Tokyo, Japan) at wavelengths 620 and 650 nm, since PC has a maximum absorbance in the visible range between 610 and 620 nm that is proportional to its concentration and 650 nm is the wavelength where the class of APC absorbs (Eq. 2) (Bekhit et al., 2019; Kissoudi et al., 2018). The PC recovery (Yield) as mg/g_{dw} of *Spirulina* was calculated using the initial mass of *Spirulina* used in the extraction process (Eq. 3).

$$\text{Concentration}\left(\frac{\text{mg}}{\text{mL}}\right) = \frac{((\text{A}_{620})\times \text{DF}) - (0.474 \times (\text{A}_{650} \times \text{DF})}{5.34} \quad (2)$$

$$\text{Yield}\left(\frac{\text{mg}}{\text{g}_{\text{dw}}}\right) = \frac{\text{Concentration}\left(\frac{\text{mg}}{\text{mL}}\right) \times \text{V}_{\text{solution}}}{\text{m}_{\text{Spirulina}}} \quad (3)$$

Where, A_{620} is the absorbance at 620 nm, A_{650} the absorbance at 650 nm, DF is the dilution factor, $V_{solution}$ and $m_{Spirulina}$ are the solution volume and mass of dry *Spirulina*, respectively.

6.2.7.5 Total phenolic compounds and carbohydrates quantification

The TPC content was determined as previously described (Ferreira-Santos et al., 2019b) using the Folin-Ciocalteu colorimetric assay, which is based on an oxidation-reduction reaction between the Folin reagent with the reducing phenolic compounds. For the analysis, 5 µL of each sample were mixed with 60 µL of Na_2CO_3 (75 g/L), 15 µL of Folin-Ciocalteu reagent and 200 µL of ultra-pure water and the mixture was kept at 60 °C for 5 min. The absorbance was measured at 700 nm using a UV/Vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., U.S.A.). The phenolic compounds content was calculated as GAE using a calibration curve prepared with

standard gallic acid (1500 to 50 mg/L, R^2 = 0.99). The concentration of the extracts was used to calculate the extraction yield expressed as mg GAE/g_{dw} *Spirulina*).

Total carbohydrates (TC) content was measured using the phenol-sulfuric acid method. For this, 50 µL of sample were mixed with 150 µL of sulfuric acid (96-98 % (v/v)). Then, 30 µL of 5 % phenol reagent were added and the final solution was heated for 5 min at 90 °C. After cooling at room temperature for 5 min, the absorbance was measured at 490 nm by microplate reader (Synergy HT, BioTek Instruments, Inc., U.S.A.) (Masuko et al., 2005). The carbohydrates quantification was made using a calibration curve prepared with glucose (600 to 10 mg/L, R^2 = 0.99). The concentration of glucose equivalents (GLcE) was used to calculate the extraction yield based expressed as mg GLcE/g_{dw} *Spirulina)*.

6.2.7.6 Optical and fluorescence microscopy

After the extraction process, fresh *Spirulina* samples were observed with an epifluorescence microscope BX51 (Olympus, Japan) using brightfield and fluorescence light. TRITC (tetramethylrhodamine), with 530 nm and 550-590 nm excitation and emission filters to visualize the PC pigment. Images were captured with DP72 digital camera (Olympus, Japan) at magnification 100X.

6.2.8 Statistical analysis

The extractions and analyses were performed in triplicate and the data is presented as mean ± SD values. GraphPad Prism® software (version 6.0; GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analyses. The ANOVA and the least significant difference test were used to determine statistically different values at a significance level of $p < 0.05$.

6.3 RESULTS AND DISCUSSION

6.3.1 Treatments of c-phycocyanin solutions

6.3.1.1 Fluorescence determination

The proteins exposure to different time-temperature combinations and electric fields may lead to irreversible changes in their secondary structure, which are reflected in changes in their physicochemical properties (e.g., intrinsic fluorescence).

Tryptophan (Trp) and Tyrosine (Tyr) are two amino acids that have intrinsic fluorescence properties and in the native folded state of a protein they are generally located within the core of the protein, whereas in a partially folded or unfolded state, they become exposed to solvent. Therefore, this indicates that they are sensitive to their environment changes and allows to know if proteins are folded or unfolded, associated with a decrease in the quantum yield and successive decrease in fluorescence caused by protein denaturation. Different excitation wavelengths were used: 280 nm, related to Trp and Tyr fluorescence; 295 nm that selectively excites Trp residues and 609 nm which could appear if some quenching process occurs (Rodrigues et al., 2019; Vernès et al., 2015). The emission wavelength was set between 615-795 nm, since PC emits fluorescence around 640 nm (Vernès et al., 2015). Thus, the fluorescence spectra of PC were determined after the application of thermal treatments using the thermal conventional and OH methods. The solution containing the untreated protein was used as a control.

For 280 and 295 nm wavelength, the fluorescence spectra (data not shown) presented a large amount of noise and it was not possible to visualize the peaks relative to the Trp and Tyr amino acids. On the other hand, a peak between 620-650 nm was observed in all samples, possibly due to the quenching process.

Figure 1 shows the evolution of fluorescence spectra as a consequence of thermal treatments with conventional or OH. The initial fluorescence of the untreated protein is approximately 9000 a.u. For conventional heating, it was verified that an increase in the severity of the treatment, in terms of both exposure time and temperature, leads to a decrease in the intrinsic fluorescence of PC. This decrease is clearly more accentuated for temperatures above 37 °C, particularly for higher times (60 min), which corroborates data from other authors who describe that the temperature of denaturation of PC is approx. 44 °C (Falkeborg et al., 2018; Jespersen et al., 2005). Martelli, Folli, Visai, Daglia, & Ferrari, (2014) have assumed a two-state protein folding and obtained a calculated denatured temperature of 57.5 ºC at pH 7, though changes in the normalized OD were visible after

45 ºC, in the conditions used. Conversely, for OH this significant reduction in the fluorescence intensity with temperature increase was not observed, leading to a protective effect of MEF against heating. For this reason, OH can be used as a promising tool for PC recovery, allowing the use of higher temperatures without affecting the protein physicochemical properties.

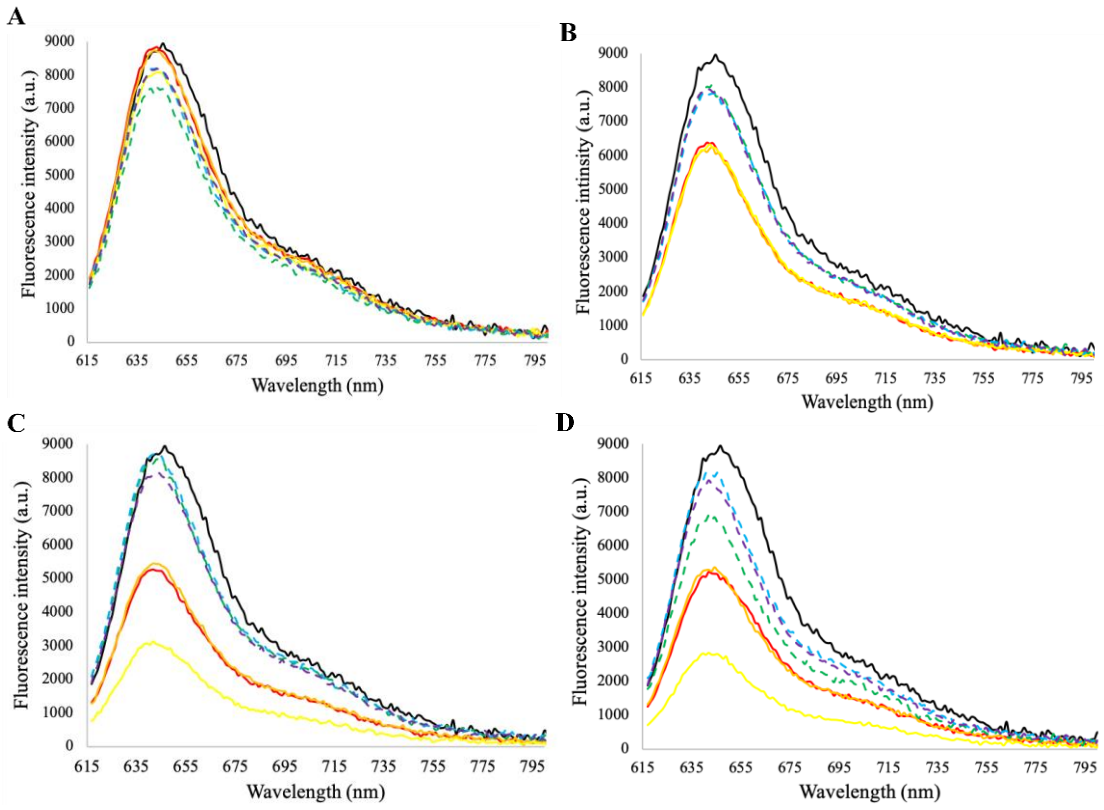


Figure 1. Fluorescence spectra of C-phycocyanin before (untreated) and after thermal treatments with or without the application of an electric field (conventional and OH, respectively) at different exposure times (15, 30, 60 min) and temperatures (30 °C: **A**; 37 °C: **B**; 44 °C: **C**; 51 °C: **D**). Excitation wavelength was 609 nm. ▬ Untreated; ▬ Conv 15 min; ▬ Conv 30 min; ▬ Conv 60 min; ▬ OH 15 min; ▬ OH 30 min; ▬ OH 60 min.

Other researchers have previously studied the effect of OH on milk protein stability. Rodrigues et al. (2019) studied the EF effects on β-lactoglobulin thermal unfolding and demonstrated that the OH treated samples presented significantly higher fluorescent intensities when compared to the conventional treatment, which reinforce our hypothesis that OH increases the thermal proteins stability. However, eventually OH applied at higher temperatures for shorter times may lead to similar or better results.

These tests with the commercial PC allowed to validate the experimental temperatures and times to be tested in the subsequent extraction assays.

6.3.1.2 Circular dichroism (CD) spectroscopy

It is expected that for temperatures above its denaturation temperature (45 °C), the PC secondary structure suffers irreversible changes (Jespersen et al., 2005). However, the application of PC as a colorant in food industry often requires protein to withstand the high temperatures and long times involved in the production processes. Thus, the application of electric fields using OH can be potentially used as an emerging technology to preserve protein secondary structure at elevated temperatures, that cannot be maintained when conventional treatments are used (Rodrigues et al., 2019).

CD spectroscopy was used to assess PC secondary structure and see how it changed with conventional and OH conditions. In this sense, CD spectrum of native PC in the UV region (190 to 260 nm) was used to monitor conformational changes in the polypeptide backbone major elements (i.e., α-helix and β-sheet) which have characteristic CD spectra.

The CD spectra profile for the protein in its native form showed a negative peak at 210 nm related to the α-helix structure and an intense peak at 190 nm related to the β-sheet configuration (de Figueiredo Furtado et al., 2018; Saraiva et al., 2017). The temperature increase caused changes in its secondary structure identified by the decrease in peak sharpness at 210 nm. On the other hand, it was observed an increase in the peak at 190 nm both in conventional and ohmic treatment (**Figure 2**). These results were reinforced by quantifying the protein secondary structures using the online software, which gave the conversion between α-helix and β-sheet structures over time. From **Table 1** it is observed that with conventional heating, both temperature increase and exposure time cause an increase in the percentage of irregular structures, which may occur due to a possible protein denaturation caused by a decrease in its stability. However, by applying a MEF to achieve heating, there is a decrease in irregular structures, an increase of β-sheet content and a decrease/absence of α-helix. Thus, it can be hypothesized that the use of OH gives greater protein stability (supported by the presence of a more defined secondary structure) in higher temperature environments, which under conventional heating would cause its denaturation.

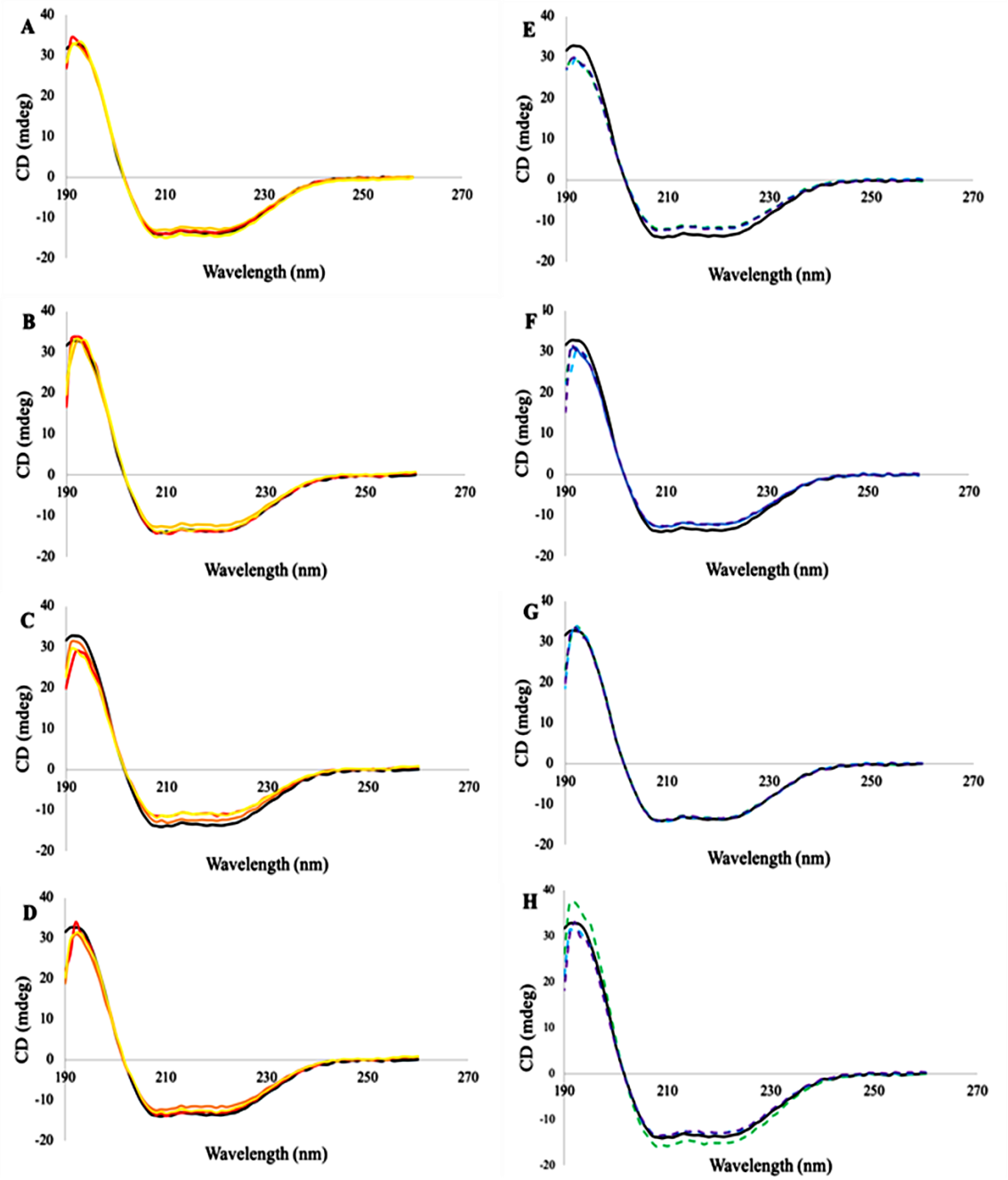


Figure 2. Circular dichroism spectra of C-phycocyanin before (untreated) and after thermal treatments with or without the application of an electric field (conventional treatment: **A**, **B**, **C**, **D** and ohmic heating: **E**, **F**, **G**, **H**) at different exposure times and temperatures (30 °C: **A**, **E**; 37 °C: **B**, **F**; 44 °C: **C**, **G**; 51 °C: **D**, **H**). ▬ Untreated; ▬ Conv 15 min; ▬ Conv 30 min; ▬ Conv 60 min; ▬ OH 15 min; ▬ OH 30 min; ▬ OH 60 min.

Table 1. C-phycocyanin secondary structure (α-helix, β-sheets and irregular structures) before (untreated) and after the application of thermal treatments (conventional treatment and ohmic heating) with different exposure times (15, 30 and 60 min) and temperatures (30, 37, 44, 51 °C).

Temperature (°C)	30			37			44			51			
Time (min)	15	30	60	15	30	60	15	30	60	15	30	60	
						Conventional							*Untreated*
α-helix	0.06	0.04	0.02	0.01	0.02	0	0	0.03	0.02	0.03	0.03	0.07	0.01
β-sheets	0.47	0.49	0.5	0.48	0.5	0.46	0.47	0.47	0.5	0.47	0.49	0.49	0.51
Irregular	0.51	0.51	0.53	0.5	0.54	0.53	0.54	0.53	0.49	0.54	0.52	0.52	0.52
						Ohmic heating							
α-helix	0.02	0	0	0	0	0	0	0	0	0.02	0	0	
β-sheets	0.52	0.57	0.61	0.55	0.59	0.64	0.52	0.58	0.62	0.53	0.57	0.62	
Irregular	0.5	0.5	0.49	0.48	0.47	0.44	0.5	0.47	0.47	0.51	0.51	0.44	

6.3.2 Extraction of c-phycocyanin from *spirulina*

6.3.2.1 Nutritional characterization of *Spirulina*

Spirulina is commonly used in various industrial sectors due to the compounds that it can supply, mainly because its high protein content and the ease that it is digested and absorbed by the organism (Vernès et al., 2015).

Table 2 summarizes the nutritional and elemental composition of *Spirulina* used in this research study. In addition to proteins, which represent about 57 % of its composition, *Spirulina* has other compounds of interest: lipids (11 %); carbohydrates (15 %); fiber (3 %) as well as minerals (K, P, Ca, Fe, Mg), so it is commonly used as a dietary supplement (Soni et al., 2017; Vernès et al., 2015). These results are in accordance with other chemical studies conducted on *Spirulina* grown elsewhere. In a study by Andrade et al., 2019 it can be observed that *Spirulina sp*. LEB 18 is a rich source of protein (aprox. 50 %), carbohydrates and lipids, obtaining values similar to those used in our study. In another work, about the nutritional composition of *Spirulina*, it was shown that it is not only a rich source of protein, but also in minerals, fatty acids, carotenoids and vitamins (Gutiérrez-Salmeán et al., 2015).

Table 2. Proximal (chemical and nutritional) and elemental (minerals) composition of *Spirulina platensis* used in this study.

Proximate composition (g/100g *Spirulina*)		Elemental composition (mg/g *Spirulina*)	
Protein	56.39 ± 0.22	0.99 ± 0.00	Calcium
Total carbohydrates	14.82 ± 0.20	3.40 ± 0.01	Magnesium
Dietary Fibre	3.08 ± 0.38	10.39 ± 0.22	Phosphorous
Lipids	11.32 ± 0.65	30.82 ± 0.31	Potassium
Ash	7.74 ± 0.09	17.45 ± 0.07	Sodium
Moisture	9.73 ± 0.03	0.02 ± 0.00	Zinc
		<DL	Copper
		0.36 ± 0.00	Iron
		0.02 ± 0.00	Manganese
		ND	Selenium

<DL, below detection limit; ND, not determined

6.3.2.2 C-phycocyanin extraction

As previously mentioned, the main objective of this study is to promote the efficient extraction of PC (blue colorant) without changing the characteristics of this compound/product, enhancing its application in the food industry.

The extraction of PC from microalgae has already been performed by various methods (e.g., physical, mechanical, chemical and biological). However, their total extraction is usually hampered by the rigidity of the cell wall (Vernès et al., 2015). Therefore, the application of strategies that allows full access to the intracellular medium is essential to facilitate the extraction of specific components. Although traditional methods (e.g., sonication) induce cell wall disruption, they have some drawbacks because of the use of a high energy consumption and long extraction time (Kissoudi et al., 2018; Vernès et al., 2015). On the other hand, cell disintegration should be done avoiding the use of processing conditions that could negatively affect the quality of the extracts and have a negative impact in the product value (Postma et al., 2016). Currently, one of the frequently used methods is the freeze-thawing process, in which the sample is subjected to successive freeze/thaw cycles that promote cell wall disruption, being a simple technique that does not imply the use of organic solvents. In the case of PC extraction, this method becomes even more advantageous as this is a water-soluble protein. Freeze-thawing extraction was then used as a conventional reference methodology, providing the expected maximum extractable amount of PC from *Spirulina* (41.90 ± 0.23 mg/g_{dw} *Spirulina*) (**Table 3**).

Table 3. Biocompounds recovery from *Spirulina* with freeze-thawing extraction method.

Compounds	Quantity
PC (mg/g_{dw} *Spirulina)*	41.90 ± 0.23
TPC (mg GAE/g_{dw} *Spirulina*)	9.08 ± 0.33
TC (mg GLcE/g_{dw} *Spirulina*)	26.01 ± 1.63

PC, C-phycocyanin; TPC, total phenolic compounds; TC, total carbohydrates

When conventional heating was applied as extraction method (**Figure 3.A**), yield of PC was always lower than with freeze-thawing. At 30 °C and 37 °C protein extraction increases as the exposure time increase ($p < 0.05$). At 44 °C, the maximum PC yield (29.36 ± 10.12 mg/g_{dw} *Spirulina*) is achieved in the first 30 min and does not significantly increase over time, which indicates that higher temperatures allow protein extraction in shorter time intervals (due to higher diffusion

coefficients). However, at 51 °C (above the denaturation temperature) a decrease in the protein yield is observed, probably due to protein denaturation.

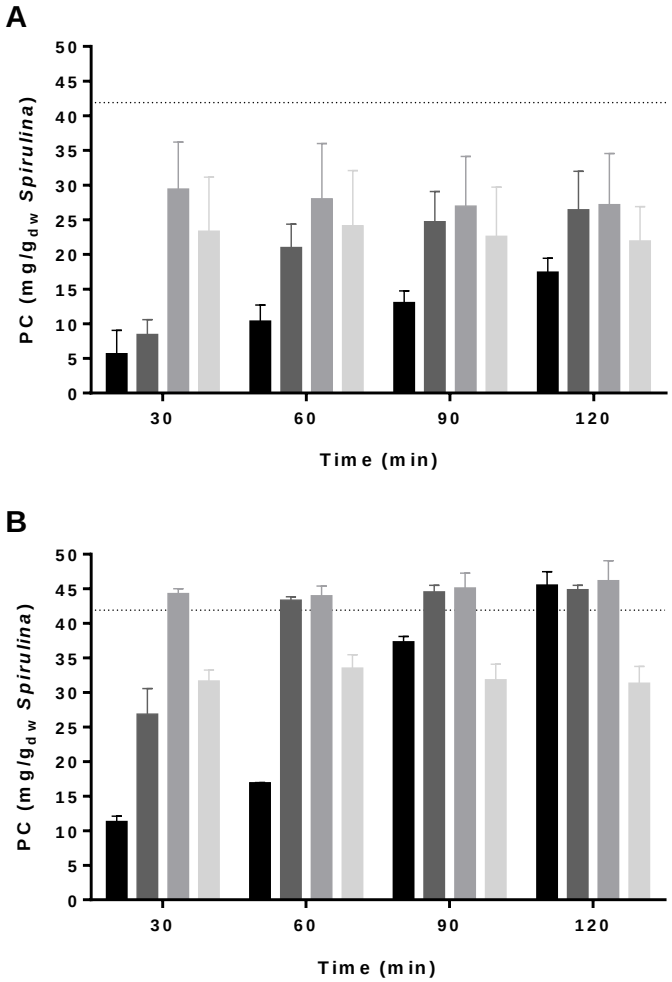


Figure 3. C-phycocyanin recovery (mg/g_{dw} of Spirulina) for conventional heating **(A)** and ohmic heating **(B)** according to different temperatures (30, 37, 44, 51 °C) and exposure times (30, 60, 90, 120 min). Error bars represent mean ± SD and the line in the graphs indicates the concentration value obtained using the freeze-thawing method. ■ 30 °C; ■ 37 °C; ■ 44 °C; ■ 51 °C.

Using OH (**Figure 3.B**) at 30 °C, the PC extraction increased with an increase in the exposure time and the maximum concentration (45.54 ± 1.93 mg/g_{dw} *Spirulina*) was achieved after 120 min and was above the value obtained with reference method. However, at 37 and 44 °C the maximum PC yield was achieved in the first 60 and 30 min, respectively. After 30 min at 51 °C, which is above PC denaturation temperature, the maximum extraction yield was 31.67 ± 1.59 mg/g_{dw} *Spirulina* and remained constant over the experiment time. This yield is lower compared to results at 37 and 44 °C, due to protein denaturation. However it is only around 25% lower when compared to freeze-

thawing extraction (where no denaturation problems are expected as no high temperatures where applied) and significantly higher than the obtained with conventional heating. PC is a phycobiliprotein, with two sub-units (α and β chains) and a chromophore (phycocyanobilin) is covalently attached to the apoprotein via cysteine residues (Martelli et al., 2014). PC is usually arranged in large granules and the position and intensity of the absorption maximum (that will also determine the overall color) is strongly dependent on the state of aggregation, that in turn is also dependent on pH and protein concentration (Jespersen et al., 2005). Therefore, it is expected that the protein denaturation will strongly influence the state of aggregation, on the one hand, and the protein solubility on the other (as more hydrophobic zones of the protein will be exposed). Therefore, this increase in the extraction yield observed for OH can be caused both by higher protein solubility in comparison with the CE, and by a higher amount of extracted protein with the blue coloring functionality active (as thus a higher amount of protein detected), as the quantification is colorometric. Furthermore, an electroporation effect may also be present, thus facilitating the migration of the extracted compound towards the solvent.

Looking at the obtained results, it is important to appreciate that in all the tested conditions, OH showed higher efficiency in protein extraction, compared to conventional heating, mitigating the negative effects caused by the temperature applied during the process.

The OH treatment increase the protein concentration with less time consumption and promotes the protein stability compared to the other studied extraction technologies (Figure 3). Moreover, it is known that OH technology reduces energy consumption by approximately half compared to the traditional heating process, demonstrating that OH is a more energy efficient process. These data have been previously reported by our group, both on a laboratory and industrial scale (Ferreira-Santos et al., 2019b; Pereira and Vicente, 2010). Freeze-thawing method is traditionally used, but it is a process that requires a lot of manpower and time/energy-consuming. On the other hand, the enzymatic process (with lysozymes) is efficient in the rupture of cyanobacteria cell wall and is suitable for large scale industrial protein extraction, but it is an expensive process (Moraes et al., 2011; Zhu et al., 2007).

Our results have been compared with those obtained in the literature with other methods such as sonication, glass bead homogenization and enzymatic action using lysozymes (Table 4). However, differences between methods and publications should be regarded with extra care. Extraction conditions are usually quite different and differences, for instance, in biomass to solvent ratios, type of solvent and type of biomass used (fresh *vs.* dried) can lead to huge differences in the overall

extraction results. Furthermore, the initial extractable amount of PC in the cells depend on the growth conditions and harvest stage, and absolute values may not be comparable. İlter et al. (2018) studied the PC extraction from *Spirulina platensis* using ultrasonication and MW extraction methods and obtained higher yields compared with our work (92.48 ± 0.35 and 96.65 ± 015 mg/g_{dw}, respectively). However, biomass to solvent ratios used were much lower (optima achieved with values ranging between 1 and 1.7 g/100 mL). Other researchers used electric technology (PEF) for PC recovery obtaining a maximum yield of 151.94 ± 14.22 mg/g, with fresh biomass and 1 g:1000 mL of biomass to solvent ratio (Martínez et al., 2017) and 25-85 mg/g (Jaeschke et al., 2019), depending on the EF applied, also with fresh biomass. These contents are in the range of those obtain in our work, being differences ascribed to the voltage intensity applied in the extraction process (25 kV/cm to 40 kV/cm used in PEF and a maximum of 7 V/cm in OH) and to different biomass processing and biomass to solvent ratios. However, these comparisons should be carefully made as it would be necessary to take into account the chemical composition of used *Spirulina* and express the results also as percentage recovery of total protein content.

Table 4. Comparation of literature C-phycocyanin extraction methods in terms of extract concentration and overall yield.

Extraction method		PC concentration (mg/mL)	PC yield ($mg/g_{spirulina}$)	Temperature (°C)	Time (h)	Solid:liquid ratio (w:v)	References
Biological	Lysozyme	4.08 ± 0.08	———	RT	24-30	1:40	Zhu et al., 2007
		———	< 2	RT	ND	ND	Moraes et al. 2011
		0.230	———	30	25	1:25	Izadi & Fazilati, 2018
Chemical	Sodium phosphate	0.60 ± 0.04	———	ND	24	1:25	Nagar, Sharma, & Kumar, 2018
		———	137 ± 0.27	ND	ND	ND	Saran, Puri, Jasuja, & Sharma, 2016
		4.20 ± 0.72	———	RT	24	1:25	Silveira et al. 2007
	Sodium chloride	3.32 ± 0.26	———	30	24	1:25	Silveira et al. 2007
	Distilled water	3.73 ± 0.12	———	30	24	1:25	Silveira et al. 2007
		———	88.00 ± 0.11	ND	ND	ND	Saran et al., 2016
		0.55 ± 0.03	———	42	ND	1:25	Nagar et al., 2018
	Calcium chloride	3.48 ± 0.74	———	30	24	1:25	Silveira et al. 2007
	Sodium acetate	1.84 ± 0.23	———	30	24	1:25	Silveira et al. 2007
	Hydrochloric acid	———	< 2	RT	ND	ND	Moraes et al. 2011
Physical	Maceration (with sodium phosphate)	0.61 ± 0.03	———	4	24	1:25	Kamble et al.2013
	Maceration (with water)	0.57 ± 0.05	———	4	24	1:25	Kamble et al. 2013
	Microwaves	———	96.65 ± 015	40 ± 2	0.5	1:100	İlter et al. 2018
	Sonication	2.13 ± 0.09	———	RT	0.66	1:40	Zhu et al., 2007
		———	92.48 ± 0.35	25 ± 2	0.25	1.100	İlter et al., 2018
		0.25 ± 0.04	———	ND	0.67	1:25	Nagar et al., 2018
		0.26 ± 0.05	———	ND	0.67	1:25	Kamble et al. 2013
		0.238	———	4	>25	1:25	Izadi & Fazilati, 2018
		———	0.57	ND	ND	ND	Moraes et al. 2011
	Glass bead grinding	2.25 ± 0.10	———	RT	4	1:40	Zhu et al. 2007
	Pulsed electric field	———	151.94 ± 14.2	40	2.5	1:1000	Martínez et al. 2017
		———	25-80	25	30	ND	Jaeschke et al., 2019
	Homogenization	———	94.13 ± 0.61	25 ± 2	0.33	1:100	İlter et al. 2018

		———	19.66	ND	ND	ND	Saran et al., 2016
	Freeze-thawing	———	146	-20/4	9	ND	Saran et al., 2016
		———	29.66 ± 0.52	-20/RT	72	1:50	Chittapun et al., 2020
	Water batch	1.83 ± 0.54	29.36 ± 10.12	44	0.5	1:20	
	Freeze-thawing	2.10 ± 0.01	41.90 ± 0.23	-20/RT	22	1:20	Current study
	Ohmic heating	2.28 ± 0.10	45.54 ± 1.93	44	0.5	1:20	

RT, room temperature; ND, not defined.

6.3.2.3 Total phenolics and carbohydrates extraction

Though the extraction of PC to be used in food or pharmaceuticals formulations as natural dye was the main focus of this study, it is interesting to study also the effect of the extraction technology in other compounds with potential high-added value present in this cyanobacteria. The extraction and quantification of these compounds, such as phenolics and carbohydrates, provides information on the potential use of OH to extract also other compounds using *Spirulina* as raw material, considering the concept of circular economy and zero waste generation.

Phenolic compounds and carbohydrates (in addition to proteins) are also found in the intracellular environment ('t Lam et al., 2017). Thus, the disruption and extraction process would allow to obtain not only proteins, but also other intracellular compounds, that can also be interesting. The extraction results of TPC and TC for the freeze-thawing, conventional thermal and OH-assisted methods, are presented in **Table 3**, **Figure 4** and **Figure 5**.

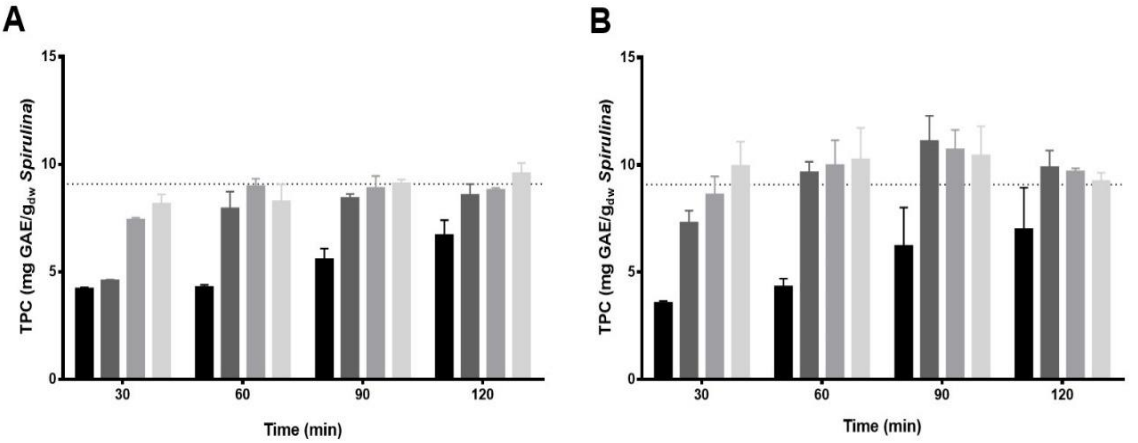


Figure 4. Total phenolic compounds (TPC, mg GAE/g_{dw} Spirulina) for conventional thermal treatment (**A**) and ohmic heating (**B**) at different temperatures (30, 37, 44, 51 °C) and exposure times (30, 60, 90, 120 min). Error bars represent mean ± SD and the line in the graphs indicates the concentration value obtained using the freeze-thawing method. ■ 30 °C; ■ 37 °C; ■ 44 °C; ■ 51 °C.

It is evident that the amount of TPC extracted by freeze-thawing method is similar to that obtained with conventional thermal extraction. The maximum phenolic compounds recovery (11.11 ± 1.17 mg GAE/g_{dw} *Spirulina*) obtained using OH at 37 °C for 90 min, was 24 % and 18 % higher than using conventional thermal treatment at same conditions (8.43 ± 0.18 mg GAE/g_{dw} *Spirulina)* and freeze-thawing method (9.08 ± 0.33 GAE/g_{dw} *Spirulina)* ($p < 0.05$), respectively (**Figure 4**).

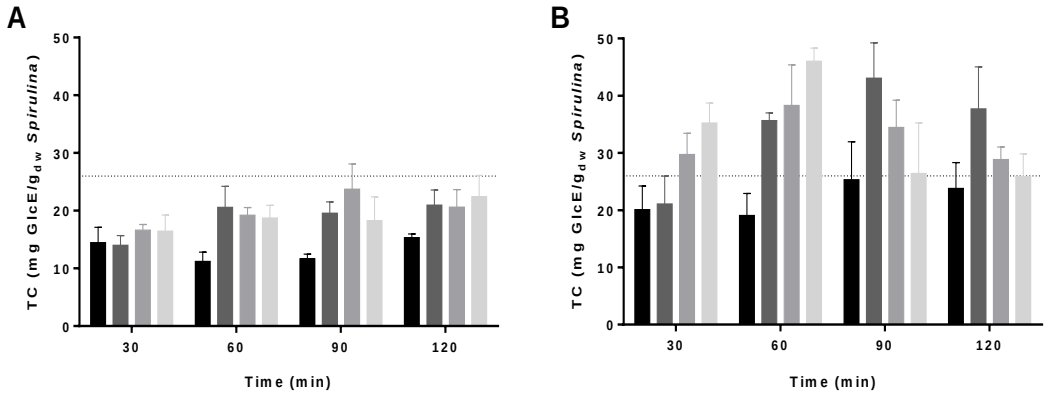


Figure 5. Total carbohydrates quantification (TC, mg GLcE/g_{dw} Spirulina) for conventional thermal treatment (**A**) and ohmic heating (**B**) subjected to different temperatures (30, 37, 44, 51 °C) and exposure times (30, 60, 90, 120 min). Error bars represent mean ± SD and the line in the graphs indicates the concentration value obtained using the freeze-thawing method. ■ 30 °C; ■ 37 °C; ■ 44 °C; ■ 51 °C.

Furthermore, the thermal treatment using electric fields (OH assisted extraction) also extracted higher amounts of TC at 51 °C for 60 min (46.01 ± 2.31mg GLcE/g_{dw}) comparatively with the maximum extracted for conventional thermal and freeze-thawing methods (23.71 ± 4.38 and 26.01 ± 1.63 mg GLcE/g_{dw} *Spirulina*, respectively), representing an increase of approx. 73 % (**Figure 5**). In view of this, OH assisted extraction demonstrates a significant increase ($p < 0.05$) in both carbohydrates and phenolic compounds recovery when compared with the conventional methods. The application of electric fields for biocompounds recovery from microalgae similar to cyanobacterium *Spirulina* was already studied by Postma et al. (2016), using PEF . Their results showed that a small fraction (< 5 %) of the TC present in the microalgae *Chlorella vulgaris* was released between 25-55 °C using conventional treatments, while using PEF treatment the TC yields were between 22-25 %. Also, Chaiklahan *et al.* (2013) studied the optimization of polysaccharides extraction from *Spirulina sp.* and revealed that the main sugar present in *Spirulina* extracts is glucose.

In this paper the TPC was also quantified, resulting in 12.88 ± 0.14 mg GAE/g sample for extracts produced at 50 °C, values in accordance with our results. On the other hand, İlter et al. (2018) studied the influence of different methods in the recovery of phenolic compounds from *Spirulina platensis* and obtained a concentrations of 226.40 ± 7.35 mg GAE/L using an Turrax homogenizer, 368.24 ± 0.49 mg GAE/L using ultrasounds and 183.12 ± 1.75 mg GAE/L using MW. These results are lower than the maximum concentration obtained in this study: 453.92 ± 16.67, 484.89

± 53.17 and 699.54 ± 166.06 mg GAE/L using freeze-thawing, conventional thermal treatment (at 44 °C, 60 min) and OH (at 37 °C, 120 min), respectively.

It is worth mentioning that when phenolic compounds and carbohydrates are analyzed by UV-visible spectrophotometry, they absorbe at wavelengths below 600 nm (Cheung et al., 2019; Olennikov and Tankhaeva, 2006). As mentioned previously, PC obtains its maximum absorption between 610 and 620 nm, which is proportional to its concentration and 650 nm is the wavelength at which the class of APC absorbs (Vernès et al., 2015). In this sense, it is assumed that the presence of these biocompounds does not affect the quantification of PC.

On the other hand, with these results (PC, TPC and TC), we can propose the use of multi-functional extracts, or after a separation process, take advantage of the various fractions for industrial applications. For example, phenolic compounds are widely used in the food industry as preservatives because they are antioxidant and antimicrobial agents. On the other hand, carbohydrates have prebiotic capacity and can be used as nutraceuticals.

Thus, it can be commented that OH is a versatile and suitable technique for obtaining various types of biocompounds from these cyanobacteria, allowing to reduce the extraction time and increase the concentrations, compared to the traditional methods used.

6.3.2.4 Microscopy analysis

Spirulina in its natural form (wet state) presents multicellular filaments like regular spirals wrapped. However *Spirulina* is usually processed in the dry form. Dry *Spirulina* presents shorter filaments due to fragmentation after the harvest treatment, without affecting the cell structure. Before the application of thermal treatments, which always involve the rehydration of *Spirulina*, slight filaments fragmentation occurs, leading to a loss in the regular and complete spirals structure and giving origin to filaments with different sizes. Furthermore, prior to thermal extraction, some membrane proteins may solubilize, facilitating the exit of compounds (Vernès et al., 2019).

In this work, this was our starting point for processing, as all treatments were tested in rehydrated samples **(Figure 6. A** and **E)**. From the optical microscopy images (**Figure 6. A**, **B**, **C** and **D**) we can observe a fragmentation of filaments and also a clear disintegration of the *Spirulina* cell wall, especially when the freeze-thawing method is applied (process that takes a long time; in this case it took approx. 22h) and OH (30 min at 44 °C), represented in **Figure 6. B** and **D**, respectively. The appearance of much smaller and less colored (less greenish) fragments is visible and this should facilitate the process of intracellular biocompounds extraction. This breakdown in the cell

structure is also observed, although less intense, after conventional heating at 44 °C for 30 min in comparison with the original *Spirulina* structure (**Figure 6. A**).

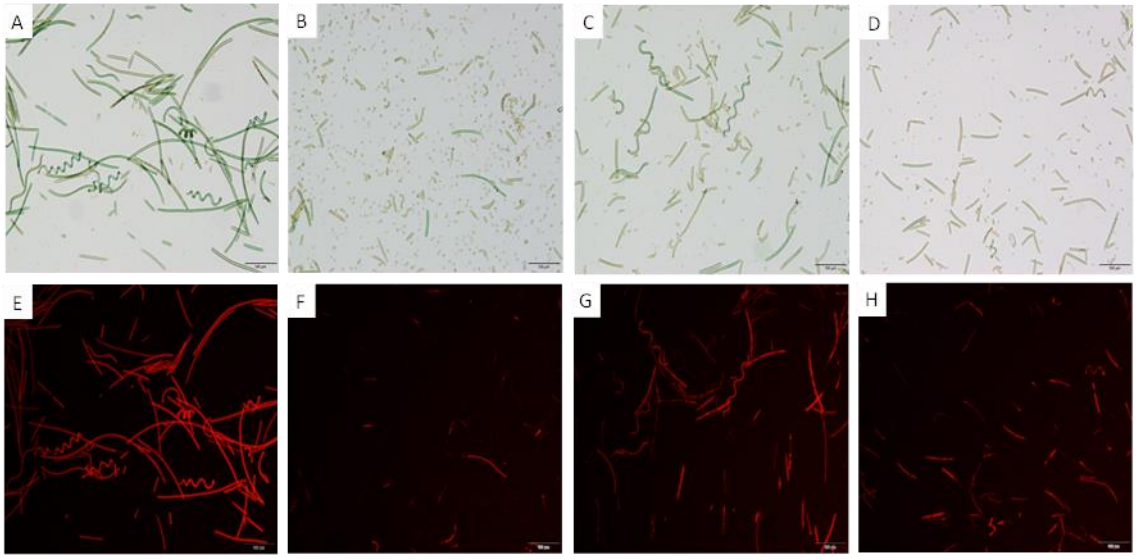


Figure. 6. Images of optical (**A**, **B**, **C**, **D**) and fluorescence (**E**, **F**, **G**, **H**) microscopy (100X) of untreated (**A**, **E**), freeze-thawing treated (**B**, **F**), conventional thermal treated (**C**, **G**) and OH treated (**D**, **H**) cells of *Spirulina platensis*. Red images represent the cyanobacteria autofluorescence using TRITC filter. Scale bar of 100 µm applies to all images.

When aqueous *Spirulina* solutions are observed by fluorescence using a bright orange-fluorescent dye (TRITC) (**Figure 6. E**, **F**, **G** and **H**), we can observe that the characteristic cyanobacteria autofluorescence (in red) caused by the existence of phycobilins in the cell cytoplasm, is altered after different thermal processes which cause the extraction of PC and other intracellular compounds. Clearly, untreated *Spirulina* has a much higher fluorescence intensity than samples treated with thermal methods (see **Figure 6. F**, **G** and **H**). These results confirm the above reported data of C-phycocyanin, which corroborate that the OH and the freeze-thawing method demonstrate greater efficiency in the extraction yield when compared to the conventional thermal method.

In comparison with the conventional thermal heating, OH clearly provoked a higher degree of disruption and disintegration, indicating that temperature alone is not the only cause for this and an electric effect must be present. Furthermore, this disruption resulted in much smaller and more homogeneous filamentous fragments, which may indicate that the electric current may have destabilized the cyanobacterium macrostructure also at specific more susceptible sites, and not only by non-specific thermal effects. This effect may also be the cause for the differences between the freeze thawing and OH. In fact, though freeze thawing seems to induce higher disintegration

degree, OH disintegration seems to be slightly more homogeneous, with a narrower range of fragments size.

Other authors have also studied processes to promote *Spirulina* cell wall rupture. Recently, Martínez et al. (2017) used PEF and observed by light microscopy that the *Spirulina* cell wall is fragmented when the applied energy is high (25 kV/cm), obtaining extracts rich in PC. They have also concluded that, while other technologies induce unspecific and, sometimes complete, cell destruction, PEF treatment caused only the destruction of the macrostructural cylindrical filaments of *S. platensis* (trichomes), but not so much of the cells itself, which may also be the case here. If this is the case, then there is also probably an electroporation effect on the cells as OH drove to higher extraction yields, under certain conditions, of PC in comparison with freeze thawing, though the structural disintegration was much higher in this last case.

Thus, OH method can be used to promote mild disruption, fragmentation and disintegration of cyanobacteria cell's structure while inducing electro-permeation effects, and clearly shows very promising results for using this technique to obtain high value-added products (such as natural colorants).

6.4 CONCLUSIONS

This study investigated the possibility of using OH assisted extraction for the recovery of PC *Spirulina.* Results showed that MEF associated to OH and extraction temperature below 51 °C, do not induce deleterious effects on PC, providing indeed an additional protection against protein denaturation. Therefore, they could be successfully applied in the extraction of this compound from *Spirulina*, with increases of ca. 36 % in the protein yield comparatively to conventional heating.

Using OH the PC stability increases when subjected to adverse temperature conditions without affecting its physicochemical properties (i.e., fluorescence and secondary structure). The PC was recovered with high yield (45.54 ± 1.93 mg/g_{dw} *Spirulina*) over a short period of time (30 min at 44 °C or 60 min at 37 °C) when compared to the conventional heating (maximum of 29.36 ± 10.12 mg/g_{dw}) and freeze-thawing (41.90 ± 0.23mg/g_{dw}) methods. The application of MEF allowed also the extraction of other valuable intracellular compounds, such as phenolic compounds and carbohydrates. Microscopic observation confirmed the effect of temperature associated with MEF promotes cell structure disintegration and electroporation effects facilitating the extraction of intracellular compounds.

Thus, it can be concluded that the OH treatment may improve intracellular compounds extraction, and reduce the process time, water and energy consumption compared to freeze-thawing and conventional heating based methods. This technique shows potential use at industrial scale to obtain high value-added products from cyanobacterium/microalgae species.

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CHAPTER VII

SEQUENTIAL MULTI-STAGE EXTRACTION OF BIOCOMPOUNDS FROM *SPIRULINA PLATENSIS*: COMBINED EFFECT OF OHMIC HEATING AND ENZYMATIC TREATMENT

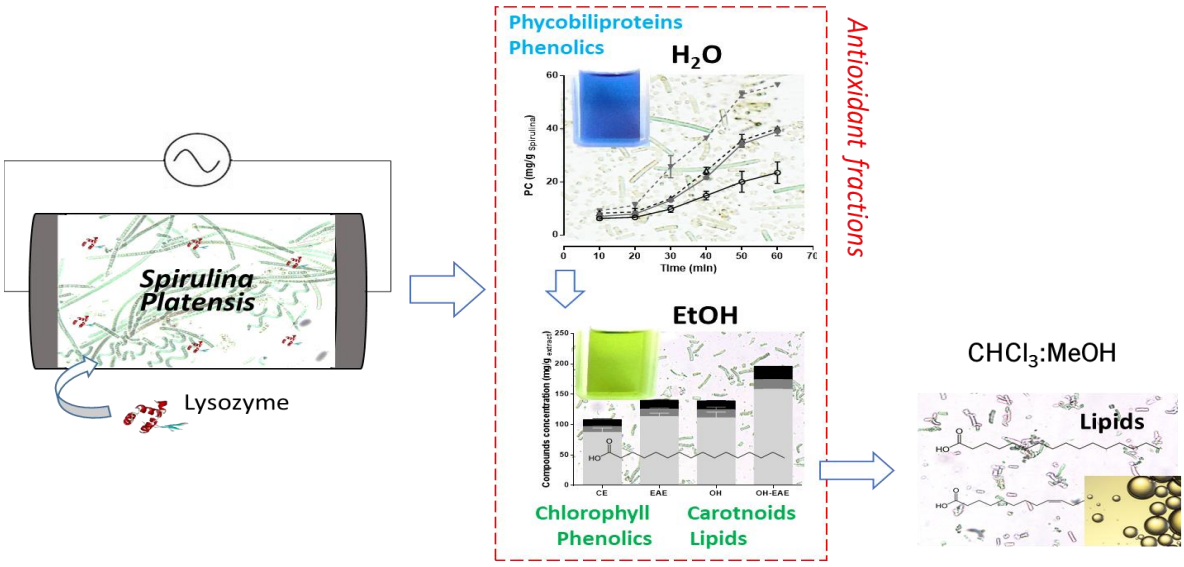
This chapter was based on the following submitted paper:

Pedro Ferreira-Santos, Sílvia M. Miranda, Isabel Belo, Giorgia Spigno, José A. Teixeira, Cristina M.R. Rocha. Sequential multi-stage extraction of biocompounds from *Spirulina platensis*: combined effect of ohmic heating and enzymatic treatment. *Innovative Food Science and Emerging Technologies* (submitted)

Abstract

A sequential multi-stage aqueous and non-aqueous extraction procedure was applied to recover biocompounds from *Spirulina platensis*, known as a source of protein, natural food-grade blue colorant and food supplements. The process consisted in three steps: an aqueous thermal treatment, using conventional extraction (CE, 0 V/cm), ohmic heating (OH, 7 V/cm), enzymatic treatment (EAE, 0.8 mg Lysozyme/mL), or their combination (1st step); ethanolic extraction (2nd step); $CHCl_3$/MeOH extraction (3rd step). The results evidenced that the combined OH and EAE extraction allowed selective recovery of phycobiliproteins in the 1st step, with increments of more than 100% in yields in comparison with CE, and more than 40% in comparison with non-combined OH and EAE. Pigments, such as chlorophyll and carotenoids were selectively extracted with 95% ethanol, as well as the lipid fraction of the microalgae. Moreover, the combination of OH and EAE in the first phase resulted in higher levels of extracted compounds in the following phases compared to processes using non-combined technologies. Overall, our results demonstrate that the intensification and integration of extraction steps facilitates the use of environmentally friendly technologies and non-toxic (bio)solvents in a multi-stage process capable of recovering and isolating different fractions with bio-functional properties, targeting zero waste and circular economy. The obtained fractions can be used as natural technological additive (e.g. blue colorant or antioxidant) and/or functional ingredient for food applications.

Graphical Abstract



Keywords: electric fields; lipids; lysozyme; phycobiliproteins; antioxidant activity; pigments

7.1 INTRODUCTION

Microalgae use have recently emerged in response to the uprising energy crisis, climate change and depletion of natural sources. Moreover, microalgae are a potential biomass feedstock due to their capability of producing compounds of great economic value, including antioxidants, dyes, proteins, phycocolloids, lipids, carbohydrates and vitamins with applications in foods, cosmetics, pharmaceuticals and biofuels industries. Microalgae can be used in a biorefinery process, exploiting sequentially different valuable fractions and thus contributing to the circular economy (Chew et al., 2017).

Spirulina (Arthrospira) platensis is a photosynthetic cyanobacterium (usually considered a microalgae) that is produced commercially for food use, as a dietary supplement and food additive due to its particular composition (*e.g.* higher content in protein, carbohydrates, lipids, etc) (Ferreira-Santos et al., 2020b). Furthermore, *Spirulina* proved biological functionality such as antiviral, anti-inflammatory, anti-cancer and AA and supposed health benefits (Karkos et al., 2011). Because of its physiological benefits and nutritional potential on people and animals, *Spirulina* is claimed to be an ideal food and dietary supplement in the 21st century by Food and Agriculture Organization of the United Nations and World Health Organization (Wang and Zhao, 2005).

In the last decades, the recovery of dyes with high pharmaceutical and food potential and lipids for the production of biofuel has been one of the main attractions for both research and the industry using microalgae as feedstock. In particular, PC from *Spirulina platensis* is commercially available as a natural blue colorant.

Concomitantly, alternative and more environmentally friendly methods should be considered to intensify the processes for obtaining microalgae (bio)products, taking into account the quality of the final product of interest. Several "green techniques", such as MW, US, electrotechnologies, enzymes, etc., have been applied to facilitate the cell wall disruption and obtaining biocompounds from different species of microalgae (Azmi et al., 2020; Lee et al., 2017). Enzyme-assisted extraction (EAE) with lysozyme and a combination with mechanical grinding has been used to disintegrate cell walls and improve the efficiency of pigment extraction (Sobiechowska-Sasim et al., 2014). Moreover, in a work by Chia et al. (2019) the influence of different cell disruption methods were studied, such as sonication, microwave, homogenisation and freeze-thawing, to maximise the recovery and purity of PC from *Spirulina* sp. Recently, our research group has proposed the use of OH, as a promising technology for obtaining PC and other biocompounds from *Spirulina* powders (Ferreira-Santos et al., 2020b).

Combination of different technologies has emerged to increase and accelerate the extraction of different intracellular compounds and make the process more efficient and with less energy consumption (Wen et al., 2020). In this sense, sequential extraction of different recoverable fractions, in a perspective of the full use of the bioresources, with minimal or zero waste generation is the objective to be achieved. However, at present time the available information about the application of selective multi-stage processes for integral extraction of biocompounds from *Spirulina platensis* is still scarce.

Thus, the objective of the present work was to investigate the influence of OH, EAE and combination of these two technologies on the selective recovery of phycobiliproteins from *Spirulina platensis* as a first stage of a multi-step extraction process for the integral valorization of the remaining fractions. The efficiency of recovery of bioactive and functional phycobiliproteins, phenolics, pigments and lipids at different stages of the extraction process was evaluated.

7.2 MATERIALS AND METHODS

7.2.1 Raw materials and Chemicals

Spirulina platensis biomass used in this work was grown, microbiologically controlled and dried at low temperatures in Azienda Agricola Prato della Voja (Bergamo, Italy). Folin-Ciocalteu reagent, sodium chloride, ethanol, sodium carbonate, sulfuric acid, phenol reagent, glucose, chloroform, methanol, DPPH, Lysozyme from chicken egg white (≥ 40,000 units/mg protein, EC number 3.2.1.17) and all standards (purity level above 94%) for HPLC and GC were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). Reagents were analytical grade, and ultra-pure water was used throughout the experiments.

7.2.2 Experimental plan

Figure 1 presents schematic of multi-stage extraction procedures for biocompounds recovery from *Spirulina platensis*. The multi-stage extraction procedure included three steps, using solvents with different polarity to extract different fractions. The 1st step included aqueous extraction assisted by electric fields (OH), enzyme (EAE) and OH combined with EAE treatment (OH-EAE). Treatments conducted without the electric and enzyme influence were used as control experiments (CE). The 2nd step included ethanolic (95%, v/v) extraction using dried residual biomass from 1st step. The 3rd

extraction step was done using chloroform/methanol ($CHCl_3$:MeOH) mixture (2:1, v/v) using dried residual biomass from 2nd step.

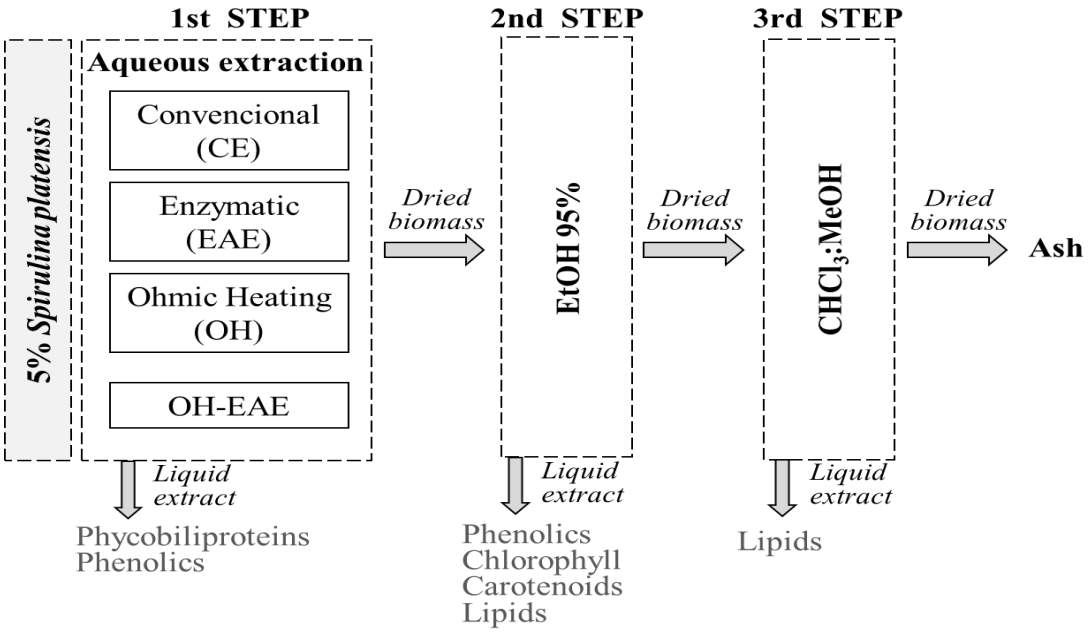


Figure 1. Schematic presentation of sequential multi-stage extraction procedures.

7.2.2.1 1st Step

7.2.2.1.1 Extraction reactor

The extraction was conducted in a double-jacketed glass cylinder (3 mm of internal diameter and 100 mm height) containing a stainless-steel electrode at each edge (distance between electrodes is 2.8 cm) with overall volume of the reactor is 25 mL (Pereira et al., 2016). For OH experiments, the power source working with a sinusoidal wave at 20 kHz (Agilent 33220A, 1 Hz-25 MHz and 1-10 V; Penang, Malaysia) allowed changing the voltage. For the conventional thermal treatment (without the electric influence), a thermostatic circulator water system (F25-ED, Julabo, Seelbach, Germany) was used to get the same heating rates in all types of treatments. Temperature was recorded with a type-K thermocouple (temperature precision of ±1 °C; Omega Engineering, Inc., Stamford, CT, USA), located in the geometric center of the reactor. The thermocouple was connected to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA) and Lab View 7 Express software (National Instruments, NI Data logger) was used to extract the data. A portable oscilloscope (ScopeMeter® 125/S, Fluke, WA, USA) was used to measure electrical frequency, voltage and current intensity during OH treatments.

The reactor was covered properly to avoid the direct incidence of light.

7.2.2.1.2 Extraction conditions

For extractions, 2 g of dried *S. platensis* was mixed with 40 ml of distilled water in a reactor (described above) with an agitation rate of 170 rpm. This powder concentration ensured an electrical conductivity of 2 ± 0.2 mS/cm at room temperature.

The optimal parameters of the extraction procedures were selected using the previous results (Ferreira-Santos et al., 2020b) and preliminary EAE experiments for PC recovery (data not shown). The OH treatments were conducted with the electric influence (7 V/cm) and CE conducted without the electric influence (0 V/cm). For EAE, 0.8 mg/mL of lysozyme was used. The extraction process was performed at 37 °C for different times (10, 20, 30, 40, 50 and 60 min). All extracts were centrifuged (9500 xg for 10 min) and the content of phycobiliproteins, soluble phenolics and carbohydrates were analyzed. The remaining exhausted biomass was then freeze-dried and subjected to a subsequent step.

7.2.2.2 2nd Step

For this step, the extractions were carried out using 1.5 g of dried biomass from 1st step mixed with 30 ml of ethanol 95% (v/v) for 60 min at 60 °C in a cylindrical reactors duly protected from light. A thermostatized water bath (SW22, Julabo, Seelbach, Germany) with shaking (170 rpm) was used to maintain the extraction temperature. All extracts were centrifuged (9500 g for 10 min), filtered (Whatman n° 2) and the content of chlorophyll (a and b), carotenoids, polyphenols, carbohydrates and residual lipids were analyzed. Lipids were re-extracted with a modification of the Bligh and Dyer method (Bligh and Dyer, 1959) using 2 mL of each ethanolic extract previously dried by nitrogen atmosphere.

The remaining exhausted biomass was then freeze-dried and subjected to a subsequent step.

7.2.2.3 3rd Step

The remaining lipids extraction was carried out by applying a modification of the Bligh and Dyer method (Bligh and Dyer, 1959), using 300 mg of the dried biomass from 2nd step with 5 mL of $CHCl_3$:MeOH (2:1 v/v) for 6 h at room temperature and agitation of 200 rpm. The lower organic phase was collected and and re-extracted 3 times with the same ratio of chloroform-methanol. Finally, the resulting organic phase was filtered and the total lipid content was determined gravimetrically after evaporation of organic solvents using a nitrogen atmosphere.

7.2.3 Extracts analysis

7.2.3.1 Extraction yield

The extraction yields of the various steps is a measure of the solvent efficiency to extract specific components from the original dry material. Yield calculation (presented in %) was based on the cumulative mass of extract with the following equation (Eq. (1)):

$$\text{Yield } (\%) = \frac{\text{extracted solids (g)}}{\text{initial dry material (g)}} \times 100 \quad (1)$$

7.2.3.2 Phycobiliproteins quantification

Phycobiliproteins concentration was determined using a UV-Vis spectrophotometer (V-560, Jasco Inc., Tokyo, Japan) at wavelengths 562, 615 and 652 nm, since PC has a maximum absorbance that is proportional to its concentration in the visible range between 610 and 620 nm and 652 nm is the wavelength where the class of APC absorbs. The amounts of PC, APC and PE were calculated according to Eqs. (2), (3) and (4), respectively (Arashiro et al., 2020).

$$PC(mg/mL) = [A615 - (0.474 \times A652)]/5.34 \quad (2)$$

$$APC(mg/mL) = [A652 - (0.208 \times A615)]/5.09 \quad (3)$$

$$PE(mg/mL) = \frac{A562 - (2.41 \times PC) - (0.849 \times APC)}{9.62} \quad (4)$$

where A562, A615 and A652 are the absorbances measured at the respective wavelengths.

7.2.3.3 Phenolic compounds

The concentration of total phenolic compounds (TPC) was measured using the Folin–Ciocalteu method, which is based on the colorimetric reduction/oxidation reaction of phenols (Ferreira-Santos et al., 2019). For all analyses, 5 µL of extract (water or ethanol 95 % for control) was mixed with 15 µL Folin–Ciocalteu reagent and 60 µL of Na_2CO_3 (75 g/L). The prepared solution was kept at 60 °C for 5 min. Absorbance was measured at 700 nm by an UV/vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). A calibration curve was prepared using a standard solution of gallic acid (1500 to 50 mg/L, $R^2 = 0.99$). Final values were expressed as milligram GAE per gram of dry material (mg GAE/g *Spirulina*).

The identification and quantification analysis of phenolic compounds present in *S. platensis* extracts were performed as described previously (Ferreira-Santos et al., 2019) using a Shimatzu

Nexpera X2 UPLC chromatograph equipped with DAD (Shimadzu, SPD-M20A). Separation was performed on a reversed-phase Aquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size; from Waters) and a pre-column of the same material at 40 °C. The HPLC grade solvents used were water/formic acid (0.1%) and acetonitrile as eluents and the flow rate was 0.4 mL/min. Phenolic compounds were identified by comparing their UV spectra and retention times with that of corresponding standards. Quantification was carried out using calibration curves for each compound analyzed using concentrations` between 250-2.5 mg/mL (250, 125, 100, 50, 25, 10, 5, 2.5 mg/mL). In all cases, the coefficient of linear correlation was $R^2 > 0.99$. Compounds were quantified and identified at different wavelengths (208–370 nm).

7.2.3.4 Chloropyll and carotenoids

Chlorophyll-a (Chl-a), Chlorophyll-b (Chl-b) and carotenoids content was determined using a UV-Vis spectrophotometer (V-560, Jasco Inc., Tokyo, Japan) at wavelengths 470, 649 and 664 nm. The amounts of Chlorophyll-a, Chlorophyll-b and carotenoids were calculated according to Eqs. (5), (6) and (7), respectively (Lichtenthaler and Wellburn, 1983).

$$\text{Chl} - \text{a}\ (\mu g/mL) = (13.36 \times \text{A664}) - (5.19 \times \text{A649}) \quad (5)$$

$$\text{Chl} - \text{b}\ (\mu g/mL) = (27.43 \times \text{A649}) - (8.12 \times \text{A664}) \quad (6)$$

$$\text{Carotenoids}\ (\mu g/mL) = \frac{(1000 \times A470 - (2.13 \times \text{Chl} - \text{a}) - (93.63 \times \text{Chl} - \text{b})}{209} \quad (7)$$

7.2.3.5 Fatty acids

In addition to fatty acids, the lipidic fractions obtained with mixture of polar and non-polar organic solvents contains pigments (carotenoids, chlorophylls, etc.), proteins, amino acids and others compounds presents in the microalgae biomass.

The analysis of fatty acid composition of *S. platensis* lipidic fractions was performed after purification of the extracted lipids by liquid-liquid microextraction in organic solvent (chloroform/methanol) adding water to form a biphasic system and simultaneous methylation with a mixture of methanol acidified by sulfuric acid (15:85, v/v) (Lopes et al., 2019). The fatty acid methyl esters (FAMEs) in organic phase were quantified by gas chromatography in a CP-3800 gas chromatograph (Varian Inc., USA) fitted with a FID detector and a TRACSIL TR-WAX capillary column (30 x 0.25 x 0.25mm, Teknokroma, Spain) using pentadecanoic acid (C15:0) as internal

standard. The injector and detector temperatures were 220 and 250 °C, respectively, and helium was used as carrier gas at 1 mL/min. The initial oven temperature was 50 °C, maintained for 2 min, followed by a 10 °C/min ramp up to 225 °C, and the final isothermal conditions were maintained for 10 min. A mixture of fatty acid methyl ester standards was used for identification purposes, comparing the respective retention times. Relative amount of each long chain fatty acid (%) was expressed as the ratio between its concentration (g/L) and the sum of the concentrations of all fatty acids analyzed.

7.2.3.6 Determination of antioxidant activity

Two different methods of measuring the AA were used: DPPH and FRAP assay. The assays were determined as described previously by (Ferreira-Santos et al., 2019). In each assay, Trolox was used as a standard reference.

DPPH assay consists in the reduction of the DPPH• radical in the presence of hydrogen-donating antioxidant and in the formation of the non-radical DPPH-H form at the end of the reaction. The scavenging effect was calculated by the equation Eq. (8):

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A0 - A1)}{A0} \times 100 \quad (8)$$

where A0 is the absorbance of blank control, and A1 is the absorbance of sample at 515 nm.

FRAP assay consists in the ability of extracts to reduce ferric ions (Fe^{3+} to Fe^{2+}), in the form of TPTZ. FRAP values are expressed as micromoles of ferrous equivalent per g of dry extract (μmol Fe^{2+}/g extract).

7.2.4 Optical microscopy

After the each extraction process, *Spirulina* biomass samples were observed with a microscope BX51 (Olympus, Japan). Images were captured with DP72 digital camera (Olympus, Japan) at magnification 100X.

7.2.5 Statistical analysis

The extractions and analyses were performed in triplicate and the data is presented as mean ± SD values. GraphPad Prism® software (version 6.0; GraphPad Software, Inc., San Diego, CA,

USA) was used for statistical analyses. The ANOVA and the least significant difference test were used to determine statistically different values at a significance level of $p < 0.05$.

7.3 RESULTS AND DISCUSSION

7.3.1 Extraction yields

The influence of the extraction steps conditions on the extraction yields is shown on **Table 1**. The application of physical/thermal (CE, 0 V/cm and OH, 7 V/cm) and/or biological (EAE, 0.8 mg of lysozyme/mL) treatments (1st step) can significantly increase of the extraction yield values compared to CE treated samples ($p < 0.05$). Similarly, in the second extraction step, where ethanol is used as solvent, the pretreatments applied in the first step with enzyme and/or electric fields resulted in significant differences in the extraction yields, also in the subsequent steps, recovering mostly non-polar molecules (such as chlorophyll, carotenoids and lipids). In addition, the combination of OH and EAE, as a treatment in the aqueous extraction (1st step) or pre-treatment for the 2nd step, resulted in the greatest increase in the extraction yields ($p < 0.01$). These results suggest that treatments using electric fields and lysozyme cause lysis of the cell wall of *Spirulina*, promoting the extraction of intracellular compounds (shown below in the microscopy results, section 3.7).

In the 3rd step, the biomass was subjected to an extraction with chloroform-methanol and the extraction yield was significantly lower compared to the previous extraction steps. This may be due to the high amount of extractives obtained in the previous stages of extraction, recovering the majority of the lipids available with ethanol 95% (results reported in section 3.5), meaning that, unless the extract profile in this 3rd step is unique and potentially with very high added value, it can be removed from a sequential extraction approach to recover the different algae fractions.

The ash content (inorganic fraction) was determined in the biomass residue from the 3rd step. The results showed that the final biomass pre-treated with CE, presents a greater quantity of inorganic matter compared to biomass pre-treated with OH and EAE. Interestingly, the combination of technologies has also been shown to be effective in recovering inorganic compounds, such as minerals, throughout the process, with less quantity in the final residue.

Table 1. Extraction yields (%) of different extraction steps from *S. platensis*.

Treatment	1st Step (H_2O)						2nd Step (EtOH 95%)	3rd Step ($CHCl_3$:MeOH)	Ash (Final residue)
Time (min)	10	20	30	40	50	60			
CE	20 ± 2	26 ± 1	28 ± 0	31 ± 2	33 ± 1	35 ± 1	9.6 ± 0.2	1.9 ± 0.2	2.44 ± 0.14
EAE	23 ± 5	27 ± 3	28 ± 0	32 ± 1	35 ± 1	38 ± 2	10.7 ± 0.7	2.2 ± 0.1	2.21 ± 0.03
OH	27 ± 1	29 ± 1	31 ± 0	33 ± 1	37 ± 2	38 ± 0	10.9 ± 0.1	2.6 ± 0.2	2.22 ± 0.02
OH-EAE	28 ± 2	29 ± 1	35 ± 2	39 ± 3	43 ± 1	43 ± 2	12.2 ± 0.1	2.8 ± 0.1	2.04± 0.05

Values are expressed as mean ± SD of three experiments.

7.3.2 Phycobiliproteins quantification of aqueous extracts (1st step)

The main objective of this study is to promote the efficient extraction of high added value intracellular compounds from *Spirulina* without changing the characteristics of compounds, enhancing its application in the food, nutraceutical and pharmacological industries.

In this sense, the recovery of phycobiliproteins (pigments used as natural colorants) from microalgae is extremely valuable due to its high commercial value. The extraction of these molecules has already been carried out by various physical, mechanical, chemical and biological methods (Izadi and Fazilati, 2018; Jaeschke et al., 2019; Kissoudi et al., 2018; Pan-utai and Iamtham, 2019). However, their total extraction is usually hampered by the rigidity of the cell wall, as well as by the extraction conditions (biomass to solvent ratios, type of solvent and previous biomass processing). Furthermore, the initial extractable amount of phycobiliproteins in the cells depend on the growth conditions and harvest stage, and absolute values may not be comparable (Vernès et al., 2015). Therefore, the application of strategies that allows full access to the intracellular medium is essential to facilitate the extraction of specific components. Although traditional methods (e.g., sonication and freeze-thawing methods) induce cell wall disruption, they have some drawbacks because of the use of a high energy consumption and long extraction time (Kissoudi et al., 2018; Vernès et al., 2015). Moreover, cell disintegration should be done avoiding the use of processing conditions that could negatively affect the quality of the extracts and have a negative impact in the product value (Postma et al., 2016).

Figure 2 presents the contents of phycobiliproteins (PC, APC and PE) after water extraction for 10 to 60 min (1st step). The data is presented for CE (without electric fields and enzyme influence), OH (electric fields influence), EAE (enzyme influence) and OH combined with EAE. The obtained data shows that the most representative phycobiliprotein in *S. platensis* is PC followed by APC and PE, ranging from 25.5–56.6, 5.4–14.7, 1.9–8.6 mg/g, respectively. These results are in agreement with those reported by Arashiro and co-workers (Arashiro et al., 2020), also confirming the commercial potential of *Spirulina* as a source of natural blue dyes.

Furthermore, the highest amounts of phycobiliproteins were obtained when the combination of the enzymatic treatment with the influence of electric fields (OH-EAE) was used, presenting significantly higher amounts ($p < 0.01$) compared to the treatments in which the techniques were used separately. In addition, it is possible to observe that the OH and EAE treatments without being combined, already lead to a significant increase of phycobiliproteins recovery in less time when compared to CE.

Recently, the use of green methodologies such as electrotechnologies (PEF, HVED) and OH to obtain high value products from microalgae has been the focus of research to allow the reduction of amount of toxic solvents, times and increasing extraction efficiency, in addition to targeting its industrial application (’t Lam et al., 2017; Ferreira-Santos et al., 2020b; Jaeschke et al., 2019; Käferböck et al., 2020; Postma et al., 2016; Zhang et al., 2020). Moreover, it is known that this electrotechnology reduces energy consumption compared to the traditional processes, demonstrating that OH is a more energy efficient process (Ferreira-Santos et al., 2019; Pereira and Vicente, 2010).

Jaeschke *et al.* (Jaeschke et al., 2019) applied PEF treatment with higher intensities (E = 40 kV/cm and W_{spec} = 112 kJ/kg) and used sodium phosphate buffer instead of tap water as solvent in subsequent extraction, yielding 76% of PC (corresponding to 94 mg/g of *Spirulina*). In addition, EAE with lysozyme and a combination with mechanical grinding has been used to disintegrate cell walls and improve the efficiency of pigment extraction (Sobiechowska-Sasim et al., 2014). These results are in agreement with those obtained in our work, in which the use of enzymes or electric fields leads to an increase of extraction efficiency of phycobiliproteins, which can be used in the industry as a natural blue colorant. Further, the combined action of both technologies has a very positive effect.

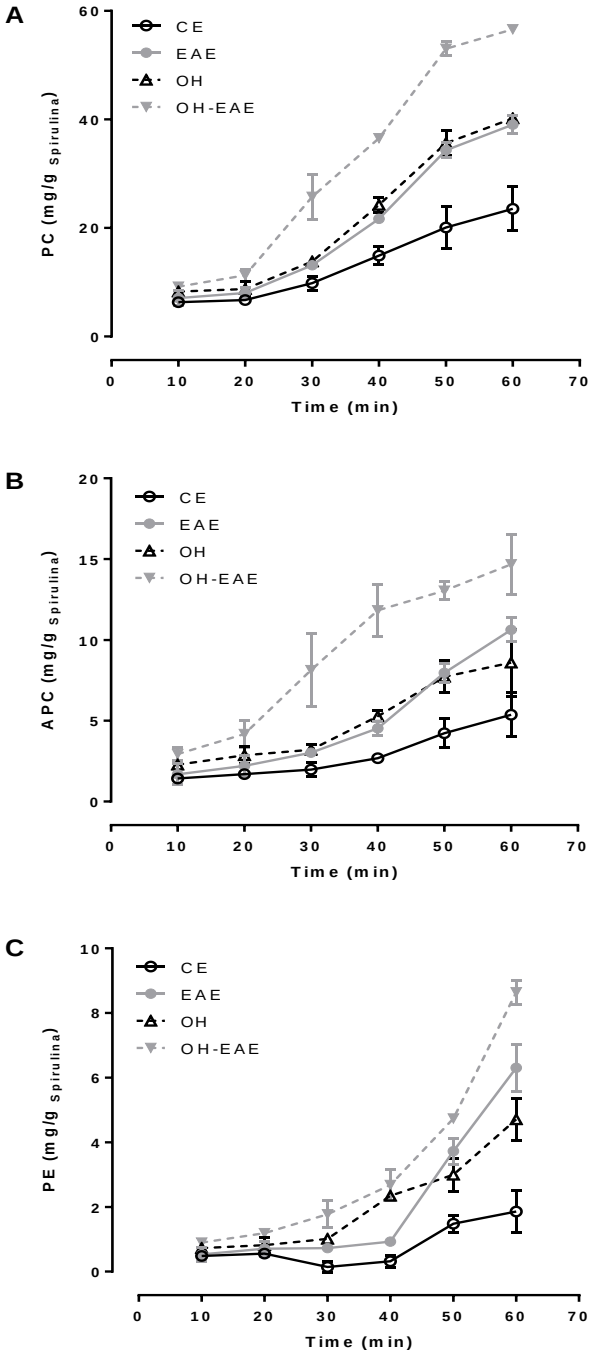


Figure 2. Phycobiliproteins recovery (mg/$g_{dw\ Spirulina}$) for 1st step extraction at different exposure times (10 to 60 min). C-phycocyanin (**A**), allophycocyanin (**B**) and phycoerythrin (**C**). Error bars represent mean ± SD of three experiments.

7.3.3 Total phenolic compounds of aqueous and ethanolic extracts (1st and 2nd step)

Phenolic compounds are known to have important bioactive and functional properties. For example, some phenolic compounds show potent activities such as antioxidant, anti-microbial, anti-cancer, among others, which make these compounds interesting for commercial applications in the food, health and pharmaceutical industries (Dai and Mumper, 2010; Ferreira-Santos et al., 2019; Mena-García et al., 2019).

As mentioned before, pigments, phenolic compounds and proteins are some of the metabolites presented in cytoplasm or internal organelles which makes difficult their extraction due to the resistance of the cell wall (Chen et al., 2020; Moraes et al., 2011; Zhu et al., 2007). In this sense, in our study, the better efficiency in recovery of phenolic compounds was observed for OH and EAE treated biomass as compared with CE treated samples (**Figure 3**).

For example, in the 1st step, the recovery of phenolic compounds were ≈0.8-fold increased by OH, and ≈0.9-fold increase by EAE, respectively, in comparison to CE (Figure 3A). Moreover, in the 2nd step, the physical (HO) and the enzymatic pre-treatment (EAE) before ethanolic extraction also shows a significant increase in the extraction of phenolic compounds ($p < 0.05$) (Figure 3B). These results can be explained by the affinity to the solvent used, in which some of the phenolic compounds have solubility in water and ethanol. In addition, the technology used for extraction helps to diffuse these compounds across cell membranes. However, in this case the global extraction of phenolics was not much influenced by the solvent used (because there are phenolics of polar character and others non-polar; however differences exist in the specific phenolic compounds profile), but it was significantly affected by the technology used in the extraction.

Furthermore the combination of the OH and EAE significantly enhanced the extraction process of phenolic compounds (1st step, 15.0 ± 0.6 mg GAE/g and 2nd step, 10.05 ± 0.5 mg GAE/g *Spirulina*) when compared to techniques applied without combination, as already happened for phycobiliproteins.

In the literature it is possible to observe that the application of electric fields for biocompounds recovery from microalgae was already studied, but mostly with pulses and rarely with OH. Postma *et al.* (Postma et al., 2016) used PEF (at 25-55 °C) for carbohydrates extraction, and their results indicate that a small fraction (< 5 %) of the carbohydrates present in the *Chlorella vulgaris* was released using conventional treatments, while using PEF the carbohydrate yields were significantly higher (22-25 %). Recently, our research group optimized the extraction process of high value

biocompounds from *Spirulina* powder using OH in which the values obtained were similar to those obtained in this work (Ferreira-Santos et al., 2020b).

The use of enzymes is considered a green process that reduces extraction time and solvent use, improving the extraction yield of specific biocompounds (Rajha et al., 2018, 2015). The combination of OH with enzymatic extraction promotes the fragilization of the structure of the *Spirulina* cell wall, ameliorating thus the release of biocompounds. Therefore, the presence of electric fields by OH enhances the extraction process of biocompounds by increasing the exchange surface area with the solvent containing the enzymes. These facts have been reported by other authors, showing that the combination of emerging technologies increases the extraction of biocompounds, improving the conditions of extraction. Rajha et al. (Rajha et al., 2018) have been reporting the selective multistage extraction process by combination of electrotechnologies (*e.g.* HVED) with enzymes, favoring the process of phenolic compounds recovery from vine shoots. Recently, Zhang and co-workers (Zhang et al., 2020) proposed a multi-stage aqueous and non-aqueous extraction of biomolecules (carbohydrates, protein, lipids and pigments) from microalga *Phaeodactylum tricornutum* using emerging technologies like HVED and high pressure. As a result they concluded that extraction in multi-stages and several solvents benefits the integral recovery of bioactive compounds with high commercial interest.

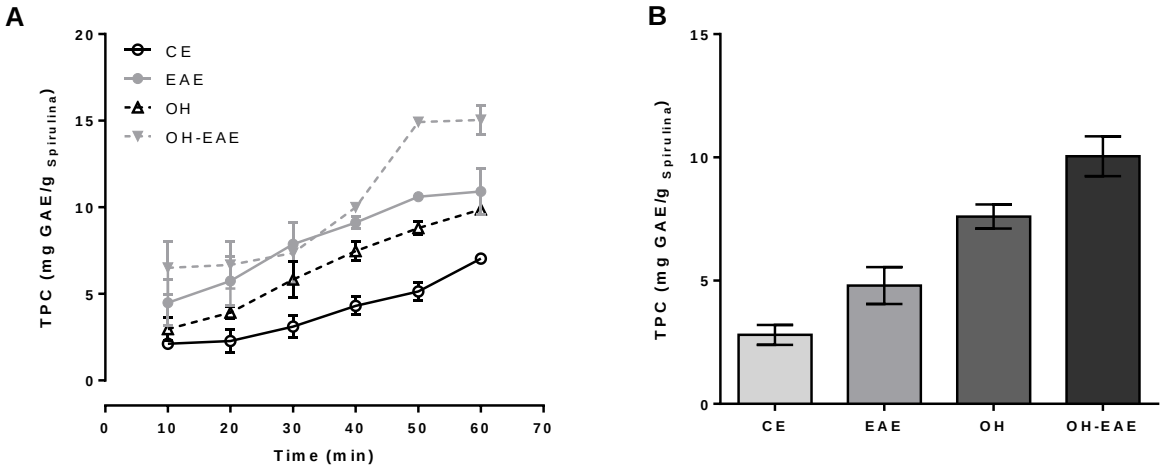


Figure 3. Total phenolic compounds (TPC, mg GAE/$g_{dw\ Spirulina}$) recovery for 1st step extraction (**A**) and 2nd step extraction (**B**). Error bars represent mean ± SD of three experiments.

Liquid chromatographic analysis was applied in order to identify and quantify the main individual phenolic compounds and to evaluate influence of the technique and the selectivity of each solvent used in the extraction process over the chemical profile of the *S. platensis* extract (see **Table 2**). In total, ten phenolic compounds were tentatively identified in the extracts of *S. platensis* according to their corresponding standards.

The 1st extraction step (aqueous extraction) resulted in higher extraction of phenolic compounds. Chlorogenic acid (131.3 to 154.8 μg/g of dry extract) was only detected in the ethanolic extracts resulting from the 2nd extraction step as well as kaempferol and hesperidin that were extracted between 2.4 to 2.7 times and 4.5 to 7.7 times more, respectively, in the 2nd extraction step compared to the extract obtained in the 1st extraction step. Ellagic acid, ferulic acid, taxifolin and quercetin were compounds found only in aqueous extracts. Ellagic acid was found in concentrations ranging from 424.7 to 502.2 μg/g of dry extract, showing to be the most representative compound in the aqueous extract. On the other hand, kaempferol is the most representative phenolic compound in the ethanolic extract resulting from the 2nd extraction step (244.0 to 375.6 μg/g of dry extract). All other identified compounds were quantified with a concentration between 11.0 to 181.8 μg/g of dry extract (see **Table 2**).

Previous studies carried out by other authors have shown that *Spirulina* contains phenolic compounds with a high antioxidant capacity (Goiris et al., 2014; Machado et al., 2019; Matos et al., 2020; Seghiri et al., 2019). Casazza *et al.* (Casazza et al., 2015) reported that the maximum phenolic yields was 33.2 mgGAE/g *Spirulina*, and Matos *et al.* (Matos et al., 2020) showed concentrations of approximately 3.34 mgGAE/g *Spirulina* for aqueous extracts and 2.05 mgGAE/g *Spirulina* for ethanolic extracts. Authors showed that phenolic acids are the main group of phenolics present in *Spirulina* (Goiris et al., 2014; Machado et al., 2019; Seghiri et al., 2019). These data are in accordance of those obtained with the results of this work.

Table 2. Phenolic compounds identification and quantification from *S. platensis* extracts.

Compounds (μg/g $_{extract}$)	1st Step (H_2O)				2nd Step (EtOH 95%)			
	CE	EAE	OH	OH-EAE	CE	EAE	OH	OH-EAE
ferulic acid	26.8 ± 1.3	25.3 ± 0.2	25.4 ± 1.4	21.3 ± 0.7	n.d.	n.d.	n.d.	n.d.
ellagic acid	502.2 ± 16.2	455.6 ± 4.1	462.3 ± 9.3	424.7 ± 2.9	n.d.	n.d.	n.d.	n.d.
chlorogenic acid	n.d.	n.d.	n.d.	n.d.	148.2 ± 3.0	154.8 ± 1.7	131.3 ± 0.4	138.4 ± 3.4
naringin	24.4 ± 5.4	24.2 ± 4.1	20.2 ± 3.8	18.5 ± 2.7	n.d.	n.d.	n.d.	n.d.
hesperidin	64.1 ± 7.0	75.3 ± 3.4	70.9 ± 6.4	70.6 ± 0.1	173.5 ± 0.5	181.8 ± 13.2	172.5 ± 11.4	178.1 ± 1.1
taxifolin	13.3 ± 1.9	15.9 ± 1.5	15.1 ± 2.1	11.0 ± 0.7	n.d.	n.d.	n.d.	n.d.
quercetin	61.9 ± 2.4	54.8 ± 0.4	54.8 ± 0.0	49.0 ± 0.0	n.d.	n.d.	n.d.	n.d.
kaempferol	56.4 ± 0.6	54.2 ± 5.2	48.7 ± 0.9	51.0 ± 2.4	308.3 ± 37.6	244.0 ± 2.8	375.6 ± 1.2	320.4 ± 3.2
3,4-hydroxybenzoic acid	153.5 ± 4.7	158.1 ± 1.6	133.1 ± 7.8	130.5 ± 0.2	52.2 ± 5.2	56.9 ± 2.2	72.1 ± 5.5	63.1 ± 0.1
aloin	152.7 ± 4.1	145.6 ± 8.3	151.7 ± 1.6	120.8 ± 2.0	161.4 ± 8.1	161.5 ± 7.2	142.9 ± 5.1	136.3 ± 5.9
TOTAL	1055 ± 44	1009 ± 29	982 ± 33	898 ± 12	844 ± 54	799 ± 27	894 ± 23	836 ± 14

Values are expressed as concentration (μg/g dry extract) mean ± SD of three experiments. n.d.: not detected.

7.3.4 Chlorophyll and carotenoids quantification of ethanolic extracts (2nd step)

Chlorophylls and carotenoids are lipophilic compounds authorized in food applications such as colouring in the manufacture of cold drinks, ice creams, among others ("Directive 94/36/CE of the European Parliament and the Council, 30 June 1994, about Dyes used in Food Products.," n.d.). The consumption of these compounds, has been linked with a number of health benefits, including anti-inflammatory activity, cancer chemoprotection, prevention of cardiovascular and metabolic diseases (e.g. cholesterol, hypertension, diabetes, *etc.*) and degenerative diseases (e.g., Alzheimer's disease) (Amorim-Carrilho et al., 2014; Ferreira-Santos et al., 2020a). Almost all carotenoids, to a greater or lesser degree, show scavenging properties against excessive numbers of free radicals (Santocono et al., 2007). This antioxidant capacity has been the most investigated and it has been suggested as the main mechanism of action of the carotenoids (Amorim-Carrilho et al., 2014).

Figure 4 presents the content of photosynthetic compounds (chlorophylls and carotenoids) for 2nd step of extraction. These compounds were not quantified in the 1st extraction step because they are non-extractable with water.

Our results clearly show that chlorophyll-a is the most prevalent photosynthetic compound in ethanolic extracts, recovering 8.4 to 19.1 mg/g from *Spirulina* and obtaining concentrations of 87 to 158 mg/g dry extract. Based on the obtained results, it is possible to observe that previous biomass treatments from 1st step aided to increase the extraction efficiency of chlorophyll and carotenoids in the 2nd step. For chlorophyll-a, the use of OH and EAE significantly (p <0.001) increases the amount of this green pigment obtaining concentrations 44.5% and 44.8% higher compared to biomass pretreated with traditional methodology (CE). Moreover, when biomass was pretreated with lysozyme (EAE) combined with OH the recovery was 128% more efficient.

Similar results were obtained for chlorophyll-b and carotenoids, in which the combined pretreatment of EAE and OH, showed larger efficiency in the recovery of these compounds when compared with the extracts obtained from the biomass pre-treated by the CE. For example, the amount of recovered carotenoids was 2 times higher in the extracts obtained from the biomass pre-treated by EAE-OH, when compared with CE.

In a recent study, authors showed that some microalgae/cyanobacteria, such as *Chlorella vulgaris* and *Spirulina platensis,* contain high concentrations of chlorophylls, mainly chlorophyll-a, and carotenoids (zeaxanthin, lutein, astaxanthin, canthaxanthin, β-carotene) (Hynstova et al., 2018).

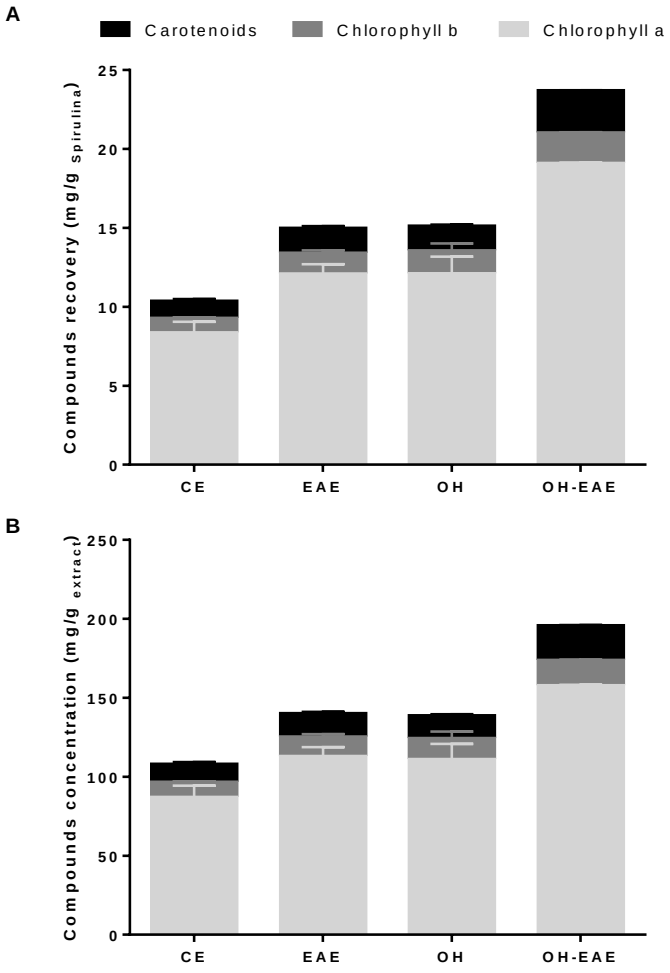


Figure 4. Chlorophyll and carotenoids recovery ($mg/g_{dw\ Spirulina}$) (**A**) and concentration ($\mu g/g_{dw\ extract}$) (**B**) for 2nd step extraction. Error bars represent mean ± SD of three experiments.

7.3.5 Total lipids and fatty acid determinations of ethanolic and choroform/methanol resulting extracts (2nd and 3rd step)

A number of methods can be used to achieve oils extraction from microalgae, including solvent extraction, supercritical extraction, pyrolysis, enzymatic hydrolysis, MW, US, among others. Moreover, the development of an efficient method for lipid extraction and purification from microalgae biomass is one of the critical steps in the downstream processes (Jeevan Kumar et al., 2017). Organic solvent extraction (e.g. hexane, ethanol, methanol, acetone, petroleum ether, and a mixture of chloroform and methanol) is one of the most well-known methods for the extraction of lipids from vegetable, flower, and oil seeds. Furthermore, various organic solvents have been used for extraction of lipids from microalgae (Chaiklahan et al., 2008). It has been found that mixture of non-polar and polar organic solvents will ensure complete extraction of all neutral lipids. On the

other hand, in addition to the solvent, the use of pretreatments to facilitate disintegration of the microalgae cell wall has been used to enhance the recovery of lipid fractions (Cho et al., 2013). As far as we know, the recovery of *Spirulina* lipids using environmentally friendly pretreatments is quite limited. Furthermore, the previous recovery of other fractions using mild processing may allow to remove contaminant (but valuable) compounds prior to the lipid extraction, allowing the recovery of a more lipid-concentrated fraction without deleterious effects in the lipidic profile, while simultaneously damaging the algae structure, enabling easier lipid recovery.

In our work, lipids were extracted after aqueous pre-treatment by CE, OH, EAE or OH-EAE. Ethanol 95% (v/v) was used followed by re-extraction with chloroform/methanol (2nd step), and in the 3rd step prolonged extraction (6h) with chloroform/methanol was done. The results of the global extraction yields of *Spirulina* are shown in Table 1 and the fatty acid content in **Table 3**.

The yield of lipids in the 2nd stage of extraction was 6.7 ± 0.3%, 7.4 ± 0.2%, 7.6 ± 0.4% and 8.4 ± 0.5% for resulting extracts from CE, EAE, OH and OH-EAE pre-treated biomass, respectively. Comparing these results with total yields (**Table 1**) we can observe that the fraction resulting from the 2nd extraction step is rich in lipids, presenting about 49-72% of lipids. The lipid yields of the 3rd step correspond to the overall yields shown in **Table 1**.

In a previous study conducted by us, the same *Spirulina* was used and chemically characterized (Ferreira-Santos et al., 2020b). Therefore, comparing the results of total lipids present in *Spirulina* (11.32 ± 0.65%), it is possible to state that the combination of technologies (OH-EAE) maximizes lipid extraction.

According to the results of GC-FID analyses, palmitic acid (approx. 45% + 47% in 2nd and 3rd step, respectively), linoleic acid (approx. 19% + 18% in 2nd and 3rd step, respectively) and γ-linolenic (approx. 17% + 15% in 2nd and 3rd step, respectively) were determined as the major components in fatty acids of lipid extracted fraction of 2nd and 3rd step, without influence of pre-treatment. The recovery of lipids in the 3rd step was clearly lower than in the 2nd step. These results indicate that most of the lipids were extracted using 95% ethanol as extraction solvent. However, it is possible to observe that despite the low amount of lipids recovered in the 3rd step of the sequential extraction, all of their content is in FAMEs (extraction yield similar to FAME content), being this an almost 100 % lipidic fraction (see **Table 1** and **3**).

Several studies have been studying processes for the recovery of lipids from microalgae, such as *Spirulina*, and their industrial applications (Chew et al., 2017; Shankar et al., 2017; Yang et al., 2020). Taken together, our results suggest that *S. platensis* may provide human beings with

beneficial fatty acids in their diets if used as nutrient sources in food products. Furthermore, after a refined purification process, some of the fatty acids can be used for medical applications, such as in the treatment of dermatitis, diabetes, and inflammatory diseases (Yang et al., 2020). On the other hand, our results demonstrate that *Spirulina* lipids can have great relevance for the industrial production of biodiesel.

Table 3. Fatty acid composition of *Spirulina* fractions.

Fatty acid (mg/g $_{extract}$)		Palmitic (C16:0)	Palmitoleic (C16:1)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	γ-Linolenic (C18:3)	TOTAL
CE	2nd step	325.3 ± 1.1	35.8 ± 0.6	15.0 ± 0.3	81.3 ± 0.5	137.1 ± 2.2	128.4 ± 4.2	720.5 ± 8.9
	3rd step	507.8 ± 26.5	57.0 ± 1.3	32.4 ± 3.7	129.7 ± 5.3	203.1 ± 11.6	153.9 ± 8.7	1083.9 ± 57.1*
EAE	2nd step	283.5 ± 16.8	25.5 ± 6.1	13.0 ± 0.5	58.7 ± 3.5	97.6 ± 6.3	133.2 ± 9.15	611.5 ± 42.4
	3rd step	514.7 ± 1.5	50.3 ± 8.3	60.0 ± 15.4	106.1 ± 28.1	181.1 ± 28.4	177.9 ± 0.8	1090.1 ± 82.5*
OH	2nd step	196.0 ± 4.4	21.6 ± 3.9	10.2 ± 0.9	49.0 ± 1.4	83.7 ± 2.8	130.0 ± 12.5	490.5 ± 25.9
	3rd step	506.6 ± 22.2	56.2 ± 4.9	70.4 ± 4.1	135.8 ± 3.3	204.6 ± 16.6	158.3 ± 10.3	1131.9 ± 62.4*
OH-EAE	2nd step	215.8 ± 1.3	23.1 ± 1.7	13.7 ± 0.0	54.6 ± 0.9	91.2 ± 0.3	127.5 ± 3.7	525.9 ± 7.9
	3rd step	484.5 ± 0.1	50.7 ± 2.8	63.2 ± 10.5	131.7 ± 16.5	180.3 ± 8.4	153.2 ± 1.3	1063.1 ± 39.5*

Values are expressed as concentration (mg/g dry extract) mean ± SD of three experiments. *Overestimated values.

7.3.6 Antioxidant activity of extracts

The AA results for aqueous and ethanolic extracts of the studied microalgae *S. platensis* are presented in **Figure 5**. Our results show that the AA increases over the 60 min for the first extraction step. These data are directly related to the amount of phycobiliproteins and phenolic compounds present in the aqueous obtained extracts.

The AA varied significantly with the type of technology used, as well as the solvent used in each extraction step. Aqueous extracts of *S. platensis* (1st step) yielded maximum DPPH scavenging activity of 87.3 ± 0.09% for OH-EAE, 86% for both OH and EAE, and a lower activity ($p < 0.05$) for extracts obtained by CE (81.9 ± 0.02%). Similar results were observed with FRAP assay, since the lowest activity was measured for the final aqueous CE extracts (1193.7 ± 21.7 µmol Fe^{2+}/L) compared to extracts obtained by EAE (1444.2 ± 28.9 µmol Fe^{2+}/L), OH (1383.7 ± 20.6 µmol Fe^{2+}/L) and OH-EAE (1580.3 ± 12.7 µmol Fe^{2+}/L), respectively.

For the 2nd extraction step, in which ethanol was used, the results showed that the pre-treatment of the biomass influences the AA of the obtained extracts. For example, radical inhibition for the DPPH assay was 9% higher ($p < 0.05$) and antioxidant reduction power (FRAP) was approximately 41% higher ($p < 0.01$) for obtained extracts when the *Spirulina* biomass was pre-treated with OH-EAE compared to CE.

Our results also showed that the presence of non-polar compounds such as chlorophylls, carotnoids and lipids, in addition to some phenolic compounds, have a high reducing antioxidant power activity, showing significant differences between the aqueous (1st step) and ethanolic extracts (2nd step) (see **Figure 5 A** and **B**). It has been concluded by several authors worked with different species of microalgae that phycobiliproteins, phenolic compounds, along with carotenoids and chlorophylls contribute significantly to the antioxidant capacity of these aquatic organisms (Goiris et al., 2012; Matos et al., 2020; Pan-utai and Iamtham, 2019). On the other hand, the obtained extract from the 3rd extraction step did not show any AA using the DPPH and FRAP methods (data not shown).

In a recent study carried out by Matos *et al.* (Matos et al., 2020), *Spirulina* ethanolic extracts also showed greater AA assessed by the FRAP method compared to aqueous extracts, but no activity was observed by the DPPH method. In another study, the authors obtained results that clearly showed that both the highest antiradical and AA, determined by the DPPH method, were highly correlated with aqueous extract of *S. platensis* (Shalaby and Shanab, 2013).

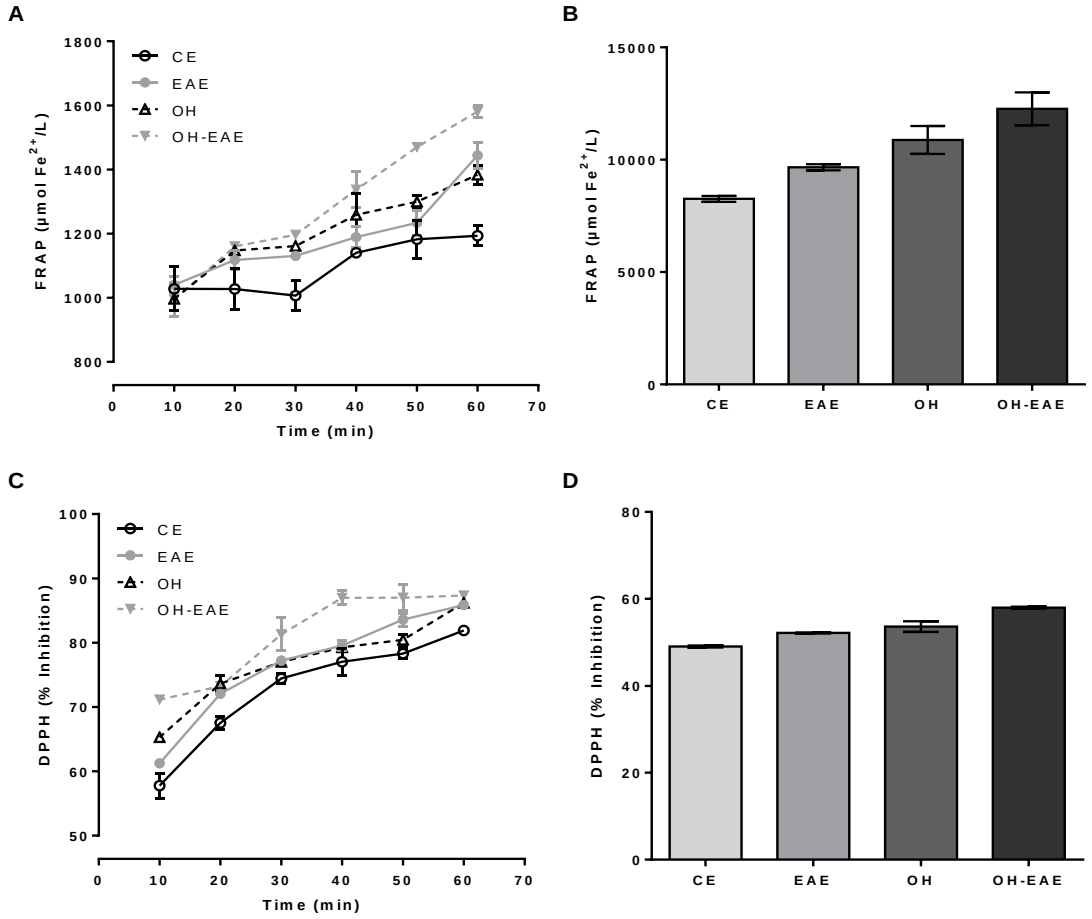


Figure 5. Antioxidant activity measurement by FRAP (**A**,**B**) and DPPH (**C**,**D**) in extracts obtained over time (10-60 min) from 1st step (**A**,**C**) and for 2nd step extraction (**B**,**D**). Error bars represent mean ± SD of three experiments.

7.3.7 Microscopy analysis

The typical morphology of *Spirulina* is characterized by its regularly coiled trichomes and arrangement in spirals. However, *Spirulina* can occur with abnormal morphologies, such as irregularly curved and even linear shapes, in laboratory and industry cultures (Wang and Zhao, 2005).

In this work, dry spirulina was rehydrated as shown in **Figure 6 A**, and we can observe green spirals, irregularly curved and even linear shapes. Moreover, when treatments are applied, a clear disintegration and discoloration of the cells is observed (**Figure 6 B** to **G**).

Concomitantly, the treatments performed with electric fields and lysozyme showed more efficiency in the disintegration and rupture of the biomass cell wall, facilitating the extraction of intracellular compounds (see **Figure 6 C**, **D** and **E**). These results corroborate the data obtained previously in this work.

In the **Figure 6 F**, we can see that the biomass subjected to pre-treatment and later treated with ethanol undergoes cellular dehydration, decreasing the size of the fragments, which indicates the extraction of non-polar compounds soluble in ethanol. This phenomenon is intensified with the subsequent extraction with chloroform/methanol, in which the cells are practically discolored and apparently in smaller fragments (**Figure 6 G**).

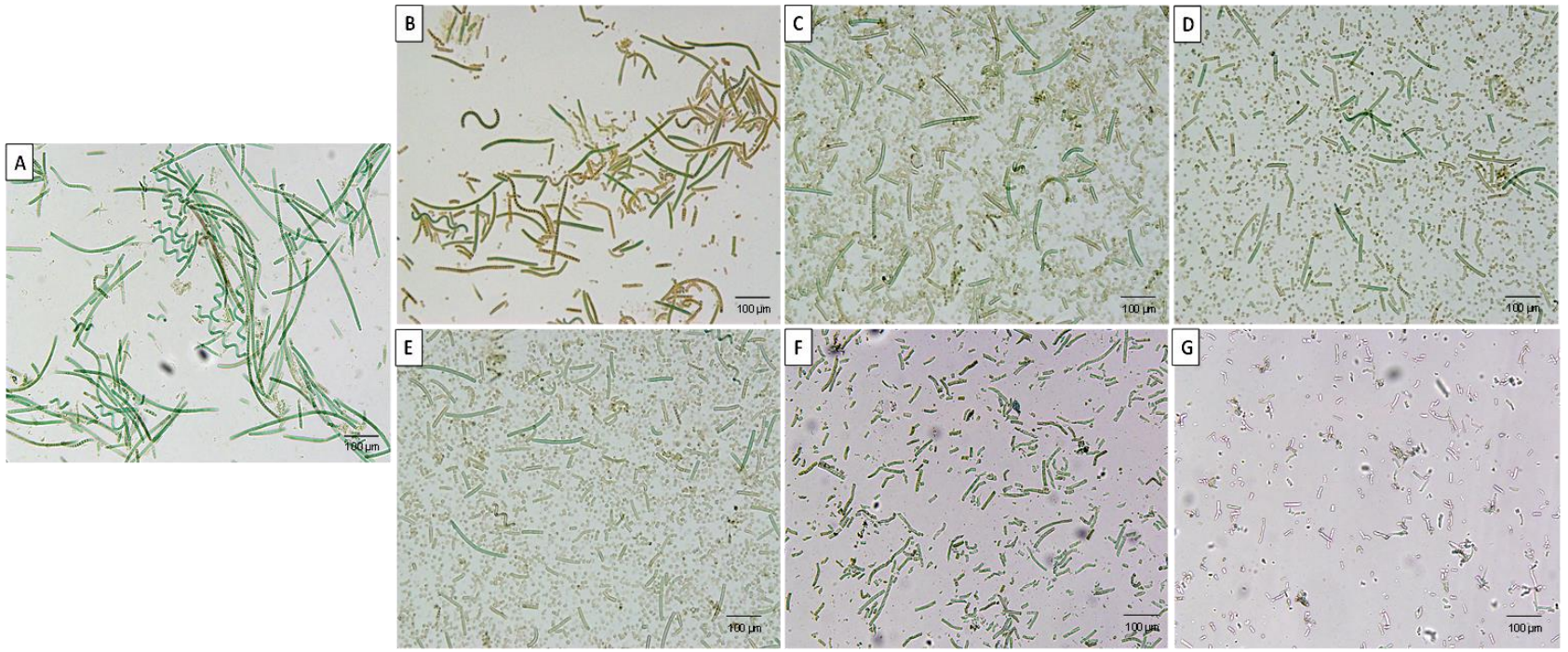


Figure 6. Microscopic images of *S. platensis* biomass (100X). Image **A** represent untreated cells, images from **B** to **E** correspond to aqueous treated cells of 1st extraction step (CE (**B**), EAE (**C**), OH (**D**) and OH-EAE (**E**)), image **F** represent the cells after ethanolic extraction (2nd step) and image **G** represent the cells after extraction with chloroform/methanol (3rd step). Scale bar of 100 µm applies to all images.

7.4 CONCLUSIONS

The efficiency of multi-step extraction procedure for the valorization of *S. platensis* biomass has been investigated. The use of combined physical/thermal (OH) and enzymatic treatments in as a first step in the extraction favors the disintegration and rupture of the spirulina cell wall and improves significantly the recovery of phycobiliproteins. The release of chlorophylls, carotenoids and lipids in the second extraction using ethanol was also significantly improved.

Overall, our results demonstrate that the intensification of the proposed process facilitates the use of alternative and environmentally friendly technologies (and their combination) and non-toxic (bio)solvents in a multi-stage extraction process capable of recovering and isolating different classes of molecules with biofunctional properties, according to the concept of biorefinery. In addition, the safe and inexpensive disposal of all waste products generated during the process must be considered. Therefore, a portion of the residual biomass can go to an anaerobic digester to generate biogas, and the rest can be used as nutrients, after a hydrolysis step to liberate fermentable sugars to feed the photobioreactor again. Finally, the proposed approach saves energy and time, facilitating scale-up and industrial application to obtain products with high added-value from natural resources such as microalgae.

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CHAPTER VIII

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

This chapter includes the final remarks, general conclusions and some suggestions for future activities on the bases of the obtained results.

8.1 FINAL REMARKS

The present thesis aimed to develop efficient and environmentally friendly methodologies involving low cost and non-toxic solvents for the reuse of undervalued bioresources, as *Pinus pinaster* bark and *Spirulina platensis* microalgae. The developed work follows the biorefinery concept to obtain bioactive and functional products with industrial interest.

In this work, it was evaluated the possibility of applying OH, an economically and environmentally viable emerging technology, for the extraction of phenolic compounds from the pine bark, as well as the combination between OH and other technologies for a integral recovery of the different intracellular compounds from the *Spirulina* microalgae. In addition, different solvents and their mixtures, such as water and ethanol, were applied to enhance the productivity and selectivity of the extraction process. The chemical and bioactive characterization of the extracts was carried out focusing on PB as a source of bioactive phenolic compounds. *Spirulina* extraction experiments took in account its use as source of a blue colorant (PC) or protein for human or animal food. In addition, the encapsulation process to protect extracted phenolic compounds from PB was optimized and their influence in bioaccessibility and bioactivity were studied.

Therefore, the main **conclusions** drawn from this thesis are summarized below:

- *P. pinaster* extracts revealed the presence of a wide range of phenolic compounds: phenolic acids, flavonoids, flavonols and stilbens. The extracts showed different phenolic profiles, depending on the solvent used.
- Extracts obtained with a hydroethanolic solvent (50% and 70% of ethanol) showed a higher concentration in phenolic compounds and promising biological activities, such as antioxidant, antidiabetic and antimicrobial.
- PBE have low cytotoxicity and show greater selectivity for cancer cells.
- OH-assisted extraction caused a significant increase in yields and phenolic compounds concentration in *Pinus pinaster* bark extracts in comparison with conventional heating. The use of ethanol in combination with OH altered the process selectivity, further boosting the extraction of phenolic compounds and increasing the antioxidant activities.

- OH causes significant cell permeabilization, facilitating the access of the solvent to the intracellular medium, allowing the use of lower amounts of solvent to reach the same productivity in the extraction of phenolic compounds.
- Polyphenolic PBE can be effectively encapsulated in MD through spray-drying, resulting in low moisture content and high encapsulation efficiencies, preserving antioxidant and antimicrobial activity of these extracts.
- The encapsulation demonstrated a protective effect on the phenolic compounds degradation (increased stability and bioaccessibility) during the digestion process
- PBE provided efficient protection of Caco-2 cells against oxidative stress, and their antioxidative activity was positively affected by encapsulation, increasing the possible therapeutic effects.
- Moderate electric fields, directly related to OH, do not induce deleterious effects on PC physicochemical properties, providing indeed an additional protection against protein denaturation by adverse temperature conditions.
- OH promotes greater recovery of PC and other bioactive compounds, such as phenolics and carbohydrates, present in the cytoplasm of Spirulina cells, a high yield over a short period of time being obtained when compared to the conventional heating and freeze-thaw methods.
- Microscopic analysis confirmed that the effect of temperature associated with MEF caused by OH and enzymes (like lysozyme) promotes cell structure disintegration and electroporation facilitating the extraction of intracellular compounds from *Spirulina platensis*.
- The combination of OH and enzymatic treatments in the extraction process of *Spirulina* biomass favors the recovery of phycobiliproteins and carbohydrates in aqueous extraction and subsequent release of lipophilic compounds (chlorophylls, carotenoids and lipids) by organic non-toxic solvents. These results allow the sequential obtaining of two interesting fractions in a perspective of circular economy and "zero waste".
- OH technology holds the potential to be a “green” technique for the extraction of bioactive, functional and technological compounds, with significant improvements in extraction yields and reduced energy consumption.

In general, the results of this work contribute significantly to the full enhancement of PB and *Spirulina* application potential. The recovery processes proposed are efficient and with expected decreased ecological footprint. These extracts have compounds (such as phenolic compounds,

natural colorants, among others) with high biological, functional and technological potential making them an attractive alternative to synthetic compounds for application in foods, cosmetics, pharmaceuticals and biofuels industries. In addition, the solids resulting from these processes can be subjected to post-treatments, such as fermentation, pyrolysis, *etc.*, in which fractions obtained can be directed to various industrial sectors, increasing the added value of these bioresources according to the concept of biorefinery, thus contributing to a circular economy.

8.2 FUTURE PERSPECTIVES

This thesis demonstrated different alternatives for the valorization of two bioresources that are generally under-exploited or discarded as wastes, undervaluing their potential. However, more studies a more detailed characterization of the extracts is need in order to establish the best strategy for its industrial application. It would be interesting to do fractionation and isolation of the phenolic compounds derived from the PB extraction process, in order to gather more information on their activities or functionalities and to establish their mechanisms of action. On the other hand, the integral use and analysis of synergies between isolated or purified compounds must be taken into account in the sense that it will be more feasible to use strategies that involve less purification due to the associated costs, decreased ecological footprint and loss of functionality due to overprocessing.

Given the great bioactive potential of PB phenolic extracts obtained by OH treatments, further studies are needed to explore their applicability in different industrial sectors. As shown in this work, PBE have antioxidant, antidiabetic and antimicrobial activity and, as clean solvents were used, can be incorporated into food products such as functional foods, packaging films and as preservatives and natural antioxidant thus increasing product shelf life. Recent studies show a great interest for new effective anticancer agents derived from natural products. Based on the selective growth inhibitory activity in cancer cell lines by the PBE, further studies are suggested to evaluate the effects of the extracts on cell functions, apoptosis induction and cell cycle distribution in tumor cells.

In the case of *Spirulina*, additional studies will be necessary to assess the stability of the extracted/purified molecules and their biological or technological potential for possible industrial applications.

Furthermore, up-scaling of the proposed extraction processes and integrated strategies for the valorization of the different fractions may be a challenge and should be considered to a future production of natural bioactive and technological compounds from PB and *Spirulina*. It is envisaged that these processes may be applied for the extraction of products with high added value from other bioresources.