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Rafael Alexandre Faria da Costa Félix

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Processing and characterization of seaweed phenolic extractives: a systematic approach

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Doutora Sónia Andreia Oliveira Santos, investigadora do Departamento de Química da Universidade de Aveiro, e do Doutor Armando Jorge Domingues Silvestre, Professor associado com agregação do Departamento de Química da Universidade de Aveiro.

o júri

presidente

Doutora Sónia Patrícia Marques Ventura
Investigadora do Departamento de Química da Universidade Aveiro

Prof.ª Doutora Sílvia Maria da Rocha Simões Carriço
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

Doutora Sónia Andreia Oliveira Santos
Investigadora do Departamento de Química da Universidade de Aveiro

palavras-chave

Bioprospeção marinha; Macroalgas; Compostos fenólicos; Florotaninos; HPLC-MS; Extração assistida por microondas

resumo

As políticas industriais do século XXI têm sido pressionadas para uma mudança que torne sustentável o atual paradigma dos mercados e do desenvolvimento económico. A bioeconomia – assente no aproveitamento integral, na valorização e na recirculação das matérias primas utilizadas na indústria, tanto quanto possível de origem biológica - tem sido vista como uma abordagem promissora. A bioprospecção da biomassa para o desenvolvimento de novas aplicações, nomeadamente no isolamento de compostos bioativos, representa uma das linhas de investigação e desenvolvimento (I&D) para a concretização deste objetivo global.A biomassa de origem marinha (e em particular as macroalgas) tem ganho reconhecimento como uma excelente fonte de produtos de valor biotecnológico acrescentado, e a sua exploração constitui a chamada Biotecnologia Azul; para países como Portugal (detentor da 11ª maior zona económica exclusiva do mundo), a valorização estratégica dos mares constitui um importante pilar para o desenvolvimento económico sustentável. Apesar de existirem no mercado produtos derivados de extratos de algas, apenas alguns compostos foram valorizados na sua forma isolada (essencialmente polissacarídeos). Não obstante, os Compostos Fenólicos de Macroalgas (CFMs) – e em especial os florotaninos - são alvo de elevadas expectativas dadas as suas promissoras propriedades biológicas. A valorização destes metabolitos tem ocorrido, contudo, a um ritmo inferior ao expectável, devido à dificuldade em extraí-los de modo sustentável e em caracterizá-los estruturalmente, permanecendo a maioria dos estudos baseada em ensaios espetrofotométricos (pouco específicos).

Esta tese propõe, assim, numa primeira fase, uma análise crítica da literatura, de modo a valorizar a informação existente, atualmente dispersa e pouco clara. Como tal, os dados publicados sobre a extração, separação e análise dos CFMs foram compilados e discutidos de forma sistemática. A aplicação de extração assistida por microondas foi revista em maior detalhe, e o uso de cromatografia-espetrometria de massa adequada à análise de florotaninos foi abordada de forma crítica. Nesta secção clarificou-se uma clara deficiência na obtenção e processamento inicial de extratos polares de macroalgas, com o objectivo de estudar os CFMs. A presença de outros metabolitos em quantidades superiores aos de interesse torna-se impeditiva no isolamento e caracterização de CFMs, em contexto académico, e potencia o uso de extratos brutos em vez de frações de valor acrescentado, na indústria. Por isso, numa segunda fase, o problema da co-extração de polissacarídeos com os CFMs é abordado, através do estudo de um processo de adsorção, do qual resultados preliminares são apresentados. Para tal, padrões de compostos modelo (fucoidano e alginato *vs.* ácido gálico, floroglucinol e catequina) foram usados, e a sua partição entre o solvente e o adsorvente (filtros de celulose *vs.* celulose microcristalina) avaliada por gravimetria, espetrofotometria e FT-IR. Os dados já obtidos por FT-IR da matriz de celulose após adsorção de uma mistura de polissacarídeos e CFMs revelam que uma maior afinidade da celulose para os polissacarídeos de algas (mantendo a maioria dos CFMs em solução), confirmando o potencial da técnica para uma rápida e eficiente separação destas duas familias.

keywords

Marine bioprospecting; Seaweed; Phenolic compounds; Phlorotannins; HPLC-MS; Microwave-assisted extraction

abstract

Industrial and economic politics of the XXI century are urging a change towards a more sustainable market and economic development paradigm. Bioeconomy – a paradigm based on integral use, valorization and recirculation of the materials used in industry, as much as possible of biological origin – has been recognized as a potential approach. Bioprospecting biomass for the development of alternatives to current models of human activity, and particularly in the isolation of novel bioactive compounds, is one of the research and development trends towards such global objective. Marine biomass – particularly seaweed - is increasingly considered an excellent source of added-value bio-based assets, and its exploration has given rise to the field of Blue Biotechnology; for countries like Portugal (holding the 11th largest exclusive economic zone), the strategic valorization of the seas constitutes an important part of sustainable economic growth. Despite the existence of some seaweed-derived nutraceuticals and cosmetics in the market, only a few isolated, structurally characterized extractives have been developed so far (essentially polysaccharides used in the food industry). Much potential is, nonetheless, attributed to Seaweed Phenolic Compounds (SPCs) - specially on phlorotannins – due to their diverse, promissing biological properties. However, a gap between the expected potentials of these metabolites and their actual rate of implementation exists, which is mainly a consequence of SPCs being difficult to be sustainably extracted and structurally analyzed. Thus, the majority of the literature in extraction and characterization of SPCs is rather speculative (based on spectrophotometric, non-specific assays.

In the first part of this thesis, a critical analysis of the literature is presented, with the goal of making sense of the already existent information, since it is highly dispersed and nuclear. The application of MAE to seaweed bioactives components was deeply dissected, and the use of chromatographic-mass spectrometric setups adequate for the analysis of phlorotannins was critically reviewed. In this part, it could be observed that the problems retarding the study and valorization of SPCs lie on the extraction and initial processing of the polar extracts of seaweed. The presence of other metabolites, more abundant than the target SPCs, impairs a thorough isolation and characterization of these compounds, in an academical context, and promotes the exploration of crude extracts rather than added-value phenolic fractions, in an industrial environment. Thus, in the second part of the thesis, the problem of co-extraction of polysaccharides and SPCs is being addressed by an adsorption-based process, for which preliminar results are here reported. Such experiments were conducted using standard compounds (fucoidan and alginic acid *vs.* gallic acid phloroglucinol and catechin) to analyze their partition between the solvente and the adsorption matrix (celulose filters vs microcrystaline celulose) by means of gravimetry, spectrophotometry and FT-IR. Data obtained so far from FT-IR of the dried adsorbent after contact with a mixture of polysaccharides-SPCs suggest revealed the higher affinity of celulose for the sugars (with most SPC remaining in solution), confirming the suspected potential of the technique for the separation of these two classes of biomolecules.

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List of Abbreviations/Acronyms

(F)MASE - (Focused) Microwave-Assisted Soxhlet Extraction

(t)CFs – (triturated) Cellulose Filters

(V)MHG – (Vacuum) Microwave Hydrodiffusion and Gravity

AA – Antioxidant Activity

ASE - Accelerated Solvent Extraction

ATP - Adenosine Triphosphate

CAMD - Compressed Air Microwave Distillation

CC – Column Chromatography

CE - Capillary Electrophoresis

CMAE - Continuous-Flow Microwave-Assisted Extraction

CPC - Centrifugal-Partition Chromatography

DAD - Diode-Array Detector

DMAE - Dynamic Microwave-Assisted Extraction

DMBA - 2,4-dimethoxybenzaldehyde

DMSO - Dimethyl Sulfoxide

DP - Degree of Polymerization

EAE – Enzyme-assisted Extraction

EEZ - Exclusive Economic Zone

EI – Electron Impact

EMEPC – Mission's Structure for the Extension of the Continental Shelf, from its portuguese name 'Estrutura de Missão para a Extensão da Plataforma Continental'

ESI – Electrospray Ionization

FAB – Fast-Atom Bombardment

FMASE - Focused Microwave-Assisted Soxhlet Extraction

FT-IR – Fourier-Transform Infrared Spectroscopy

GC – Gas Chromatography

GDP - Gross Domestic Product

HILIC - Hidrophilic Interaction Chromatography

HPLC - High-Performance Liquid Chromatography

ILs – Ionic Liquids

LMWP - Low-Molecular-Weight Phlorotannins

LOD - Limit of Detection

LOE – *Laminaria ochroleuca*'s Extract

LOQ – Limit of Quantification

MAD - Microwave-assisted Digestion

MAE - Microwave-assisted Extraction

MAHD or MWHD - Microwave-Assisted Hydrodistillation

MDG - Microwave Dry-diffusion and Gravity

MIS - Microwave Integrated Soxhlet

MRM - Multiple Reaction Monitoring

MS - Mass Spectrometry

MSD - Microwave Steam Distillation

MWCOD - Molecular-Weigh Cut-Off Dialysis

MWh - Microwave heating

NADES - Natural Deep Eutectic Solvents

NIH - National Institutes of Health

NMAE - Negative-pressure Cavitation MAE

NMR- Nuclear Magnetic Resonance.

NOAA - National Oceans Services Agency

NP – Normal-Phase

NPCE - Negative-Pressure Cavitation Extraction

PCR - Polymerase Chain Reaction

PEG - Polyethyleneglycol

PFP - Pentafluorophenyl

PGU - Phloroglucinol Units

PLE - Pressurized Liquid Extraction

PVP - Polyvinilpyrrolidone

R&D – Research and Development

RI – Refractive Index

RP – Reversed-Phase

SEC – Size-Exclusion Chromatography

SEM – Scanning-Electron Microscopy

SFE - Supercritical Fluid Extraction

SIM - Single Ion Monitoring

SLE – Solid-liquid Extraction

SLr – Solid-to-Liquid ratio

SPCs - Seaweed Phenolic Components

SPE - Solid-Phase Extraction

TAC – Total Anthocyanin Content

TFC – Total Flavonoids Content

TLC - Thin-Layer Chromatography

TPC – Total Phenolic Content

TPtC- Total Phlorotannins Content

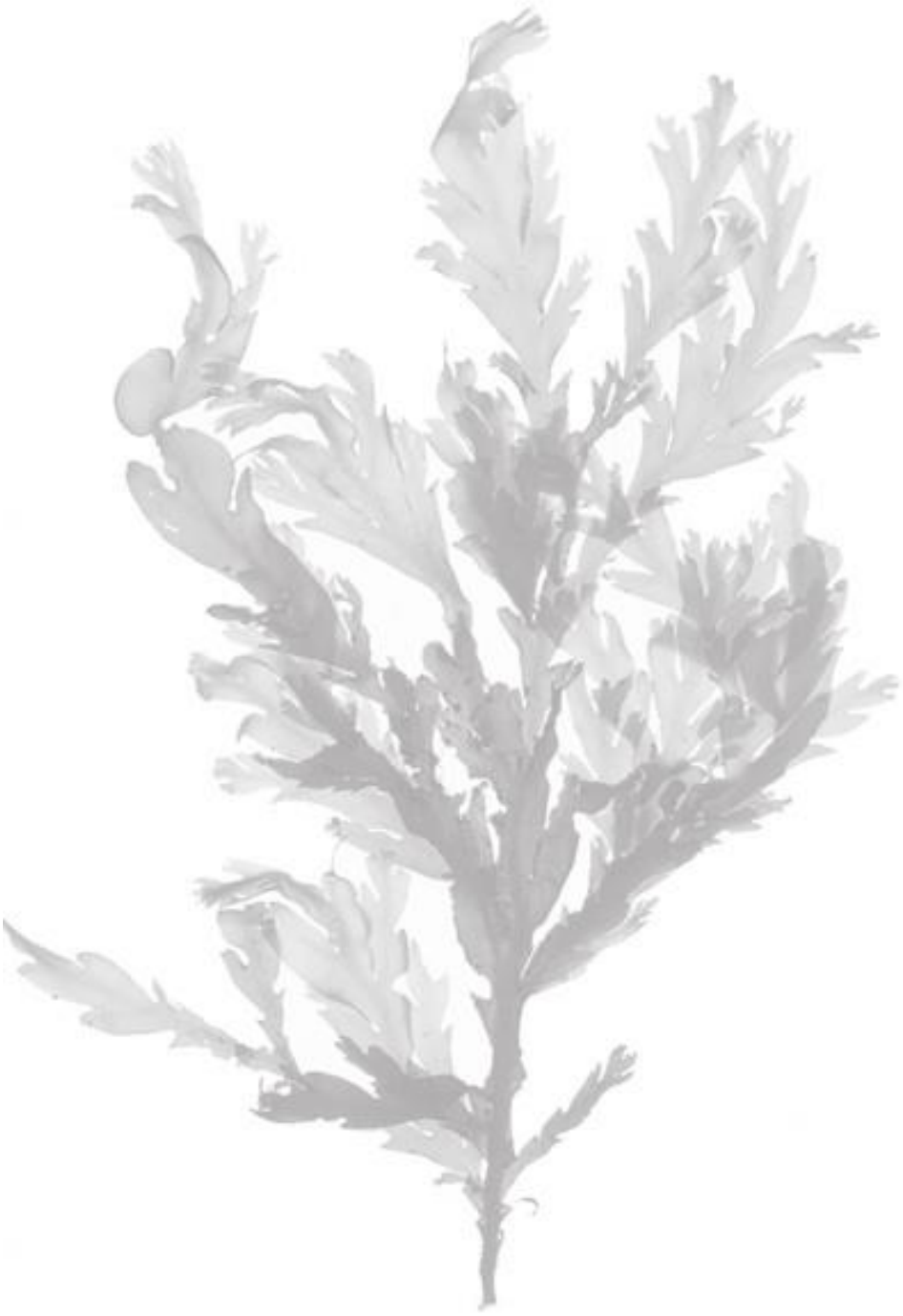
UAE - Ultrasound-assisted Extraction

UF - Ultrafiltration

UHPLC - Ultra-High Performance Liquid Chromatography

UMAE - Ultrasonic Microwave-assisted Extraction

VMWHD - Vacuum Microwave-Assisted Hydrodistillation



PART I - BACKGROUND

Biotechnology comes in many different forms, but one thing is transversal: it supports the paradigmatic changes expected for the XXI century. While highly technological biological tools such as gene editing technologies or protein engineering promise great advances in the limitations of mankind – i.e., novel medical perspectives for today's incurable diseases, or even the abrupt extension of average lifespan -, a different type of biotechnological revolution is happening regarding the industrial activity, where biological sources and processes promise to result in more efficient and ecological production-consumption cycles. This type of biotechnology will be introduced in the following topics, for a motivational contextualization of this thesis, emphasizing the importance of conducting the research that supports the implementation of such urgent modernizations.

I.a - Bioeconomy concepts

The world of the 21st century is one of multiple distresses and generalized concern of the scientific and political community regarding the sustainability of mankind – not only for humanitarian, geopolitical and societal challenges, but also for the problematics arising from planetary resources inadequate exploitation. Pollution of the ecosystems, scarcity of key resources, disruption of biotic homeostasis from biodiversity loss and the climate change crisis are examples of the drastic phenomena increasingly present worldwide [1], and driven by the economic model (economic as in 'management of production and consumption of goods and services by mankind') established, more prominently, since the industrial revolution. In general, damage for the environment can be attributed to the petrochemical-based approach to energy and materials generation (or any fossil-based one) that settled the current paradigm. Petroleum and its derivatives form the basis for most human activities, from the simple fact that it still provides the majority of energy in the world, to the myriad products and services depending on petrochemicals like organic solvents and precursors, ranging from pharmaceuticals to food packaging and many polymer-based commodities.

Bioeconomy – the rational design of an economic model of development where biomass provides for the energy and materials industries, virtually feeding every human activity - is nowadays considered a promising candidate paradigm to promote a shift towards sustainability [2]. By centering the economy in biomass, rather than in fossil resources, several improvements are achieved: total renewability; reduced pollution and ecotoxicity

(from decreased disposal of hazardous by-products, increased re-circulation of residues and overall biodegradability and biocompatibility of many bioproducts); contribution to zero-carbon net emissions, along with other renewable energy sources. Thus, a reversion of the climate crisis and biodiversity loss driven by human activity might begin to take place. Furthermore, an improved capacity to rationally design products, services and production processes due to plasticity and diversity of biological systems (i.e., large unexplored library of organic compounds, genes, etc.) makes bioeconomy a logic step for this ever-evolving civilization that is Mankind.

Bioeconomy has the potential to provide a more homogeneous, fair access to energy and products across the globe, helping the resolution of humanitarian crisis as well as reducing the incidence of armed conflicts over oil-rich territories. However, this also makes bioeconoy vulnerable, for its inconvenient impact on the well-established oil industry. With or without an active intervention of petrochemical-industry lobbyists on the retention of bioeconomy's evolution, the slow implementation of biobased-industries resides in the indirect effect of having petrochemical-based industries so well established. Decades of optimization and infrastructure development have led to the currently low prices associated with it, so much that the higher prices of bioindustry implementation keep it from attracting massive investments or thrive in markets.

For the reasons herein presented, research in chemistry and biotechnology has been marked, more than ever, by the dual attempt to reach technological progress while ensuring sustainability – both environmental and financial. Bioprocess engineering, biomaterials science, bioprospecting, and others, are fields of the natural sciences dedicated to key portions of the economic construct of bioeconomy. Moreover, the development of a biomass-based economy represents a change in paradigm for the financial system of the world, as well. Novel chains of resource harvesting or production, as well as adapted infrastructures for transport and industrial processing are required for a bioindustry to develop. Key activities (or sectors) of biotechnology exist and strategic adaptation to each will lead nations and economic groups to different specialties. One such sector – the blue biotechnology – is particularly appealing for its proven potential to generate high-value economical and social assets. An overview of blue biotechnology is provided in the next topic.

I.b - Blue Biotechnology

Blue biotechnology is the sector responsible for exploring the technological potentialities of the marine environment and the immense marine biodiversity [3]. In the past years, it has leaped from the state of a promising area to a nowadays implemented fast-growing business area - drugs, cosmetics, food, biofuels and all other sorts of chemicals have been isolated, identified and/or explored from marine sources [4]. Classical examples are Acyclovir (anti-retroviral medication, Figure 1a) and *Taq*-polymerases (the enzymes routinely used for polymerase chain reaction, PCR, Figure 1b), first discovered in a marine sponge [5] and in marine hydrothermal bacteria [6], respectively. However, the potentialities of marine resources as a source of bioactive compounds or other-purpose added-value components are far from completely identified and explored. In fact, oceans cover more than 70% of earth's surface and the United States' National Oceans Services agency (NOAA) states that less than 5% of them has been explored [7] – despite the lack of definition on "explored", it is illustrative of the undiscovered potential. Also, while 250 000 species are described for the oceans by the Census of Marine Life, one million non-microbial species plus tens to hundreds of millions of microbial species are estimated to occupy marine habitats [8]. The structural and functional diversity of biomolecules associated to biodiversity is thus presumably enormous; actually, in the first sampling expedition of the ocean exploration genome project, 1.2 million new genes have been identified, doubling the number of protein sequences in GenBank (National Institutes of Health, NIH) [9].

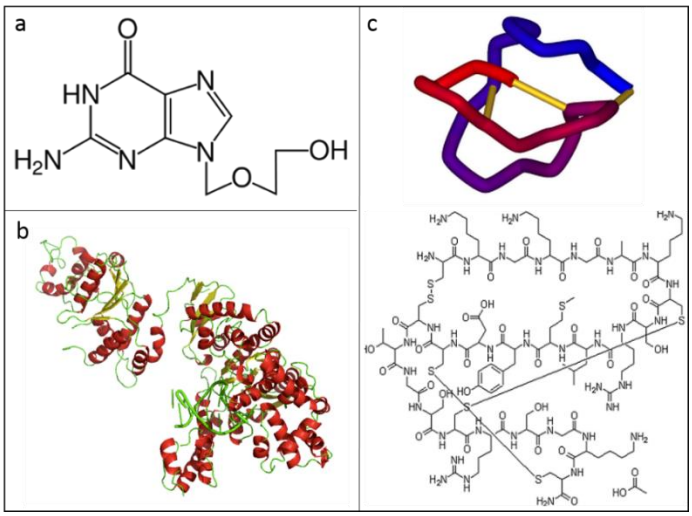


Figure 1 – Structural depiction of some different types of marine biotechnologically interesting compounds; a- acyclovir; b- *Taq*-Polymerase 3D structure; c- 3D conformation of ziconotide (upper) and its structural formula (lower).

Not only is there a large amount of compounds to be discovered, but marine natural products are actually more likely to exhibit certain types of bioactivity, e.g. 10x the anti-tumor activity of terrestrial ones [10] while being superior in terms of chemical novelty, i.e. of revealing novel scaffolds, with novel bioactivity pathways [11]. An example of excellence is ziconotide (Figure 1c), a marine natural product nowadays commercialized as an analgesic, whose mechanism of action is completely different of any other previously known, resulting in a non-opiate, devoid of tolerance development and 1000 times more potent than morphine [12]. For these and for other reasons, the marine biotechnology market is estimated to reach $6.4 Billion by 2025 [13].

Portugal has an exclusive opportunity to expand its economy on the basis of marine biotechnology: as a country, it detains the 11th biggest Exclusive Economic Zone (EEZ) of the world (see Figure 2), comprising about 1,727,408 square kilometers of marine environment; plus, since 2009, a proposal has been delivered by the Mission's Structure for the Extension of the Continental Shelf (EMEPC, from its portuguese name 'Estrutura de Missão para a Extensão da Plataforma Continental') committee to expand Portugal's EEZ in about three times [14,15].

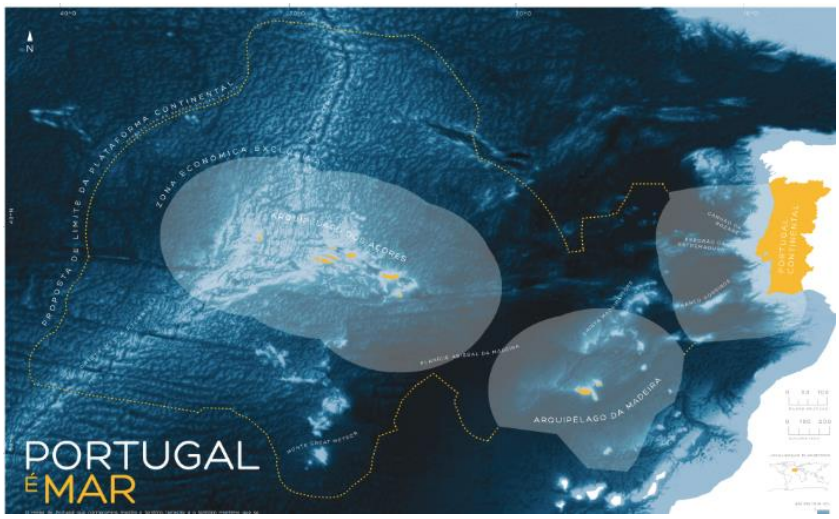


Figure 2 - Communication flyer of the EMEPC committee depicting both the actual EEZ (transparent gray areas) and the proposal of expansion (dashed yellow lines). [16]

In the near future, major benefits could advert from blue biotechnological Portuguese advancements. It is known that Portugal, as other South-European countries, are living an

economic crisis with ever-growing public debt and population poverty; smart economy politics are necessary if we are to overcome the crisis with a sustainable and profit-seeking strategy. For instance, the discovery of new drugs and bioactive compounds propels the creation of new technology-based start-ups and their role in capital dynamics [17]. Furthermore, knowing and exploring the natural resources, in contrast to importing and intensively producing non-naturally occurring products, might lead to reduction of pollution, energy and water consumption, habitat destruction and other forms of environmental hazards.

A portuguese maritime bioeconomy is under planning for a long time: until 2009, the "Hypercluster for an Economy of the Seas", and after that, the "National Strategy for the Sea 2006-2016" and the "National Strategy for the Sea 2013-2020 (ENM2020)" are the documents that systematize the potential and the handicaps of a national sea-based bioeconomy, while developing the strategic actions and changes that are meant to lead this economic evolution. Horizon 2020 – the European project for financing Research and Development (R&D) – has a considerable budget allocated only to the marine resources in Portugal: almost 400 million euros [15]. Such investment might aid the accomplishment of the gross domestic product (GDP) goals predicted in the ENM2020: an increase in the sea's contribution for GDP from the 2% direct value (5-6% indirect) observed in the first decade of the century – employing 75000 people – to 3% by 2020 [18] and 5% direct values (12% indirect) by 2025 [19]. ENM2020 clearly identifies five strategic areas of intervention for the blue economy to bloom: energy; aquiculture; tourism (both coastal and cruise); mineral maritime resources; and finally, blue biotechnology [18]. The latter – more related to this Thesis – is particularly interesting, firstly because its products – being highly technological – have the potential to generate massive revenues (however at higher risks and initial costs), and secondly, because Portugal's EEZ has a large ultra-depth domain mostly unexplored, where extremophiles, e.g. from the Azorean hydrothermal occurrences or other methane emitting structures, are assumed to prosper, creating unique opportunities for high-value molecular novelties to be discovered [19].

The value of marine biomass is related to several different potentials – it can serve as food, provide substrate for other food-producing systems, be a source of bioactive compounds, polymers and other biobased materials, among many other applications. Most societal requirements can, indeed, be directly or indirectly addressed by bio-based solutions.

Either for a bioactive compound-directed approach, or for a more generalized characterization of a given biomass potential for exploitation, bioprospecting studies are used. This area of research consists in the identification of candidate organisms and the study of their interesting characteristics, usually by chemical characterization of its (macro)molecular components, involving extraction, fractionation and structural and functional characterization. Research-scale bioprospecting intimately relates to the large-scale biorefinery (the industrial activity of actually employing bioprospecting-derived knowledge in the refining of interesting molecules from industrial by-products or fresh stock feed from selected biomass). Lab-scale experiments shall serve as an initial assessment of the feasibility of such biomass valorization from the point-of-view of procedural costs and sustainability. Thus, optimization of extraction and purification processes carefully addressing the implementation of the studied technology are often performed alongside or after the identification of biological sourced values. This role of research in blue biotechnology, and more specifically in seaweed biomass valorization, is the scope of this Thesis. The biotechnological potentials of seaweed are discussed in the next topic.

I.c - Seaweed Biotechnology

Seaweed are a polyphyletic group of marine, photosynthetic organisms. It is informally described as the group of macroscopic algae (macroalgae) from the three monophyletic groups Phaeophyceae (Class, Brown algae), Chlorophyta (Division, Green algae) and Rhodophyta (Division, Red algae) [20]. The designations of seaweed and macroalgae, as well as the color-based naming of the groups are, however, rather inaccurate and constantly target of updates and discussion by biologists [20]. Notice how red algae correspond to a division of Plantae, while green algae are a specific set of classes from Chlorophyta, a much bigger phylum encompassing many more microalgal species, and brown algae are the members of Phaeophyceae, a class from the kingdom Chromista (Figure 3). Nonetheless, it remains a useful classification for the applied sciences.

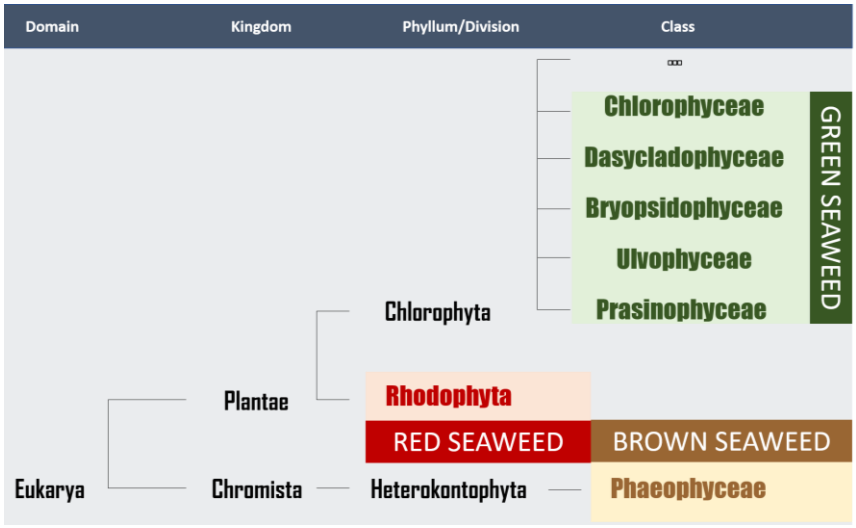


Figure 3 - Taxonomy of what is collectively described as seaweed – a polyphyletic group.

Macroalgae have world-wide distribution, and are important contributors to the ecosystems. Major roles of macroalgae in natural environment include sheltering of biodiversity and primary production, serving both atmosphere and water chemistry equilibrium and food-chain initiation [20]. Also, they have been explored by mankind in diverse ways – in China and other oriental countries, the presence of seaweed in routine, daily diet is ubiquitous and started as early as 2000 b.C., both because flavor was pleasant and because iodine levels in seaweed make it an excellent functional food regarding the obtainment of this nutrient, while in Wertern civilizations, whole seaweed is rarely eaten,

but a large number of processed food products contain seaweed extractives – such as carrageenans – for preservation or organoleptic improvements [21].

Applications for seaweed-derived technologies (such as seaweed bioactive compounds) are a research highlight of blue biotechnology – and will be detailed below. A more detailed revision will be made on the phenolic components of seaweed and on recent developments in extraction methodologies, since the identification of an added value product from seaweed biomass along with a profitable and sustainable extraction approach are crucial steps for the integration of seaweed in a future bioeconomy.

I.c.1. Seaweed bioactive compounds

Seaweed are now a recognized potential source of bioactive metabolites, applicable in diverse industries, ranging from food to cosmetics, passing through biomedical and nutraceutical technologies [22]. Such applications are potentiated by the fact that many seaweed species are edible (even though many are not yet classified as such), which constitutes an advantage for food technologies (by the direct application of seaweed or seaweed derivatives) as well as for the remaining industries, as contamination with toxins is unlikely and therefore purification procedures have less costs associated. Also, biocompatibility of seaweed-derived materials is a positive feature of macromolecules isolated from seaweed [23]. Several macromolecules and secondary metabolites with biotechnological relevance have been found in seaweed: polysaccharides, proteins and amino-acids, carotenoids and other lipids, phenolic compounds and halogenated derivatives, among others [24].

Polysaccharides have been one of the most exploited algal products, especially by the use of agar and carrageenan (red algae polysaccharides) in the scientific and food industries, respectively [25]. Both for their gelling properties and for their bioactivities, e.g. anti-inflammatory, diverse classes of sulfated polysaccharides have been studied, e.g. alginic acids and fucoidans (brown algae), or ulvans and sulfated rhamnans (green algae). Biomedical applications, ranging from wound dressing to controlled drug delivery are under development [26].

Phycobiliproteins are a family of algal proteins exhibiting optical properties that make them excellent fluorophores for application in biotechnology, such as relatively large stokes

shift, high intensity and high water-solubility [27]. Seaweed have also been found to express lectins, carbohydrate-binding proteins which have countless applications [28].

Carotenoids (such as β-carotene, lutein, violaxanthin, neoxanthin, zeaxanthin and fucoxanthin) and sterols (such as fucosterol and 24-methylenecholesterol) are two examples of biotechnologically relevant seaweed lipids, with the first being used as coloring agents and investigated for their antioxidant properties [29,30], while the latter are considered "healthy sterols", shown to have anti-hypercholesterolemic activity [31]. Other terpenoids (sesquiterpenoids) are also present in seaweed's essential oils [32]. Hydrocarbon-derivatives have also been identified in seaweed, as well as some of their halogenated derivatives, often presenting high antimicrobial and cytotoxic activities, while serving anti-predator and sexual pheromone functions in the ecology of seaweed [33,34].

I.c.1.1. Seaweed phenolic components – structural diversity

Structurally, phenolic compounds present in macroalgae vary from simple molecules, such as phenolic and cinnamic acids or flavonoids to the more complex phlorotannin polymeric structures. Hydroxybenzoic acid derivatives, such as gallic acid (Figure 4), are commonly reported as constituents of different green, red and brown macroalgae species [35–37].

Flavan-3-ol derivatives, such as epicatechin or epigallocatechin (Fig.4), have been one of the major class of phenolic components detected in green, red and brown macroalgae [35,38]. Other flavonoids, such as rutin, quercitrin, hesperidin, myricetin, morin, kaempferol and cirsimaritin (Fig.4), have been also detected in several Chlorophyta, Rhodophyta and Phaeophyta species [39,40]. Different isoflavones, such as daidzein or genistein (Fig.4), have been also identified in the red macroalgae *Chondrus crispus*, *Halopytis incurvus and Porphyra sp. and* in the brown ones *Sargassum muticum*, *Sargassum vulgare* and *Undaria pinnatifida* [41]. Halogenated derivatives of phenolic compounds have also been reported as constituents of macroalgae, including simple structures, such as brominated derivatives of hydroxybenzoic acids, already detected in the green macroalgae *Ulva lactuca* [42], to more complex classes, such as the two clorinated aurones reported in the brown algae

Spatoglossum variabile [43]. Additionally, a vast number of sulphated phenolic compounds were also detected in several Rhodophyta and Phaeophyta species [44,45].

Uncharacteristic components have been also detected in some macroalgae, such as carnosic acid (Fig.4), a phenolic abietane based diterpene commonly found in flowering plants [46], and which has been also detected in *Himanthalia elongata* [40].

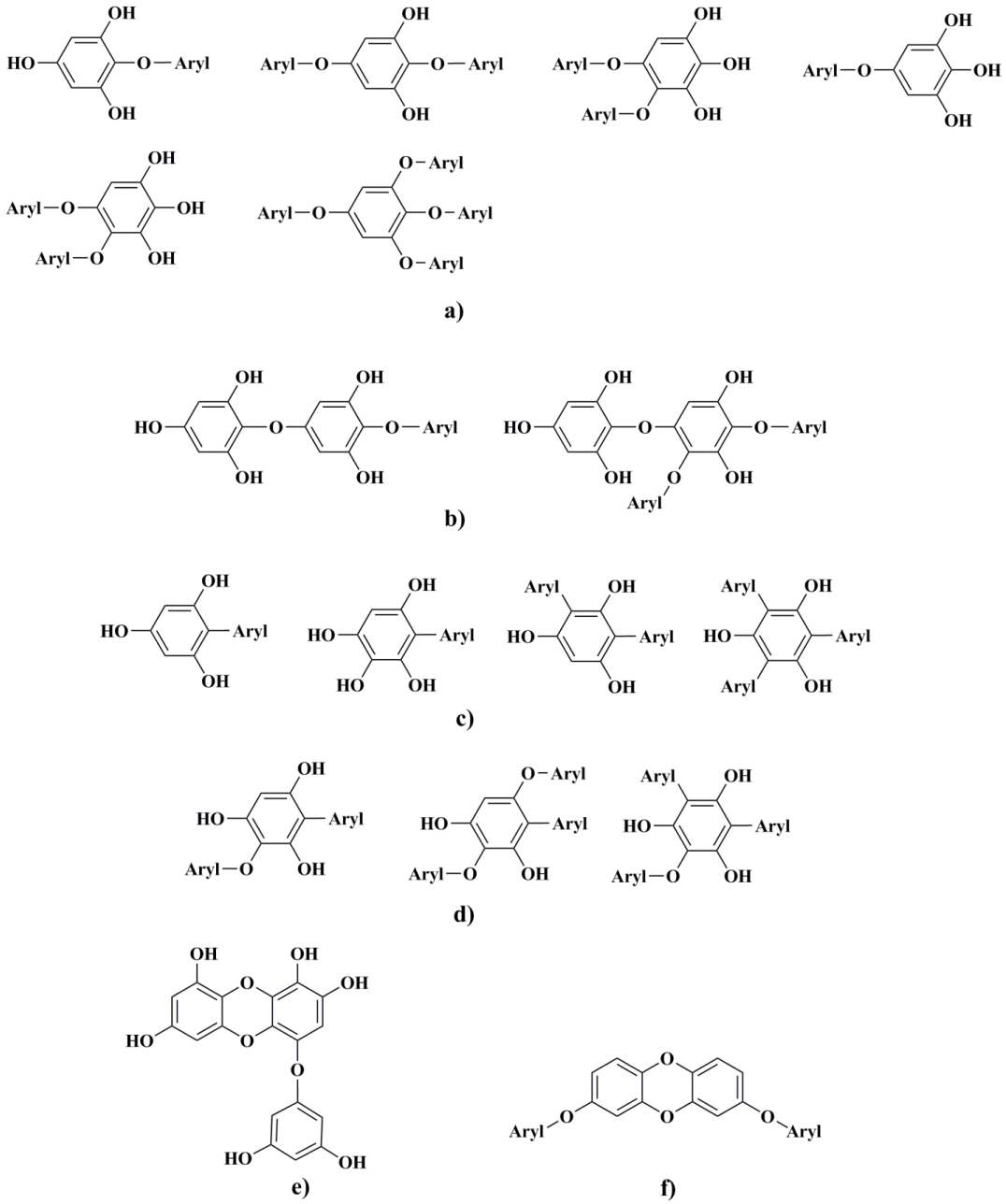


Figure 4 - Generic structure of phlorethols a), fuhalols b), fucols c), fucophlorethols d), eckols e) and carmalols f). Adapted from [47]

Notwithstanding, the often-recognized high content of phenolic compounds in brown macroalgae (as compared to green and red ones), is normally associated with phlorotannins, a restrict class of polyphenols derived from the oligomerization through diaryl ether or C-C bonds of phloroglucinol (1,3,5-trihydroxybenzene) units. This class comprises compounds with a large range of molecular sizes, ranging from 126 Da (phloroglucinol) to several kDa. Actually, there is no consensus about the highest molecular size of phlorotannins, although several authors reported values of 100 [48,49] or 650kDa [50], despite there is no evidence of such values have been confirmed. Phlorotannins can be classified into four groups depending on the type of linkage between aromatic units, namely: phlorethols and fuhalols, with ether linkages; fucols, with phenyl linkages; fucophlorethols, with both ether and phenyl linkages; and eckols and carmalols, with benzodioxin linkages [47,51], as depicted in Figure 5.

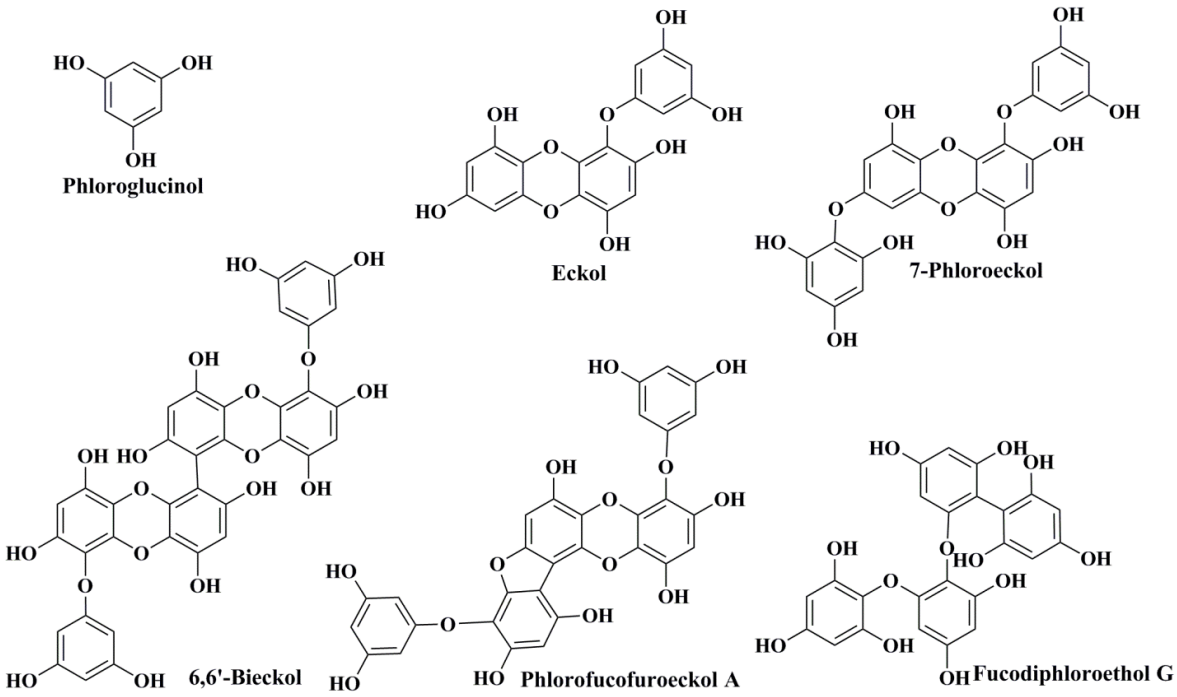


Figure 5 - Major phlorotannins identified in brown macroalgae

Although some species, such as those from *Ecklonia* [52,53] and *Fucus* genus [49,54,55], are known to contain significantly higher contents of phlorotannins, these components have been identified in a vast number of other macroalgae species [56–63], with quite variable profiles and abundances. Phloroglucinol, eckol, 7-phloroeckol, 6,6'-bieckol,

phlorofucofuroeckol A and fucodiphloroethol (Figure 6) have been the most described phlorotannins as constituents of brown macroalgae [52,53,56,58,60]. In fact, the majority of the published studies reported phlorotannins with a polymerization degree below 10 phloroglucinol units [52,53,56,58,59], however the availability, in the last years, of more advanced separation and analysis techniques have allowed to report phlorotannins structures with higher numbers of repeating units. Heffernan et al. [49] have detected phlorotannins with up to 16 units of phloroglucinol in four brown macroalgae (*Fucus serratus*, *Fucus vesiculosus*, *Himanthalia elongata* and *Cystoseira nodicaulis*) from Irish coast. Phlorotannins containing up to 17 phloroglucinol units were also identified in *Cystoseira abies-marina* [57]. Finally, the largest phlorotannins characterized so far were detected in *Fucus vesiculosus*, namely with 49 phloroglucinol units [55].

Finally halogenated and sulphated derivatives of phlorotannins are also commonly present in brown macroalgae species [61–66]. Bromo, iodo and chloro derivatives of phlorotannins are commonly composed by up to 3 phloroglucinol units, being mainly derivatives of phloroglucinol, phlorethols and eckols [63–65], although a chlorobifuhalol and a chlorodifucol have been already detected in *Carpophyllum angustifolium* [62]. A sulphated bromophloroglucinol was also identified in *Polysiphonia lanosa* [66], while 8 sulphated phlorotannins with 1 or 2 phloroglucinol units, namely phloroglucinol and difucol or diphlorethol derivatives, were detected in *Pleurophycus gardneri* [61].

I.d – Objectives

Arising from the recognition of the enormous potential of seaweed as a rich biomass for bioactive compounds fractionation, seaweed bioprospecting has become a hot-topic in biotechnology. High expectations are being put in the potential of macroalgae for rendering novel bioactive compounds, as the number of studies reporting a diverse set of bioactivities (anti-tumoral, antimicrobial, antioxidant, among others) is increasing.

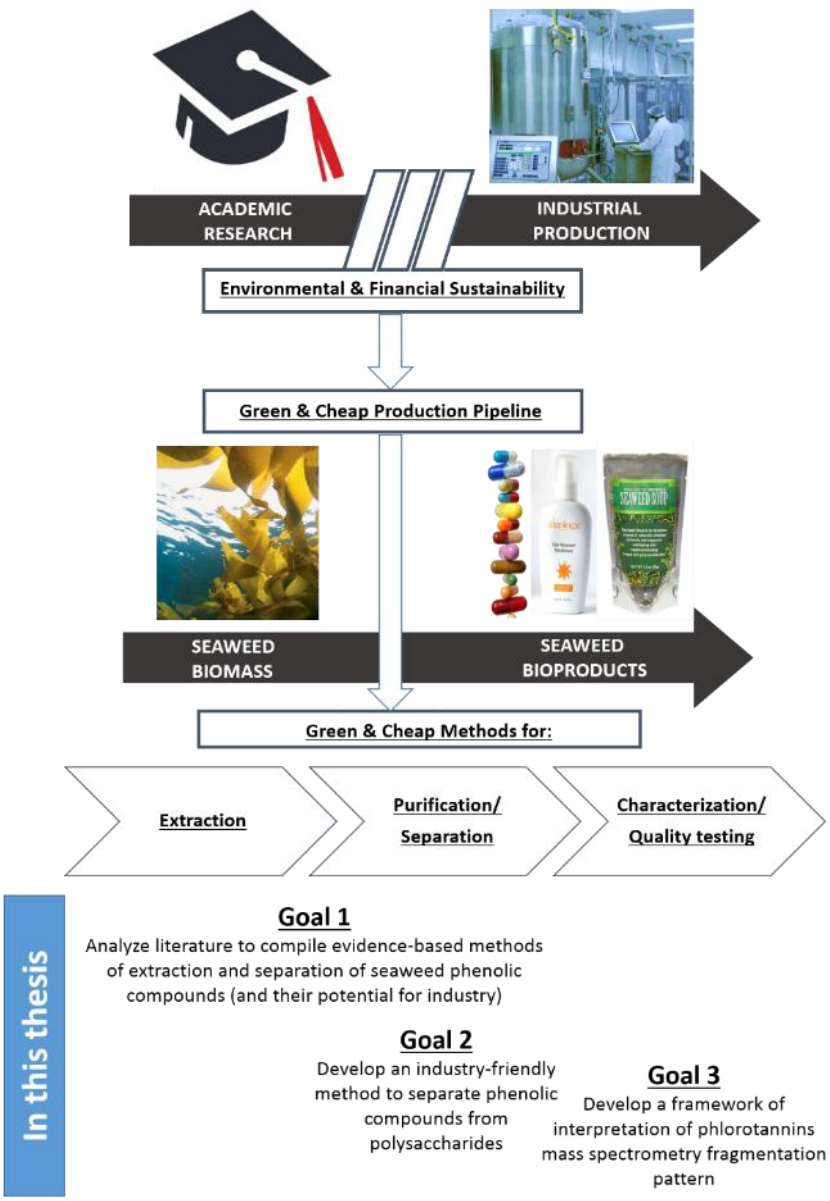


Figure 6 – Scheme of the main topics regarding Seaweed Phenolics valorization and the frame within which the goals of this thesis are placed.

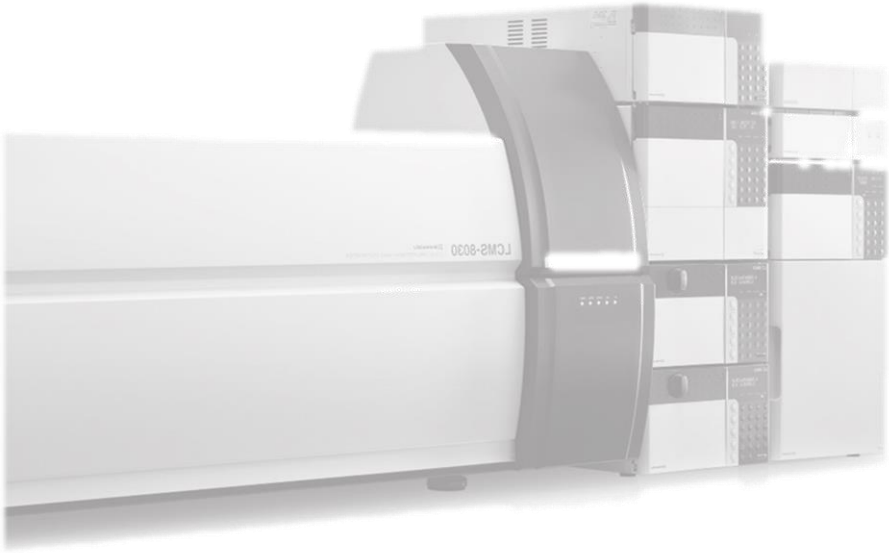
However, a gap between the expected potentials of these metabolites and their actual rate of implementation exists, which is partly a consequence of seaweed bioactives – and particularly seaweed phenolic components (SPCs) - being difficult to be sustainably extracted and structurally analyzed. These difficulties must be adressed in an academic context, and by means of a systematic approach – i.e., current knowledge, current obstacles and strategic solutions need to be thoroughly dissected under the focus of scientific rigor. The objectives of the approach used in this thesis are outlined in Figure 7.

Actually, a critical analysis of the literature on SPC bioprospecting reveals a confusing, incoherent and speculative set of methods and results across scholars. For that reason, a compilation of the literature that offered a tentative resolution of prefered practices and pinpointed supported claims has been organized and critically dissected. Not only was the state-of-the-art described concerning SPC extraction and characterization, but current challenges and obstacles to the valorization of these compounds were identified. Part II – "From Seaweed Biomass to Seaweed Bioactives" is the result of this bibliographic journey, and was adapted from two scientific pulications produced with such information.

This initial work provided an integrated view over the major problematics in SPCs research and development. From extraction to valorization, some procedural steps have been limiting factors concerning both the characterization of phenolic metabolites, in academical context, or the industrial formulation of SPC-based products. Effectively, while extraction technologies discussed in Part II may provide a solution for initial treatment of seaweed biomass, they are almost invariably impossible to be done withouth the co-extraction of polysaccharides, and these are much more abundant than SPCs (and incompatible with their analysis). A separation step for enrichment in phenolics and removal of carbohydrates is essential for the success of the bioprospecting. Despite all the published methodologies, no consensus nor a solid demonstration of reliability has been found proving any purification approach to be efficient while preserving PCs diversity and yields. For that reason, an experimental design was prepared for testing a method based on adsorption of the polysaccharides to a cellulosic matrix, with posterior recovery of the supernatant and differential elution of the adsorbed compounds. The rationale, procedural details and preliminary results of this work are reported in Part III.

Another observation can be made regarding the identification of both the already known and the unknown phenolic compounds in a given extract. Structural characterization

has been succesful, only when isolated compounds have been prepared by extensive purification methodologies and different spectroscopic methods were applied. This is, however, an impracticable approach for industrial quality control or for high-throuput bioprospecting research. Much prefered, High-Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS) provides an interesting combination of high resolution and high throughput in the analysis of biomolecules. However, mass spectrometry alone is rather unexplored for the analysis of SPCs – and particularly phlorotannnins. Despite the known limitations of this technique, we attempted the compilation of mass spectral data and development of a framework for its interpretation (a tool nowadays unaccessible). Due to the limitations encountered along this phase, the possible analysis has been included in section II.f.



PART II - FROM SEAWEED BIOMASS TO SEAWEED BIOACTIVES

A PHENOLIC-COMPOUNDS-FOCUSED STATE-OF-THE-ART ON EXTRACTION AND SEPARATION APPROACHES

Note:

An adaptation of this Part of this thesis (except II.b) is currently being arranged to be submitted as a review article, for which approval of the theme has already been obtained from the journal Algal Research (ISSN: 2211-9264).

The extraction of phenolic compounds from seaweed is impacted by different parameters, such as the sample particle size, the extraction method employed, the extraction time, the storage conditions (for both raw material and extracts) or the presence of interfering components [67]. Additionally, the extraction conditions must be carefully selected in order to avoid possible degradation of phenolic compounds. Temperature, air and light exposure are the main factors promoting the degradation reactions of phenolic compounds [68,69]. Besides, the approach of extraction of biomass directly impacts the costs and environment-degrading consequences of its industrial application, thus becoming a key aspect to be considered when valorisation of biological molecules is intended. As with most biomass extractions with an industrial application potential, SPCs extraction by conventional, solvent maceration, although being often optimized and the basis of most data available, is being increasingly avoided, and novel extraction methodologies, designed for higher efficiency and sustainability, are being tested as future substitutes. An analysis on academy- and industry-suited extraction platforms in use or in development is the content of section II.a. Of particular interest for high-throughput extractions – academically relevant - and for industrial employment due to infrastructure adaptability of current bioprocessing facilities, Microwave-assisted Extraction (MAE) is a method with great procedural and productive advantages. For that reason, MAE is the focus of a detailed examination in section II.b.

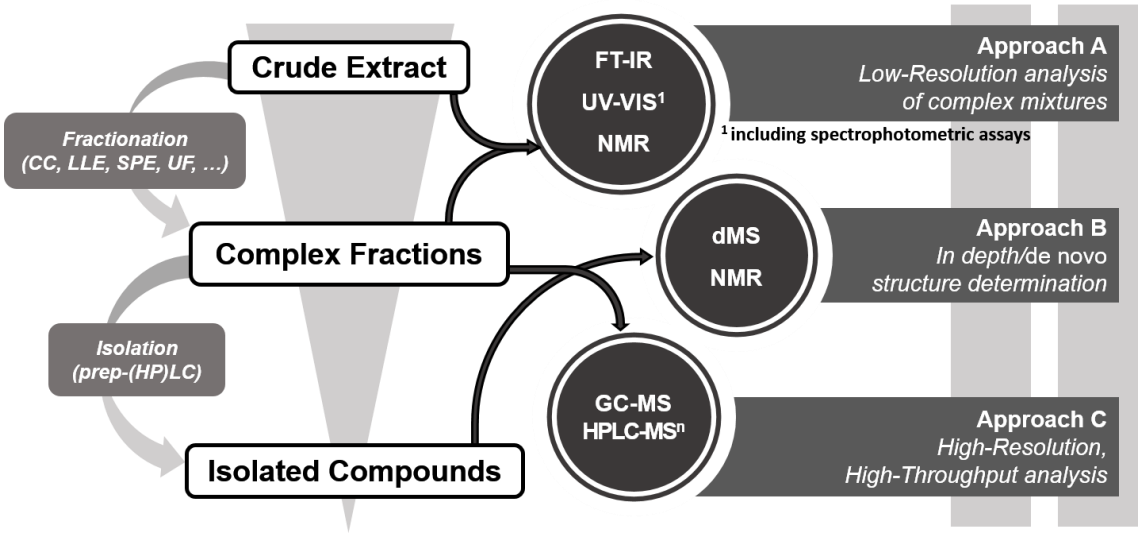


Figure 7 – Post-extraction seaweed phenolic compounds experimental approaches. Depending on how processed the extract is prior to qualitative inquiry, two major approaches are possible: a low-resolution one (direct analysis of the crude extract, Approach A) and high-resolution analysis (using separation techniques to improve compound specific data, Approaches B and C).

Further analysis of phenolic rich extracts is a complex task that has been simplified in the last decade by the technological evolution of many chromatographic and spectroscopic techniques. Nonetheless, as being mostly complex extracts, their characterization usually involves a global gravimetric and spectrophotometric analysis as well. In Figure 8, the different techniques used in SPC-rich extracts processing and analysis, as well as their phased applicability, are schematized. Different strategies might be used, mostly depending on the extent of purification of the extractives, the resolution of the analytical platforms utilized and the previous existence (or absence) of spectral data on a given class of compounds. The critical steps at such pipeline are discussed in sections II.c. to II.f.

II.a – Extraction of seaweed phenolic compounds

II.a.1 – Conventional extraction: current practices and drawbacks

Conventional solid-liquid extraction (SLE), also called maceration, in which the components are removed from a biological matrix by submersion in an appropriate solvent or solvents mixture, often aided by mechanical stirring [49,70–73], is the most traditional technique used to extract phenolic compounds [74], being also the most applied in macroalgae [60,72,75]. Apart from the solvent used, the main parameters affecting the efficiency of this technique are the temperature, extraction time and solid/liquid ratio (SLr). Different conventional solid-liquid extraction approaches employed for the isolation phenolic compounds fractions from macroalgae are summarized in Table I.

The choice of the extraction solvent or solvent mixtures is one of the main concerns for the success of any extraction process. Organic solvents are still the most successfully applied in the extraction of phenolic compounds from macroalgae. Binary aqueous mixtures with methanol, ethanol, acetone or acetonitrile have been the most often used [40,53,55,56,75]. Although there is no consensus on the best solvent/mixture of solvents used to extract phenolic compounds from macroalgae, several authors have observed better extraction efficiency with organic aqueous mixtures than the ones obtained with a single solvent [75,76], with the exception of Leyton et al. [71] which verified a higher Total Phenolic Content (TPC) in the brown macroalgae *Macrocystis pyrifera* extracts using water as solvent when compared with different organic solvents or organic aqueous mixtures. However an increase in the polysaccharides content should not be discarded to contribute to the higher TPC. Notwithstanding, Nwosu et al. [75] verified a noteworthy increase on the TPC of different extracts from edible macroalgae when these were prepared with acetonitrile:water (50:50, v:v) instead of 100% methanol.

Most of the reported studies have performed SLEs at room temperature [36,49,54–56,60,70,75–81]. Actually, the use of higher extraction temperatures has been described to promote the oxidation of phenolic compounds [69]. Notwithstanding, Kim et al. [60] have verified a positive and independent effect of the extraction temperature on the soluble phlorotannins content of *Eisenia bicyclis* extracts, reaching a maximum extraction yield at 80ºC. The extraction times reported in literature for phenolic compounds from macroalgae ranged between 5 min [72] and 24 h [76]. More importantly, a balance between temperature

and time should be acquainted, since the thermal degradation can be prevented by limiting the time of exposure to high temperatures. More consensus is observed in the SLr used, with most of the authors employing ratios (w/v) of 1:10 [49,75] or 1:20 [80][76]. Several authors have already also studied the effect of the SLr on the phlorotannins content. The optimal SLr determined by Leyton et al. [71] for the phlorotannins extraction from *Macrocystis pyrifera* was 1:15 (within a studied range between 1:10 and 1:20), while, according to the authors no SLr effect (in the range of 1:2 and 1:10) was observed on the phlorotannins content of *Eisenia bicyclis* [60].

The addition of antioxidant agents to the extraction media, has been reported to increase the stability of phenolic compounds in general and thus preserve their antioxidant potential [82], and inevitably of phlorotannins. Actually, the use of up to 0.3% of ascorbic acid (w/v) showed to enhance both the number of identified phlorotannins as well as their contents and quantity of extracted phenolic components [54]. Finally, in some of the studies, phlorotannins rich extracts have been further derivatized through acetylation in order to prevent oxidation [58,63,73].

Despite its simplicity, easy adjustment, efficiency and exhaustive reproduction in the bibliography – all of which account for conventional SLE being a first approach to study the phenolic composition of macroalgae -, its limitations have led to a pursuit of more efficient, environmentally friendly and sustainable techniques in recent years. The major concern regarding conventional SLE is the need for large volumes of organic solvents. This problem results in many different disadvantages: it increases the cost of the process, it makes the process polluting and eventually toxic for workers, and it is not compatible with most food-applications due to toxicity concerns. Having this into consideration, water based extractions are privileged, and ethanol and acetone are borderline solvents, with rather good acceptance both from security and price-related perspectives. Another fact that is responsible for the decay of SLE as an option is the much-reduced time of extraction that has been achieved when alternative methods are employed for processing biomass. Such reduction provides a very significant improvement in the economic feasibility of a given added-value potential product.

Table I - Solvent mixture, SLr, extraction time and temperature employed for conventional SLE of phenolic compounds from different macroalgae species.

Solvent mixture	SLr (w/v)	T (h)	T (ºC)	Species	Extracted Compounds	Type of characterization[a]	Ref
Methanol, ethanol, water, methanol: water (50:50), hexane:ethanol (88:12), ethanol: water (25:75 and 80:20), ethyl acetate: water (50:50), acetone: water (80:20 and 70:30), methanol:chloroform (66:33)	1:10	2	40	*Macrocystis pyrifera*	phlorotannins	TPC, AA, HPLC-MS	[71]
Water, water +HCl 5mM, water +0.2% formic acid, acetonitrile: water (50:50) + 0.2% formic acid	1:10	1	r.t.	*Ascophyllum nodosum*	phlorotannins	HPLC-MS	[70]
Methanol: water (80:20), ethyl acetate: water (80:20), acetone: water (70:30), ethanol:water (80:20 and 50:50)	1:25	24	r.t.	*Fucus vesiculosus*	phlorotannins	TPC, AA, HPLC-MS	[78]
Methanol, Acetonitrile:water (50:50)+0.2% formic acid	1:10	overnight	r.t.	*Ascophyllum nodosum,Ulva lactuca, Palmaria palmata, Alaria esculenta*	phlorotannins	TPC, AA, HPLC-MS	[75]
Water	1:100	5 min	100	*Ecklonia cava, Ecklonia stolonifera, Eisenia bicyclis*	phlorotannins	AA, HPLC-UV, NMR	[72]
Methanol	1:4	2	r.t.				
Ethanol:water	1:10	24	r.t.	*Fucus spiralis, Pelvetica canaliculata, Ascophyllum nodosum*	phlorotannins	TPC, AA, MS	[76]
Water	1:20	24	r.t.				
Water	1:20	24	60				
Acetone:water (70:30)	1:20	1(x4)	r.t.	*Cystoseira nodicaulis, Cystoseira tamariscifolia, Cystoseira usneoides, Fucus spiralis*	phlorotannins	AA, HPLC-MS	[56]
Acetone:water (70:30)+0.3% ascorbic acid	1:50	1(x4)	r.t.	*Fucus vesiculosus*	phlorotannins	TPC, HPLC-MS	[54]
Methanol:water (80:20)	1:(7.5-13.2) 1:(3.4-6.6)	0.5 + 3 (x3)	r.t.	*Pelvetia canaliculata, Fucus spiralis, Fucus vesiculosus*	phlorotannins	HPLC-MS	[55]

				Ascophyllum nodosum, Saccharina longicruris			
Methanol:water (60:40)	1:10	2	40	*Himanthalia elongata*	phloroglucinol, phenolic acids, flavonoids and phenolic terpenes	TPC, AA, HPLC-MS	[40]
Ethanol:water (80:20)	1:10	24	r.t.	*Fucus, serratus, Fucus vesiculosus, Himanthalia elongata, Cystoseira nodicaulis*	phlorotannins	TPC, AA, HPLC-MS	[49]
Ethanol	1:10	12	r.t.	*Eisenia bicyclis*	phlorotannins	HPLC-DAD	[60]
Ethanol	1:1.6	2	On ice	*Fucus vesiculosus*	phlorotannins	HPLC-MS, NMR	[73]
Ethanol	1:10	overnight	r.t.	*Fucus serratus, F. vesiculosus, F. distichus, F. spiralis, Sargassum muticum, Saccharina latissima, Laminaria digitata, Dictyota dichotoma, Enteromorpha intestinalis, Ulva lactuca, Palmaria palmata, Porphyra purpurea, Chondrus crispus, Mastocarpus stellatus, Polysiphonia fucoides, Gracilaria vermiculophylla*	phenolic acids	TPC, AA, HPLC-DAD	[36]
Water	1:20						
Water	1:20	24	r.t.	*Ascophyllum nodosum, Pelvetia canaliculata, Fucus spiralis, Ulva intestinalis*	-	TPC, AA	[77]
Ethanol:water (80:20)	1:10						
Acetone:water (80:20)	1:10						
Methanol:water (80:20)	1:10	2	r.t.	*Kappaphycus alvarezii*	-	TPC, TFC, TAC, AA	[79]
Acetone:water (70:30)	1:20	1(x4)	r.t.	*Cladostephus spongiosus, Cystoseira nodicaulis, C. tamariscifolia, C. usneoides, Fucus spiralis, Halopteris filicina, Padina pavonica, Saccorhiza polyschides, Sargassum vulgare, Stypocaulon scoparium*	-	TPtC-DMBA	[80]
Ethyl acetate, acetone, ethanol, methanol, acetone:H_2O (70:30), ethanol:H_2O (80:20), methanol:H_2O (80:20), H_2O	1:50	1(x4)	r.t.	*Fucus vesiculosus*	-	TPC	[81]

[a]TPC- total phenolic content, TPtC- total phlorotannins content, TFC – total flavonoids content, TAC – total anthocyanin content, AA – antioxidant activity, HPLC-DAD-high performance liquid chromatography with photodiode array detector. MS- mass spectrometry, NMR- nuclear magnetic resonance.

II.a.2 – Novel extraction methodologies

Several novel extraction methodologies have been applied in the extraction of phenolic compounds from macroalgae, namely enzyme assisted extraction (EAE), accelerated solvent extraction (ASE), ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE) and microwave assisted extraction (MAE), the latter explored in detail in section II.c. In addition to the application of the above mentioned novel methodologies (individually analyzed below), in the last years the use of sustainable solvent media such as ionic liquids (ILs) and natural deep eutectic solvents (NADES) to extract by both conventional or by novel methodologies valuable components from natural resources has becoming a hot topic. ILs, salts with melting points below 100°C, are usually composed of a large organic cation and an organic/inorganic anion, with a multitude of cation/anion combinations bestowing them with tunable properties (e.g. hydrophobicity, solution behavior). These "designer solvents" and particularly their aqueous solutions have shown a remarkable potential for the extraction of added value compounds [83]. Finally, NADES are emerging as remarkable solutions for bioactive compounds extraction [84] due to the diversity of combinations and their benign/sustainable nature. Notwithstanding, ILs and NADES have not yet been explored in the extraction of phenolic compounds from macroalgae.

II.a.2.1. Enzyme assisted extraction

Several authors have applied EAE in the extraction of phenolic compounds from different green, red and brown macroalgae species [85–87]. Algae cell walls are composed by fibrous composites of microfibrillar polysaccharides embedded in matrix polysaccharides and proteoglycans [88]. These components are a physical obstruction for the normal release of bioactive components, such as phenolic compounds. Additionally other aspects can difficult their extraction, such as the gelling properties of some polysaccharides, namely alginic acid and laminarin-like ones, or the strong complexes that phlorotannins can form with proteins by either non-covalent or covalent bonds [89]. Therefore, the use of enzymes to break down all these complex molecules seems to be an important step to improve the extraction of phenolic compounds. Different enzymes and EAE conditions have been used to this, as summarized in Table II. Carbohydrases and proteases have been the most commonly used enzymes, either isolated [90], combined [91] or even using multi-enzyme complexes [85–87,92]. Although some authors have combined the enzymatic treatments

with other extraction methodologies, such as conventional SLE [91], MAE [92], or ASE [86], most of the authors (Table II) have only used the aqueous medium as extraction solvent at the optimum conditions of the different enzymes used, which certainly limited the extraction of the target compounds.While Wang et al. [87] have verified an increase in the TPC of green, red and brown macroalgae extracts obtained by EAE, specially using proteases, when compared with conventional water extraction, Olivares-Molina and Férnandez [90] observed a significant decrease on the TPC of *Lessonia nigrescens*, *Macrocystis pyrifera* and *Durvillaea antarctica* extracts obtained by EAE when compared to those obtained by conventional SLE using acetone:H_2O as solvent extraction. In fact, the lower TPC is certainly related with the aqueous medium used in the EAE, and the lower affinity of macroalgae phenolic compounds for this solvent, as aforementioned. In order to increase the phenolic compounds extracted from macroalgae, some authors have studied the combination of EAE with other techniques. Charoensiddhi et al. [92] obtained *Ecklonia radiata* extracts with higher TPC and antioxidant activity using microwave-assisted enzymatic extraction than with conventional SLE or single EAE; Siriwardhana et al. [91] verified an increase in the TPC and antioxidant activity of the brown macroalga *Hizikia fusiformis* extracts when EAE is combined with conventional SLE; while Sánchez-Camargo [86] studied the effect of applying ASE (methodologies discussed in more detail below) in the biomass residue obtained after EAE. This sequential methodology leaded to obtain macroalgae extracts with lower TPC than when ASE was applied to non-hydrolyzate macroalgae. However, this should certainly be related with components that were extracted during EAE and discarded, not being accounted in the TPC determination. Finally, it should be highlighted that a limited number of studies regarding the use of EAE in the phenolic compounds extraction from macroalgae have characterized the final extracts obtained [86], just evaluating their phenolic content by spectrophotometric assays.

Table II - Conditions of EAE applied to the extraction of phenolic compounds from macroalgae

Species	Enzymes	t (h)/pH/T (ºC)	Combined with	Ref
Sargassum muticum	Endo-peptidase	24/8.0/50	-	[85]
Osmundea pinnatifida	Endo-protease and exo-peptidase	24/7.0/50		
Codium tomentosum	Cellulase	24/4.5/50		
	Multi-enzyme complex	24/4.5/50		
Lessonia nigrescens	Cellulase	17/4.5/50	-	[90]
Macrocystis pyrifera	α-Amylase	17/6.0/60		
Durvillaea antarctica				
Palmaria palmata	Endo- and exo-peptidase complex	24/7.0/50	-	[87]
	Endo-peptidase	24/8.0/50		
	Protease complex	24/6.0/40		
	Amino- and carboxy-peptidase	24/6.0/40		
	Metallo-endo-protease	24/6.0/50		
	Endo-protease and exo-peptidase	24/7.0/50		
	Multi-enzyme complex	24/4.5/50		
	β-Glucanase	24/7.0/60		
	Exo-1,4-α-glucosidase	24/4.5/60		
	Cellulase	24/4.5/50		
	α-Amylase	24/6.0/60		
Hizikia fusiformis	Endo-peptidase+β-glucanase	24/7.0-8.0/50-60	Conventional SLE	[91]
Ecklonia radiata	Multi-enzyme complex	24/4.5/50	MAE	[92]
	Cellulase	24/4.5/50		
	β-Glucanase	24/7.0/50		
	Endo- peptidase	24/8.0/50		
	Metallo-endo-protease	24/6.0/50		
	Endo-protease and exo-peptidase	24/7.0/50		
Sargassum muticum	Endo-peptidase	2 and 4/7.0/50	ASE of the biomass residue	[86]
	Multi-enzyme complex	2 and 4/4.5/50		

Table III - ASE conditions applied to the extraction of phenolic compounds from macroalgae

Solvent of extraction (v:v)	P (psi)	T (ºC)	t (min)	Species	Compounds extracted	Type of characterization[a]	Ref.
Ethanol:water (25:75; 75:25)	1500	120	20	*Sargassum muticum*	-	TPC, AA, NMR	[93]
Water	1500	120	25	*Fucus serratus* *Laminaria digitata* *Gracilaria gracilis* *Codium fragile*	-	TPC, AA	[94]
Ethanol:water (80:20)	1000	100					
Methanol:water (70:30)	1000	90					
Ethanol:water (95:5)	1500	160	20	*Sargassum muticum*	phlorotannins	TPC, TPtC, AA, LCxLC-DAD-MS	[95]
Ethanol:water (72:25)	1500	120	20	*Sargassum muticum*	phlorotannins	TPC, TPtC, AA, HPLC-DAD-MS	[86]

[a]TPC- total phenolic content, TPtC- total phlorotannins content, AA – antioxidant activity, HPLC- high performance liquid chromatography, DAD- photodiode array detector, MS- mass spectrometry, NMR- nuclear magnetic resonance, LCxLC – two dimensional liquid chromatography

II.a.2.2. Accelerated solvent extraction

ASE, also known as pressurized liquid extraction (PLE), becomes another promising process for the phenolic compounds extraction, providing lower extraction time and solvents consumption. This technique uses solvents at high pressures and temperatures, accelerating the extraction process. The high temperatures used increase the extraction kinetics, as at the same time decrease the solvent viscosity, enhancing diffusion and the sample penetration, facilitating the target components desorption [96]. Despite the high temperatures used, it is claimed that in ASE the degradation of phenolic compounds does not occurs, due to the absence of air and light [69], being one of the main advantages of this technique. Notwithstanding, a limited number of studies have applied ASE in the extraction of phenolic compounds from macroalgae [86,93–95]. Ethanol:water mixtures have been the most used extraction solvents, as showed in Table III. The pressures used by the authors ranged from 1000 [94] to 1500 psi [86,93], with extraction (static) times ranging between 20 [86,93] and 25 min [94]. Sánchez-Camargo et al. [86] optimized the ethanol percentage (95%) and temperature (160ºC) for the ASE of phenolic compounds, namely phlorotannins, from the brown macroalgae *Sargassum muticum*. These conditions were then applied by the same authors [95] to study the geographical variability of phlorotannins composition of this alga. *Sargassum muticum* was also the algae used by Anaëlle et al. [93] to compare different novel techniques in the extraction of phenolic compounds from macroalgae, verifying a higher TPC in the extracts obtained by ASE with ethanol:water (75:25, v/v). Different solvent mixtures, temperatures and pressures were applied in the ASE of phenolic compounds from *Fucus serratus*, *Laminaria digitata*, *Gracilaria gracilis* and *Codium fragile* [94], however in this case the author reported that the extracts obtained presented lower TPC than those obtained by conventional SLE with the same solvent mixtures. This indicates that more in depth studies regarding the optimization of ASE of phenolic compounds from macroalgae are needed.

I.a.2.3. Ultrasound assisted extraction

UAE, is a non-thermal extraction method in which the solid matrix is immersed in a solvent and submitted to ultrasound irradiation, by using an ultrasound bath or probe, has become an emerging technique in the extraction of phenolic compounds. UAE uses sound waves at frequencies over the human hearing values (20 kHz), which propagate by

rarefactions and compression, creating vapor bubbles. These undergo implosive collapse, known by cavitation, producing physical, chemical and mechanical effects, which results in the disruption of the biological membranes, thus enhancing the release of the target compounds [97]. The non-thermal nature of UAE is expected to circumvent the limitations associated with other extraction techniques such as conventional SLE, MAE, and ASE in which temperature plays a key role in the global extraction efficiency, but that also has a deleterious effect over phenolic composition as mentioned several times above. Some authors have already successfully applied UAE in the extraction of phenolic compounds from macroalgae. Table IV summarizes the conditions employed in these studies. The frequencies used ranged from 20 [98,99] to 60 [85] kHz, with the extraction time accounting between 5 [99] and 60 [100] min. Most of the authors performed the UAE using water as solvent, although a study using ethanol as extraction solvent to the UAE of phenolic compounds from the red macroalgae *Laurencia obtuse* can also be found [100]. The optimal SLr (1:30, w:v), temperature (50ºC) and extraction time (42.8 min) were determined in this study. Kadam et al. [98] verified that an increase in the ultrasonic amplitude (from 22.7 to 114 µm) as well as the use of diluted aqueous solution of HCl (0.03M) instead of water in the UAE of phenolic compounds from *Ascophyllum nodosum* leaded to a considerable increase in the TPC of the extracts. Later, the same authors [99] optimized by surface response methodology the extraction time (25 min), HCl concentration (0.03 M) and ultrasonic amplitude (114 µm) of this UAE. Additionally, it was verified that UAE allowed obtaining higher molecular weight phlorotannins (4-12 phloroglucinol units (PGU)) from *Ascophylum nodosum* than those obtained by conventional soli-liquid extraction (4-7 PGU). The use of ultrasound technology as a pretreatment of different macroalgae samples before the SFE of isoflavones was also accessed [41]. The sonication of the macroalgae with the SFE modifier mixture (methanol:water, 10:90/v:v) for 30 min, by both sonication bath or thorn sonication, showed to be crucial for the SFE of the isoflavones fraction, probably due to the damage of the cell walls or organelles of the matrix prior to the SFE, enhancing the mass transfer.

II.a.2.4. Supercritical fluid extraction

SFE is becoming an attractive alternative method for the extraction of high valuable compounds from natural sources [101][102].A supercritical fluid is a substance that, at

temperatures and pressures higher than its critical point shows compressibility, transportation and penetration properties of a gas and the density and solvating power of a liquid. Additionally, in comparison to common solvents supercritical fluids present higher diffusivities, lower viscosities and almost null surface tensions, which provide them exceptional solvent and operational characteristics [103]. Finally, their properties can be easily tuned by changing the temperature, pressure or even by adding a modifier (co-solvent). Supercritical carbon dioxide (SC-CO_2) (Pc =7.28 MPa, Tc = 304.1 K [103]) has been the most widely used fluid in SFE, since it is non-toxic, environmentally safe, non-flammable, low cost at high purity, and easily removed from final extracts. In addition, it allows the use of relatively low pressures and near room temperatures, which together with the absence of light and air in the process reduces the possibility of oxidative degradation [104]. Several authors have applied SC-CO_2 SFE for phenolic compounds from macroalgae (see Table V), although few have considered this technique as less promising than others. Anaëlle et al. [93], for example, observed a considerably lower TPC and antioxidant activity on SFE extracts when compared with those obtained by SLE or ASE. Notwithstanding this could means that an optimization procedure should always precede the use of these novel methodologies. S*argassum muticum*, as one of the most exploited macroalgae, have been also object of several studies concerning the use of SFE [41,93,105]. Ethanol has been the most used modifier [93,105,106]. Conde et al. [105] verified that the addition of 10% of ethanol (from a studied range of 0.5-10%) in the SC-CO_2 SFE extraction of phenolic compounds from *Sargassum muticum* increased the TPC 1.5 times. Anaëlle et al. [93] used a similar ethanol content (12%) in the extraction of phenolic compounds from the same macroalgae species. Low extraction times, namely 1 or 1:30h, has been chosen for most of the authors [41,93,105,106], although considerably higher values was adopted for the extraction of phenolic compounds from red and green macroalgae [107]. Notwithstanding kinetic studies performed for the SFE extraction of phenolic compounds from *Sargassum muticum* showed a maximum on both TPC and antioxidant activity at 40 min of extraction time [105]. The temperatures and pressures used in the SFE of phenolic compounds from macroalgae have ranged from 30 to 60ºC and from 8 to 50 MPa, respectively (Table V). Klejdus et al. [41] observed a maximum recovery of several isoflavone standards using SC-CO_2 SFE modified with methanol:water (10:90) at 40ºC (in a studied range between 35 and 75 ºC) and at 35 MPa, which was then applied successfully to extract the same isoflavones

from several macroalgae species. The optimal conditions verified for the SC-CO_2 SFE modified with 10% ethanol of phenolic compounds from *Sargassum muticum* were at 50ºC and 20 MPa [105], while the TPC of *Undaria pinnatifida* extracts obtained with SC-CO_2 SFE modified with 3% ethanol were maximized at 60ºC and 25 MPa.

Table IV - UAE conditions applied to the extraction of phenolic compounds from macroalgae

Extraction solvent	Frequency (kHz)	Conditions	Species	Compounds extracted	Type of characterization[a]	Ref
Water Water + HCl (0.03M)	20	10 min; amplitude 22.8 - 114 µm	*Ascophyllum nodosum*	-	TPC	[98]
Water + HCl (0-0.06M)	20	5-25 min; amplitude 22.8 - 114 µm	*Ascophyllum nodosum*	phlorotannins	TPC, MS	[99]
Water	50/60	60 min (pauses of 2 min every 10 min); 50ºC[b]	*Sargassum muticum* *Osmundea pinnatifida* *Codium tomentosum*	-	TPC, AA	[85]
Ethanol	40	30-60 min; 30-50ºC	*Laurencia obtusa*	-	TPC, AA	[100]

[a]TPC- total phenolic content, AA – antioxidant activity, MS- mass spectrometry. [b]UAE applied after 24h of conventional SLE at 50 ºC.

Table V - SC-CO_2 SFE conditions applied to the extraction of phenolic compounds from macroalgae

SFE modifier	FlowCO2 ($kgCO_2\ h^{-1}$)	P(MPa)/T (ºC)/t(h:min)	Species	Compounds extracted	Type of characterization[a]	Ref
-	200	50/40/5:17-13:28	*Cladophora glomerata,+ Ulva flexuosa + Ulva clathrata (mixed)*	-	TPC	[107]
Ethanol (12%)	-	15.2/60/1:30	*Sargassum muticum*	-	TPC, AA, NMR	[93]
Methanol:water (10:90) (3%)	-	35/40/1:00	*Sargassum muticum, + Sargassum vulgare + Hypnea spinella + Porphyra sp + Undaria pinnatifida + Chondrus crispus + Halopytis incurvus (mixed)*	isoflavones	Fast LC-MS	[41]
Ethanol (0.5-10%)	1.5	10-30/30-50/1:00	*Sargassum muticum*	-	TPC, AA	[105]
Ethanol (3%)	1.69	8-30/30-60/1:00	*Undaria pinnatifida*	-	TPC	[106]

[a]TPC- total phenolic content, AA – antioxidant activity, LC- liquid chromatography, MS- mass spectrometry, NMR- nuclear magnetic resonance.

II.b – A detailed look into Microwave-assisted extraction of SPCs (and other bioactives)

Microwave heating (MWh) has revolutionized the world since its discovery in the World War II period. Since then, it evolved into a widespread technology with quotidian uses spanning from domestic food and drinks heating to industrial applications and laboratory use in synthesis and extraction [108].

MWh shows advantages, among which being faster and more homogeneous, when compared to conventional heating and reduces key components degradation [109]. However, temperature distribution heterogeneity is still a problem, as factors such as volume, geometry and dielectric properties of the irradiated object may result in hot and cold spots [108]. Finally, microwaves non-ionizing character considerably reduces the safety concerns, turning them into a common domestic and laboratory technique although at industrial level they are not yet ubiquitous.

Note:

Topic II.b has been accepted for publication as a book chapter*.

*Félix, R., et al. (2018). Microwave-assisted Extraction of Seaweed Bioactive Components. In: A. K. Jaiswal (ed.), Seaweed Bioactives: Extraction and Characterization Techniques, 1st ed. CRC Press, Boca Raton, Florida (978-1138197534)

The first reports on the fractionation of organic molecules from biological matrices by MWh were a procedure for adenosine triphosphate (ATP) release from bacteria [110], and a study on the use of MWh as general purpose extraction technique [111] now commonly called Microwave-Assisted Extraction (MAE), which could improve extraction efficiency for a diverse group of matrices and target compounds. MAE potential as a viable alternative to conventional Soxhlet extraction [112], as well as for the extraction of more complex labile compounds, such as pectins [113] and bioactive compounds [114] in much shorter times was demonstrated. Finally, in 1991, a patented process entitled "Microwave-assisted natural products extraction" was published demonstrating MAE potential in biomass fractionation for both analytical and industrial purposes [115]. MAE efficiency continued being demonstrated, e.g. in the extraction of alkaloids from *Senecio spp.*, up to 8 times faster than by traditional [116], or in the accurate extraction of 3-nitro-4-hydroxyphenylarsonic acid from swine organs [117]. In 1994 a first review on MAE was published [118], becoming a widely accepted extraction method.

In this section, an overview of the principles and applications of MAE to bioactive components extraction from seaweed will be presented. The different parameters affecting MAE efficiency will be discussed in detail, first in general perspective and then applied to seaweed polysaccharides, lipophilics, phenolic components, among others.

II.b.1. MAE applied to natural components

Until today, hundreds of scientific publications on MAE were published, consistently reporting it as an efficient extraction technique and potential alternative to the conventional methods. MAE often allows the extraction of compounds in shorter times and with higher selectivity, less costs and environmental damages (e.g. from reduced solvent usage) than conventional methods [119]. However, some issues still require improvement, such as the use of organic solvents (even in much lower quantities), the low efficiency on non-polar systems, low applicability on viscous media and unsuitability in the extraction of thermally labile compounds [120]. Nonetheless, lab-scale successful extractions of alkaloids [121], polysaccharides [122], essential oils [123], phenolic compounds [124–128], sterols [129], fatty acids [130], among others, as well as emerging industrial applications in the extraction of antioxidants and colorings (such as polyphenols and carotenoids, respectively) are examples of the solid impact of MAE development [109]. The mechanisms underlying this technique, the instrumentation and a wide range of applications, as well as the key variables affecting MAE are discussed in the following sections.

II.b.1.1. Mechanisms of microwave heating and extraction

Microwaves are electromagnetic radiation that has a frequency between 0.3 and 300 GHz [131]. When electromagnetic waves pass through matter, if the quantized energy of the photons is able to excite a given physical process – such as electron ejection/excitation, covalent bond resonance or molecules rotation – it will be absorbed and the object is opaque to that specific radiation, while if no interaction takes place the body will be transparent to that specific frequency [132].

In the case of microwaves, despite their wavelength being too large to produce atomic or intramolecular interactions with the electrons, they cause small, dipole molecules to rotate millions of times per second, aiming to orient their dipole accordingly to the electrical field [133]. As a result of molecules friction, heat is produced. In water-rich materials heating, the abundance of water (an excellent dielectric material) accounts for most of the heating, but other molecules also rotate. A schematic, simplified representation of the interaction of microwaves with matter is presented in Figure 9.

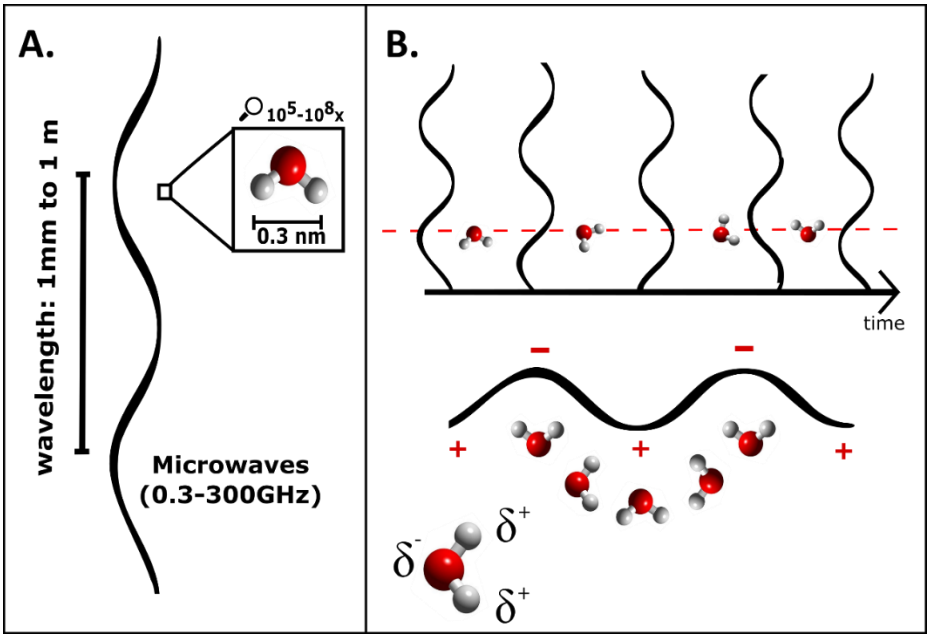


Figure 8 - Schematic diagram of the interaction between water molecules (red-and-white) and microwaves. A. Microwave interaction with water's electrons and bonds is absent because the large wavelength of this type of radiation allows it to pass through matter (even in the solid state) without significant photon absorption. B. Passage of microwave radiation generates a highly oscillating electric field, which makes water molecules (and other dielectrics) rotate frenetically, as poles are forced to orient accordingly to it. The increased kinetic energy is thus converted to heat.

In MAE, samples (e.g. biological) are microwave irradiated for typically short, repetitive periods of time, with or without an extracting solvent, in open or closed vessels, under conditions of adjustable temperature and pressure, providing a broad flexibility of conditions [109,133,134].

The physicochemical phenomena responsible for the rapid, increased extraction of compounds from their biological matrix to an extracting media were drawn from the first MAE studies. The effect of both the compound of interest and the solvent's polarity in MAE was the first to be described [114]: the more polar the molecules, the more efficient was the extraction. However, non-polar molecules are still extracted by MAE with high efficiencies (yet much faster than conventional solid-liquid extraction approaches, such as Soxhlet extraction) – which was proposed to be the direct effect of temperature increment due to sample's original moisture, essential to increase the solubility/volatility and diffusion rates of the solutes. In such cases, MWh is a faster and more homogenous heating mode, which accounts for its good performance even when the solutes are poor dielectrics. Furthermore, it was noted that the rotational nature of the heat-generating response to microwaves of

dielectric molecules should lead to the disruption of hydrogen bonds, which facilitates solvent penetration in the sample and solutes solvation (Ganzler, Szinai, and Salgó 1990). A very important MWh-derived phenomenon is the selective heating of structures by water content, which can lead to different tissue and/or cell compartments to breakdown sequentially. Such phenomenon was first described for the essential oils extraction from mint leaves by both Soxhlet and MAE using a patented process [135]; because of decreased heat exposure (6 hours *vs.* 20 seconds), the lower temperature of the solvent upon extraction (mint leaves have water and heat much faster than hexane, a poor dielectric solvent), and the localized heating of mint glands as a result of their higher water content – which leads to rupture of these cellular compartments, leaching their content before regular cells, as documented from electron microscopy – much more clean extracts, i.e. with negligible chlorophyll extraction, were obtained with MAE as compared to Soxhlet ones.

More recently, some authors proposed that the synergistic effect of simultaneous mass- and heat-transfer taking place in the same direction (from inside to outside), as opposed to typical resistance heating, might contribute to explain the faster nature of MAE [136,137].

Besides this generalized effect on samples – an improvement of extraction rate –, MAE's differential performance as a result of instrumental conditions and extraction parameters adjustments allows to obtain high-quality extracts as well, i.e., highly pure and less degraded [115], and is therefore explored in section 8.2.3 and 8.2.4.

II.b.1.2. Instrumentation and operational setups

Initial works on MAE used adapted versions of domestic ovens, a practice nowadays obsolete, as more advanced MAE apparatus are commercially available allowing more controlled and safe operation. MAE equipment are always composed by a magnetron, a waveguide, a circulator and an applicator [109], with different geometries of microwave extractors arising from variations in the applicator and in the samples vessels. An infogram on these components is presented in Figure 10.

Due to the nature of each option, multi-mode microwave ovens often operate with closed vessels (i.e., under pressure), and single-mode microwave ovens with open vessels.

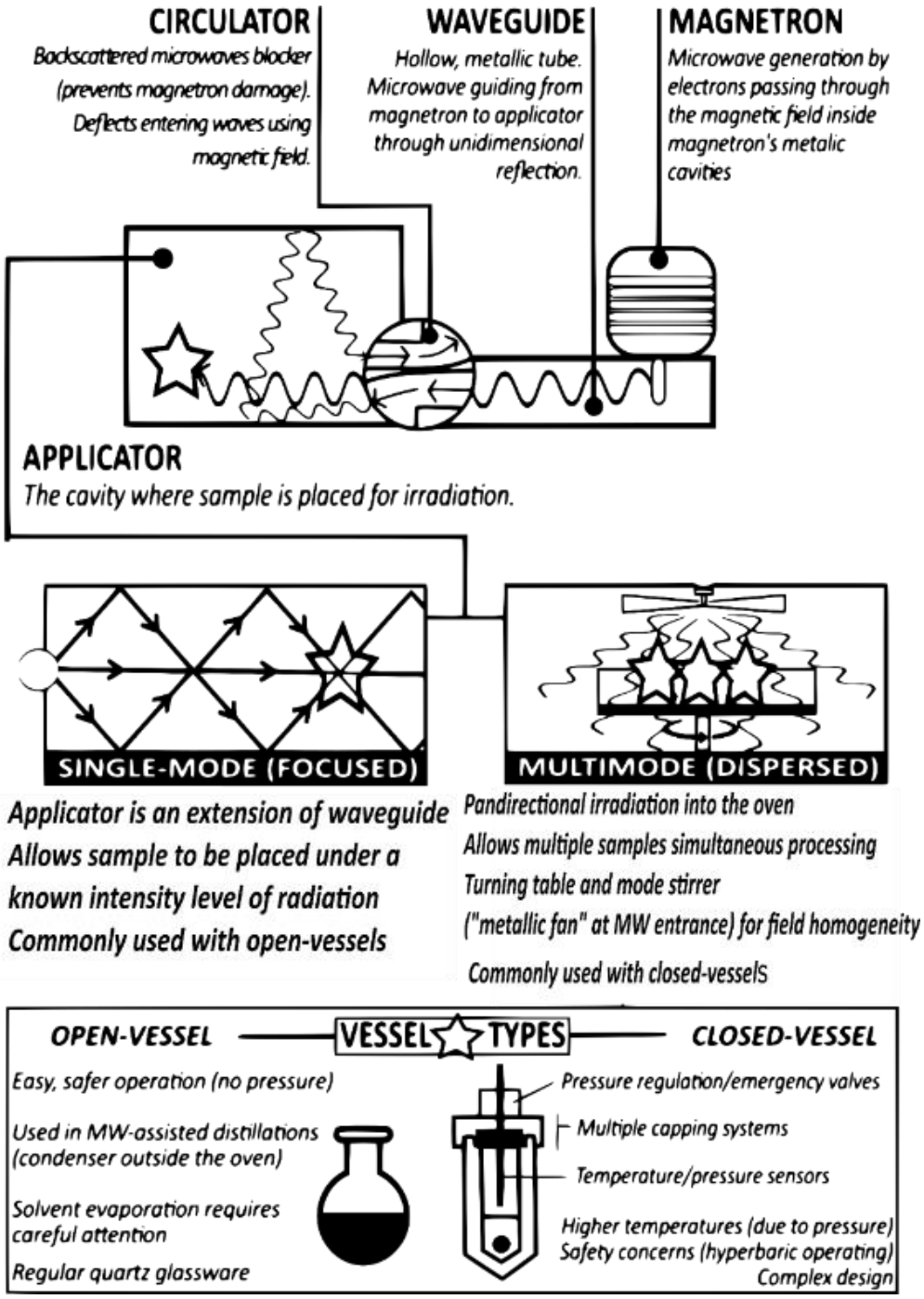


Figure 9 - Schematic representation of the instrumental backbone of a MAE apparatus (not to scale). On top, the base components of every MAE apparatus (note the three-gate, unidirectional functioning of the circulator). In the middle, the two types of microwave applicator – focused microwaves are obtained by the geometry of the applicator, allowing reflections to be calculated and symmetrical, whereas dispersed microwaves are the result of a larger, unphased oven, aided by a mode stirrer (the 'metallic fan' on the exit of the waveguide). In the bottom, the two type of vessels (open and closed) are depicted – see text for more details.

This has led literature to refer interchangeably to these definitions [138]. Although it is true that the inverse combinations are rare, they exist and discrimination of both applicator's and vessel's nature should be performed when discussing MAE in non-commercial terms.

Finally, most modern microwave extractors are equipped with temperature and pressure sensors, both for monitoring and regulation. Temperature sensors can be either infra-red sensors or fiber optic thermometers, while pressure sensors (only applicable to closed vessels) are often placed in a "control vessel" that is irradiated along with the samples, whose function is solely to monitor conditions.

Adding to the diversity of extraction methodologies powered by microwaves, different setups resulting from the incorporation of other extraction techniques might be used for specific purposes. For instance, coupling of Focused MAE with a Soxhlet-like extractor is now an established method called Focused Microwave-Assisted Soxhlet Extraction (FMASE); likewise, the use of MWh to perform hydrodistillation is now a common approach and is referred to as Microwave-Assisted Hydrodistillation (MAHD or MWHD).

Table VI summarizes some of the most relevant MAE applications described in literature.

Table VI – Specific applications of MAE developed by modification of typical extraction setups.

Name	Configuration	Applications
Microwave Steam Distillation (MSD) [139,140]	Multimode; Open-vessel; Steam produced inside or outside the oven crosses a cartridge with biomass that is under microwave irradiation	Organochlorine Pesticides; Essential oils;
Compressed Air Microwave Distillation (CAMD) [141]	Multimode; Open-vessel; Compressed air forces volatiles through a condenser outside the oven	Essential oils; Pigments
(Vacuum) Microwave-Assisted Hydrodistillation ((V)MWHD) [123,142,143]	Multimode; Open-vessel; Water and sample are heated by microwave irradiation, vapors are condensed outside the oven. (Vacuum coupling can increase tissue rupture and decrease temperature of operation)	Essential oils; Volatile Secondary Metabolites
(Vacuum) Microwave Hydrodiffusion and Gravity ((V)MHG)[a] [144–146]	Multimode; Open-vessel; Sample is heated without water or solvent, and extractives are hydrodiffused by the biological water evaporated and separated by gravity. (Vacuum coupling increases hydrodiffusion capacity and therefore diffused compounds)	Plant flavonols; Essential oils;
Microwave Dry-diffusion and Gravity (MDG) [a] [137]	Multimode; Open-vessel; Equal to MHG, but for the extraction of dry material, such as powdered spices.	Essential oils
(Focused) Microwave-Assisted Soxhlet Extraction ((F)MASE[b]) [147–150]	Focused; Soxhlet-like open-vessel; conventional heating of the solvent and simultaneous (focused) microwaves irradiation of the sample.	Lipids; Pesticides; PAHs
Microwave Integrated Soxhlet (MIS) [151–153]	Multimode; Soxhlet-like open-vessel; similar to (F)MASE, but both solvent and sample are microwave heated (solvent compartment contains a microwave-absorbing solid to improve transparent solvent heating)	Lipids; Pesticides; PAHs
Continuous-Flow Microwave-Assisted Extraction (CMAE) [154–157]	Tubular circuit with flowing sample and solvent mixture (atmospheric pressure), heated in a portion of the path by microwaves (either focused or multimode).	Antioxidants; Miscellaneous Bioactive compounds
Dynamic Microwave-Assisted Extraction (DMAE) [158,159]	Capillary circuit with sample is crossed by flowing solvent (atmospheric pressure) and irradiated with microwaves (either focused or multimode), with online filtering and analytical equipment.	Thermolabile analytes; Flavonoids
Negative-pressure Cavitation MAE (NMAE) [160,161]	Multimode; Open-vessel; MAE coupled with NPCE (Extraction assisted by cavitation of the solvent by injected nitrogen, consequent of negative-pressure).	Phenolic compounds; Miscellaneous Bioactive compounds
Ultrasonic, Microwave-assisted Extraction (UMAE) [162–164]	Focused; Open -vessel; Ultrasound and microwaves emission onto the sample for higher yield. Ultrasounds are known to aid extraction from biological matrices by cavitation.	Polysaccharides; Flavonoids
UMAE with PEG (PEG-UMAE) [165–167]	Multimode; Open-vessel; Regular UMAE exploiting low-molecular weight liquid PEG (polyethyleneglycol) as solvent (several advantages for the food industry).	Miscellaneous bioactive compounds; Protein; Polysaccharides
MAE with Ionic Liquids (ILs-MAE) [168–170]	Multimode; Open-vessel; Regular MAE exploiting Ionic Liquids as solvent (their polarity leads to very efficient temperature increase).	Flavonoids; Alkaloids; Polyphenols

MAE with Deep Eutetic Solvents (DES-MAE) [171]	Multimode; Open-vessel ; Regular MAE exploiting DES as solvent.	Miscellaneous bioactive compounds

[a] - Examples of Solvent-Free Microwave Extractions (SFME) [b] - Not to be confused with MASE: Microwave-Assisted Solvent Extraction, a designation for all non-SFME methods.

II.b.1.3. Key parameters affecting extraction efficiency

MAE's performance is affected by several parameters, which can be related to the modulation of the heat-generation phenomena, or to the solute release from the matrix (mass-transfer efficiency) or both (as chemical environment plays a key role both in the dielectric heating and the solubility of extractives). Energy-transfer is primarily modulated by microwaves irradiation (power and exposure time), resulting in a temperature (and eventually pressure) increase. Mass-transfer can be directly manipulated by system properties, such as stirring and matrix's granulometry. Both energy- and mass-transfer are yet influenced (arguably to the largest extent) by solvent and moisture content, that define both the dielectric permittivity of the media and the solubilization of the solute in the extract. Finally, the effect of each parameter will itself vary with the variations of other parameters, such that the analysis of the effect of a given factor, e.g. temperature, is performed in a multifactorial design, often by response surface methodologies, allowing to understand the effect of temperature at both fixed and varying conditions, e.g. time [172].

Temperature and pressure

Temperature is one of the main variables used to control efficiency in most extraction techniques. Extraction increment at higher temperatures results mainly from solubility increase, as well as from matrix's structural disruption with leaking of solutes, from heat-derived expanding pressure; surface tension/viscosity decrease, facilitating permeation of the matrix to be extracted by the solvent [173]. Pressure is, in MAE using closed-vessels, the result of temperature increment, and allows operation above the boiling temperature of the solvent. Therefore, pressure is a parameter that has to be considered only in closed-systems and that allows the use of higher temperatures (with corresponding advantages and disadvantages).

In both open or closed-systems, however, temperature increase doesn't necessarily result in increased yield [138]. For example, in the MAE of flavonoids from *Radix astragali*, it was shown that a maximum yield was achieved at 70 ºC, and that above 130 ºC a decrease was observed due to components degradation [174]. The extract's components thermal stability is of utmost importance since even among a given family, it might vary considerably: for example, in a study of 22 phenolic compounds (from phenolic acids to flavonoids, among others) during MAE [68], all were stable at 100 ºC, but epicatechin,

resveratrol and myricetin were degraded at 125 ºC. In the case of proteins, e.g., thermal denaturation is critical to control – an example of temperature effect on MAE of phycobiliproteins from algae will be briefly discussed in section 8.3.4. Noteworthy, thermal stability in MAE and in other (convection-heated) extraction methods behaves differently. In MAE, because the heating is associated with dipole rotation, macroscopic temperature may underestimate the amount of energy deposited onto more polar molecules (locally super-heated), thus leading to their degradation – in practice, polar compounds will degrade more easily, even if temperature reached is not as high as reported for other convection-heated experiments [174,175].

It was also shown [175] that an increase in the extraction of all matrix components occurs at higher temperatures, which means that regardless of the extraction yield increment this does not necessarily reflect an improved extraction of target compounds.

Microwave power and extraction time

Microwave power, extraction time and temperature are all interchangeable, since the choice of two values will determine the third. Power and time represent a measure of the "path" to achieve a given temperature. It is therefore reasonable to discuss power and time together. Following the previous discussion on temperature, time spent at higher temperatures also accounts for degradation, and may thus allow irradiation cycles to be drawn such that high temperatures are applied to increase extraction yield without compromising the extract. As for temperature, an increase in power of irradiation and/or in time of extraction leads to higher yields to a certain extent, and then either stabilizes, or decreases as degradation starts to occur [176–178]. For instance, power increase can lead to a more pronounced difference in the localized temperatures of different components in a matrix or of different polarity compounds [177]; therefore, high-power should be used carefully when dealing with highly polar solutes, as thermal degradation is more likely to occur. Power should also be adjusted according to the localization of the desired compounds in biomass cellular structures: if cellular leaking is beneficial for their fractionation, high-power is useful [179]; if not, high-power will lead to increased cellular leaking into the extract, thus decreasing selectivity [180].

Whenever possible, higher powers should be used in order to decrease time of extraction [133]. In closed systems, power is also adjustable when dealing with multiple

samples, as time and temperature should remain the optimal. Since high-power can lead to very-high pressure in very short periods of time, this should be taken into consideration for safety [181].

Extraction time generally leads to increased yield [182]. However, since long exposure to MWh will increase the risk of degradation and co-extraction, for example, it was demonstrated that flavonoids from *Radix astragali* were better extracted with time increase up to 25 minutes, but after that a decrease in extraction yield was observed [174].

If solvents have a high dielectric loss, long exposure will result in massive heating of the sample, because both the matrix and the solvent are heating and heat-transfer from matrix to solvent is not occurring [181]. Thus, this type of system requires shorter irradiations (as will be described below). Also, long exposures to microwaves, even if low power is used (and consequently low temperature is reached) can also lead to yield and/or activity decrease [109]. The mechanism for such disruption is not well understood, but the structural damages inflicted by low-temperature MWh on two thermostable enzymes from *Sulfolobus solfataricus*, have pointed non-thermal effects from MWh as a plausible cause [183]. Thus, a compromise between polarity of compounds of interest and of the other compounds, dielectric permittivity of the system (both solvent and matrix) and operational power (because it will define temperature) has to be made when designing MAE times. A good way to successfully provide high power/temperature and long exposure times (for better recovery of biomass's components) is by drawing time cycles, with rapid heating followed by either cooling of the system and eventually replacement of the solvent with fresh one, further improving the mass partition phenomena [138,174,179].

In such complex systems, the adequate adjustment of all parameters can be efficiently performed using Response Surface Design of Experiments approaches [184]. In this study, the extraction yields of phenolic compounds from the green algae *Chaetomorpha sp.* were shown to increase with time, except at low powers (prolonged exposure without significant desorption from matrix); interestingly, the antioxidant activity of the extracts increased at high-power, long exposure conditions, which may seem inconsistent, since in general these conditions would lead to degradation. However, phenolic compounds in particular have shown to require long exposure times to be extracted [185], which may turn the results of high-power, long exposure better than low exposure ones, in spite of the likely increased degradation of extractives.

Matrix particle size

The physical properties of the matrix, namely the surface area and thickness, will determine solvent-solute interaction and therefore mass-transfer. Particle size, modulates diffusion from internal content and solvent penetration, by determining the path necessary for solvent-solute contact (sample's thickness) and the amount of solvent in direct contact with the sample (surface area). In MAE as for other extraction methodologies in general, decreasing particle size results in shorter extraction times and often in increased yields [109]. Furthermore, it also contributes to a more homogeneous irradiation and heating of the matrix [186].

Samples are usually grinded to particle sizes of 0.1-2 mm before MAE [133]. As an illustrative example, the yield of glycyrrhizic acid MAE from *Glycyrrhizae radix* roots increased with size reduction from 4–2 mm pieces to 0.3 mm powder [187]. However, fine powders can become impractical for routine or high-throughput extractions, as the subsequent separation of the liquid extract from the solid residue becomes more laborious [188]. Also, mass-transfer can be compromised by impaction, leading to decreasing in the extraction yields as demonstrated by the decrease in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of MAE of *Monostroma nitidum* for particles smaller than 0.25 mm [189].

Stirring

In low-volume solvent systems, desorption of the solutes into the solvent can lead to a saturated microenvironment at the solvent-matrix interface (conditioning yield from insufficient mass-transfer), the reestablishment of the contact of the sample with non-saturated solvent by stirring mechanism might be necessary [179] as well as to faster desorption of bound-compounds by mechanically disturbing their weak bonds due to fluid kinetics [190]; also, the use of magnetic stirrers improves both rate and homogeneity of heating, overall leading to improved yield [191].

Solvent system

Solvent is one of the most important parameters in any extraction. In MAE, the differential impact of solvents in the extracts composition is attributed to three major solvent-influenced phenomena - the heating of the system, the partitioning and solvation of the

extracted compounds and the penetration into the matrix's [192], which are all primarily conditioned by solvent polarity [193,194].

Solvent polarity dramatically affects the heating process: solvents with high polarity (high dielectric constant) enable rapid and intense heating; those with low polarity (low dielectric constant), will barely interact with microwaves (often called "transparent"), and thus won't heat significantly (except for conductivity associated with samples moisture). Adequate combination of the two solvent types in different proportions are often used to achieve a good dielectric loss without losing selectivity [186]. The dielectric constants for the most common solvents are present in the Table VII.

Table VII – Dielectric heating related parameters for a selected list of solvents. Adapted from [195].

Solvent	Dielectric Constant (')	tan	Dielectric Loss ( )	Boiling point (°C)
Water	80.4	0.123	9.889	100
NaCl (aq. 0.5M)	67.0	0.625	41.875	100.5
DMSO	45.0	0.825	37.125	189
Acetonitrile	37.5	0.062	2.325	82
Ethylene Glycol	37.0	1.350	49.950	197
Methanol	32.6	0.659	21.483	65
Ethanol	24.3	0.941	22.866	78
Acetone	20.7	0.054	1.118	56
2-Propanol	18.3	0.799	14.622	82
Dichloromethane	9.1	0.042	0.382	40
Tetrahydrofuran	7.4	0.047	0.348	66
Ethyl Acetate	6.0	0.059	0.354	77
Chloroform	4.8	0.091	0.437	61
Benzene	2.27	0.080	0.035	80.1
Hexane	1.9	0.020	0.038	69
Toluene	2.4	0.040	0.096	111

Polar solvents allow to reach the highest temperatures (boiling point in open-vessels or higher in closed-vessels), while leading preferentially to the extraction of polar compounds. Commonly used polar solvents are water [196], alcohols [197], acetone [198], dimethyl sulfoxide (DMSO) [199], acetonitrile [200], tetrahydrofuran [201] or aqueous mixtures of the previous, allowing to successfully extract phenolic compounds [124,197,201] and polysaccharides [122], among others [202]. Even for the extraction of non-polar metabolites, the higher temperatures reached using polar solvents can make them more suited than the normally employed non-polar ones, as demonstrated in the extraction of triterpenic acids from *Chaenomeles sinensis*, using methanol [203] or in the extraction of steroidal hormones from fish using acetonitrile [200].

Transparent solvents are mostly used for the extraction of thermolabile compounds [204], since the extracted components diffuse from hot matrix into the unheated solvent. For such applications, matrix's moisture influence is high, as discussed in the next section. Non-polar solvents can also be used in the MAE extraction of lipophilic components, as more polar solvents won't be able to solubilize them. Low polarity flavonoids [197], alkaloids [205], fatty acids [150], essential oils and other terpenic compounds [206] are also better extracted using pentane [207], hexane [153] or even petroleum ether [208] or benzene [209]

Finally, mixtures of polar and non-polar solvents – aiming to achieve temperature control and selectivity – were used for the extraction of specific lipids, for which MAE is not effective unless both a non-polar and a polar solvent are used [210]. Hexane:acetone (1:1) was a frequently explored for MAE extraction of contaminants from soil and marine sediment [211]; hexane:ethanol (3:1) was optimized for the extraction of solanesol from tobacco leaves [212], and the efficient extraction of essential oils from lemon leaves was achieved with hexane:ethanol (3:2) [213]. Finally, it was shown that, compared to conventional methods, the MAE of meat with hexane:chloroform (3:1) recovered less than 10% of lipids, while ethyl acetate:methanol (4:1) recovered 100% of the lipids, which was assigned mainly to the temperature increment [210]. Nevertheless, if only heating improvement is intended, this can be achieved by the simple addition of less than 10% (v/v) of water to the non-polar media replacing hazardous solvents [214].

Along with solubility and heating capacity, matrix interaction with the solvent and its capacity to penetrate the sample is also dependent on polarity. After solubility and heating optimization, final adjustments can be done to improve the solvent-matrix interaction and thus yield. It was demonstrated that that the addition of small amounts of water to the extraction solvent leads to better yields due also to the diffusion of water into the matrix, enabling the transport of compounds into the solvent at higher mass transfer rates [109].

Solvent volume also affects MAE yield, by interfering with both the time required for heating the system and the maximum concentration of solutes extractable [215]. While higher liquid/solid (L/S) ratios are known to favor convection and the desorption of compounds from the matrix [216], leading to higher yields in most extraction setups, in MAE, convection is not generated efficiently, and unless stirring is applied, there's an L/S value above which yield decreases, due to increased time to generate heat and consequent overexposure to microwaves [174]. Because of this, and also because lower solvent volumes

is a valued trait in extraction procedures [217], low L/S are usually used in MAE, although with some variability. L/S ratios between 10:1 and 20:1 (v:w) were found to be optimal [125,218]. Nevertheless, ratios up to 50:1 were used [134], and a minimum of 2:1 was reported [133]. In closed-vessels, though, a given temperature is reported to be achieved in less time if a higher solvent volume is used – likely because of the overall dielectric loss of the vessel's inner volume increasing with a higher ratio liquid:gas [219], meaning optimal L/S ratio absolute values might be increased in pressurized MAE.

Salinity and pH also influence MAE. Ionic species strongly influence the magnitude of dielectric heating [216], as pH can also influence the solubility of the compounds and their proneness to oxidation. For example, optimal pH for MAE of flavonoids from *Perilla frutescens* was fixed at 8.4 [220], while pectin MAE from *Carcia papaya* L. [221] was maximized at pH 2, above that aggregation would difficult desorption from the matrix and below that acid hydrolysis would cause pectin depolymerization [222].

Alternative solvents, such as ionic-liquids (IL) have also been explored in MAE. ILs are, allegedly more environmentally friendly, and show interesting and tunable properties when it comes to selectivity [83]. The use of ILs in MAE (IL-MAE) was successfully reported in the extraction of alkaloids [168] and phenolic compounds [169,170,223].

Natural deep eutectic solvents (NADES) – a recent highlight in green solvents – have also been considered for MAE applications [224], namely in the extraction of both the hydrophobic and hydrophilic components from *Salviae miltiorrhizae* [225]. It was also shown that DES-MAE outperforms ethanol- or ultrasound-assisted extraction of flavonoids [171].

Other options to improve MAE include the use of surfactants as Triton-X100, as in the extraction of glycyrrhizic acid and liquiritin from *Glycyrrhiza glabra* [226], and PEG, as in the extraction of *Prunus dulcis* seeds' proteins [165], polysaccharides from *Pericarpium ganati* [167] and flavonoids from *Diospyros kaki* [166].

Matrix moisture

Finally, matrix moisture will account for a direct interaction of microwaves with the sample, generating internal heating and pressure. This will lead to an inside-to-outside energy-transfer, simultaneously transporting the compounds outwards, which can thus be collected by a cold transparent solvent [134] or even without solvent in the case of volatile

compounds [133]. Solvent-free MAE is available for fresh or moistened samples (at least 70% water content), and was shown to be efficient for essential oils extraction e.g. *Ocimum basilicum, Mentha crispa* and *Thymus vulgaris* [227]. The localization of matrix's moisture in specific areas (as glands) might result in hot spots [135], which affects the outcome of extraction depending on target compounds' characteristics. Pre-impregnation can also be used to reduce heating time, and therefore to protect thermolabile compounds, regardless of sample's moisture [228].

Notably, for poorly absorbing samples, MAE can be improved by a) the use of high dielectric permittivity solvents; b) pre-impregnation with polar solvents, allowing subsequent MAE to be applied as normal [181]; and c) addition of microwave absorbing substances, such as carbonyl iron powder, to produce localized heating [229,230].

II.b.2. Microwave assisted extraction applied to seaweed bioactive components

The MAE of bioactive compounds from seaweeds is rather unexplored, comparing to other matrices MAE or to other thoroughly characterized extraction methods. However, the efficiency of MAE in the analytical and preparative extraction of phytosterols, carotenoids, essential oils, phenolic components and, more prominently, polysaccharides, was demonstrated with a recognized potential to grow in the next years Additionally, several MAE studies of microalgae were reported, with relevant insights for its applications in seaweeds and will therefore be briefly addressed as a final subtopic. A review article has been recently published addressing, among other techniques, the use of MAE essentially in algae, and a selection of studies are presented [231]. Here, a more detailed discussion on the applications of MAE to macroalgae components extraction will be provided.

II.b.2.1. Polysaccharides

Polysaccharides are probably the most exploited macroalgae components, with well-established industries exploring agar, carrageenans and other polysaccharides for a panoply of applications, which, together with their abundance explains the higher number of studies concerning their MAE from seaweeds, as shown in Table VIII. Notably, and probably due to the need of water for solvation – present in every seaweed polysaccharide MAE, pressurized MAE has been frequently used in order to reach temperatures higher than 100ºC (see Table VIII). Red algae are the most studied by MAE, as agar and carrageenans are

industrially exploited in food processing, yet worth of optimization. MAE of polysaccharides from brown and green algae, namely fucoidan, ulvans and to some extent sulfated rhamnans was also investigated (Fig. 11).

In addition to MAE-associated parameters, a multitude of other variables can influence the extraction of polysaccharides from seaweeds, e.g. previous dehydration of the sample, pre-treatment with alkaline and/or acid solutions, purification steps before and after extraction, will affect MAE itself and consequently the extract characteristics. For example, MAE of *Gracilaria gracilis* agar [232] was tested for both native and alkali treated sample. Interestingly, an inverted effect of temperature was observed: for native agar, yield was 25.7% at 100ºC and <1% at 140ºC, while alkaline-treated agar yielded 2.1% at 100ºC and 16% at 140ºC. Similarly the MAE of carrageenans from alkaline treated *Hypnea musciformis* showed that yields drastically decreased compared to untreated samples [233].

Two noteworthy efforts of integrative, industry-friendly MAE of polysaccharides setups have been reported with seaweed: a) the extraction of carrageenans of *Kappaphycus alvarezii* and *Eucheuma denticulatum* [234], upon assemblage of a continuous, single-mode microwave extraction process, with potential for scaling-up, where time of extraction was greatly reduced and purity of the product obtained was greater than conventional, allegedly at ready-to-use levels; b) a sequential treatment of *Ascophyllum nodosum* biomass with microwaves [235] that allowed the recovery of alginic acid after fucoidan extraction, with final yield close to that obtained in a dedicated extraction – aiming at the valorization of brown seaweed industrial by-products.

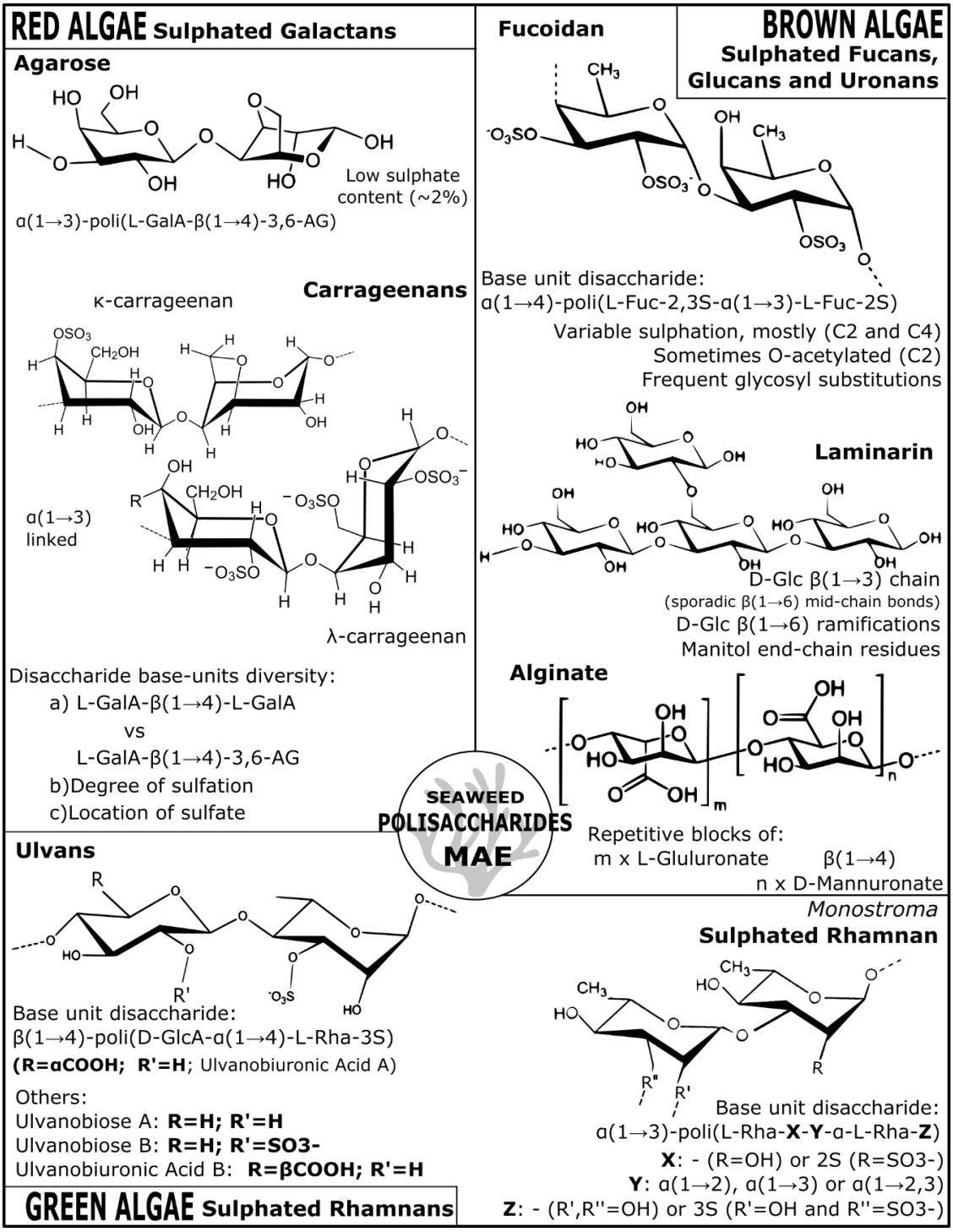


Figure 10 - Structure and general features of the most important polysaccharides extracted from seaweeds. A condensed nomenclature was used in order to maintain the readability of the image – monosaccharide named according to IUPAC recommendations, with adaptations: presence of sulfate denoted by "S" preceded of the carbon numeration of occurrence (e.g. "L-Rha-3S" means a L-Rhanmnose residue sulfated in the third carbon); R, R' and R'' were used as chemical groups of varying composition; X,Y and Z were used as variable portions in the written names of the compounds, according to varying chemical groups.

Table VIII - Extraction conditions of MAE of polysaccharides from seaweeds. Yield is presented as dry weight percentage of purified polysaccharide, except where otherwise noted.

SPECIES (BIB SOURCE)	MAE CONDITIONS Mode; Vessel	Solvent	L/S (ml/g)	Power (W)	Time (min)	T (ºC)	EXTRACT CHARACTERISTICS Yield	Composition
Fucus vesiculosus [236]	MM; CV	Water	25:1	n.d.	1	172	18.2	Fucoidan SC = 21% (w/w)
Undaria pinnatifida [237]	MM; CV[1]	Water	125:1[a]		30	140	55[c]	Fucoidan Mw = 5-30kDa
Ecklonia radiata [238]	MM; OV	HCl (aq.) pH=2	30:1[a]		6	60	3.8	Fucoidan + Laminarin SC = ca 22% (w/w) Mw = ca 400 kDa;
Ascophyllum nodosum [235,239]	SM; CV	0.1M HCl	17,65:1		15	120	16,08	Fucoidan SC = 14.71% (w/w) Mw = 9.04 kDa
						90	14,09	Fucoidan SC = 27.12% (w/w) Mw = 34.42 kDa
		0.1M Na_2CO_3			10	100	23,13	Alginic acid M/G = 1.44 Mw = 195.3
Saccorhiza polyschides [240]	MM; n.d.	Na_2CO_3 aq. pH=10	n.d.	1500	20	100	23.5	Alginic acid
Kappaphycus alvarezii *Eucheuma denticulatum* [234]	SM; OV[2]	Organic[3]	15.7:1[a]	800	2,1[b]	n.d.	n.d.	Carrageenans (k and I) (FT-IR/ NMR - ID and purity assessment)
Palmaria palmata [241]	SM; OV	Water	70:1	500	10	70	15.47	n.d.
Porphyra dentata [242,243]	MM; OV	EtOH (40%)	100:1	200	1	n.d.	80,23[d]	nd
		EtOH (44.4%)			45s x 4 (15s interval)		79,91[d]	nd
Hypnea musciformis [233]	MM; CV	KOH (aq.) 3%	50:1[a]	800	10	105	15	Carrageenan [3,6-AG]=32% (w/w) SC = ca 20% (w/w)
Gracilaria gracilis [232]	MM; CV	Water	40:1[a]	*1500*	*20*	*100*	25.7	Agar SA = 5 m^2/g V_p = 0.02 ml/g
Gracilaria vermiculophylla [172,244–247]	MM; CV	Water	20:1	1500	5	90	n.d.	Agar GS = 1319 (g/cm^2) Mw = 136 kDa SC = 2.5% (w/w) [3,6-AG]=40.7% (w/w)
			40:1[a]	1500	20	110	14.4	Agar
					15	120	15.8	Agar GS = 115.1 (g/cm^2)

								Mw = 54.0 kDa SC = 2.7% (w/w)
					15	120	n.d.	Agar SC = 3,22% (w/w) Mw = 476 kDa
	n.d.			n.d.	15	100	n.d.	Agar SC = 3.9% (w/w) [3,6-AG]=10.9% (w/w) Mw = 132.5 kDa
Enteromorpha prolifera [248]	SM; OV		40:1	700	25	70	10.85[e]	n.d.
Ulva ohnoi [249]	MM; CV	Water	20:1[a]	nd	14	160	36,50	Ulvan Rhamnose = 33.8% (w/w) SC = 5% (w/w) Mw = 2-3kDa
Monostroma latissimum [249]						140	53,10	Sulfated Rhamnan Rhamnose = 79.7% (w/w) SC = 7% (w/w) Mw = 112 kDa
Ulva meridionalis [249]						160	40,4	Ulvan Rhamnose = 54% (w/w) SC = 6.7% (w/w) Mw = 11.8-22.8 kDa

1-Sample de-oiled by SFE; 2- Continuous MAE.; 3. Methanol 45%; Ethanol 37%; Acetone 55%; 2-propanol 40%. all at 0.1M OH^-

a - On dry algae weight basis; b - Equivalent time of irradiation, calculated from the mass irradiated per unit of time due to the circulation. Total time of extraction was 30min.; c - Crude extract; d - total sulfate (ug/ml); e - total sugars extracted on a wet weight basis

MM-Multimode; SM-Single-mode; CV-Closed-vessel; OV-Open-vessel; SC: Sulfate content; Mw: Molecular weight; SA: Surface area; Vp: Pore volume; GS: Gel strength

Agar MAE

Temperature was shown to increase *Gracillaria gracillis*'s agar extraction yields until a maximum value (100-120ºC) after which degradation rapidly takes place, with an accumulation of gaseous products in the vessel indicative of pyrolytic degradation of extracts being reported [232]; however, even at maximum yields some degradation might occur, e.g. depolymerization, sulfate content decrease and gelling and melting temperature increase [172]. Temperature was also the main parameter affecting agar mechanical properties – gel strength of agar extracted by MAE from *Gracillaria vermiculophylla* increased with temperatures from 60 to 100ºC [172]. Extraction time had little effect, leading to a yield plateau at 5-15min; some degradation was only observed after 25min in *Gracilaria vermiculophylla* extraction [245]. All studies with agar MAE were performed with plain water.

Compared to conventional methods (85ºC/2 h or 120ºC/1.5 h), agar MAE (under softer conditions, see Table VIII increased yield from 21.3% to 25.7% in *Gracilaria gracillis* [232] or from 8.5% to 14.4% in *Gracilaria vermiculophylla* [172], and no significant differences in monosaccharide composition of *Gracilaria gracillis* were observed. Mechanical properties have, however, been enhanced: the use of MAE led to an overall increase in gel strength [172,232,247]. Also, MAE of agar resulted in extracts with higher molecular weight than those obtained by conventional extraction [246].

Carrageenan MAE

Carrageenans from three species of seaweed (*Palmaria palmata*, *Hypnea musciformis* and *Porphyra dentata*) have been MWh-extracted. Temperature had little effect on yields, probably because the range of temperatures adequate for extraction (70-105ºC) is quite far from degradation temperatures, reported to start at 115ºC [250]. The same applies for extraction time, L/S ratio and irradiation power, except for *Porphyra dentata* carrageenans extraction by intermittent MAE [243]. Here, low power was found to be optimal and yield increased with irradiation time up to 45 seconds. Regarding the solvent, *Palmaria palmata* extract purity and antioxidant activity were greatly improved, despite the lower yield compared to conventional extraction, by using water [241]; in *Porphyra dentata* MAE, however, it was shown that the addition of ethanol up to 40% v/v was beneficial for higher recoveries [242,243] and slightly alkaline pH values also improved yields. In *Hypnea*

musciformis carrageenan extraction [233], yield and sulfate content were reduced from conventional extraction to MAE methods; however, MAE showed some selectivity between k-carragenans and i-carragenans – which highlights the possibility for selective, intelligent extraction designs to be further developed using MAE.

Fucoidan MAE

MAE of fucoidans (sulfated fucans from brown seaweed) of the genus *Fucus*, *Ecklonia*, *Ascophyllum* and *Undaria*, showed to be a promising alternative, leading to acceptable [239] or even improved [251] extraction yields, using less solvent and shorter time. MAE optimization for fucoidans shows that time is the most important factor. The positive effect of time and temperature in extraction yield and purity of *Ecklonia radiata* polysaccharides was observed [238] only in the first 6 min (from a studied range of 0-200 min). Actually, prolonged irradiation, independently of temperature (but accentuated at high temperatures), led to chemical modifications of the fucoidan from *Ascophyllum nodosum*, with a loss of sulfated monosaccharides, as well as fucose, while the contents of galactose and mannose increased; molecular weight also decreased drastically with both time and/or temperature increment [239]. On the contrary direction, fucoidan extracted from *Fucus vesiculosus* [236] was found to contain xylose residues when extracted in 1 minute, not detected when auto-hydrolysis was used, suggesting that MAE, when adequately tuned to hamper degradation, can also reveal structural features no attainable by conventional methods. Finally, MAE can also be tuned for simultaneous controlled and rapid degradation of fucoidan, in order to obtain specific molecular weight distribution for target applications, e.g. 5-30kDa fucoidan was obtained from *Undaria pinnatifida* [237].

Alginic acid MAE

Alginic acid MAE has only been reported twice, to the best of our knowledge. The alginic acid recovery from *Ascophyllum nodosum* after fucoidan extraction was successfully reported [235], presenting a yield of 18.24% w/w,. Notwithstanding, parameter-oriented discussion/optimization or conventional techniques reference-values were not provided. In another study, alginic acid of *Saccorhiza polyschides* has been MWh-extracted [240], and although the focus of optimization was the acid pretreatment, MAE at 100ºC for 20 min after

the previously determined pretreatment yielded as much alginic acid as earlier reported in the literature, with less solvent and time consumption.

Ulvans and Sulfated Rhamnans MAE

Extraction of ulvans from *Ulva meridionalis* and *Ulva ohnoi* and sulfated rhamnan from *Monostroma latissimum* [249], showed 2.17 and 3.34-fold yield increments for ulvans and rhamnans respectively, in only 8% of the time used in conventional techniques. Yield as a function of temperature had the typical parabolic curve response, reaching maximum values at 160ºC for ulvans and at 120ºC for the sulfated rhamnan. Higher temperatures caused a drastic decrease in recovery of polysaccharides, resulting in molecular weight decrease and extensive degradation (browning was observed), resulting in the complete degradation of *Monostroma latissimum* polysaccharides at 140ºC [249]. Uronic acid, ash, sulfur and xylose contents were not affected by temperature in MAE extraction of *Ulva* polysaccharides; however, a significant decrease in rhamnose relative content, accompanied by a significant increase in the glucose content upon temperature increment suggested that starch solubilization occurs in this range of temperatures, decreasing the purity of the extract. In the extraction of *Monostroma latissimum*, uronic acid, protein and xylose remained constant, while higher temperatures resulted in a considerable increment of ash and a moderate increment of sulfur. Additionally, no evidence of rhamnose:glucose ratio changes seems to occurs between 100 and 140ºC.

A polysaccharide fraction from *Enteromorpha prolifera* was extracted using MAE [248], and although no characterization was reported, the extraction yield and antioxidant capacity were analyzed for different conditions. Compared to the other methods (UAE and HRE), MAE produced a higher-yield in shorter time [248].

II.b.2.2. Lipophilic compounds

Macroalgae lipids and other lipophilics show high potential for biotechnological and nutraceutical exploitation [252]. In algae, like in most vegetable bioprospecting, essential oils are a focus of MAE from seaweeds, followed by other lipophilics, such as carotenoids, sterols as well as fatty acids and naturally occurring hydrocarbons, as described in Table IX. The chemical structure of selected lipophilic compounds MAE extracted from seaweeds is presented in Figure 11.

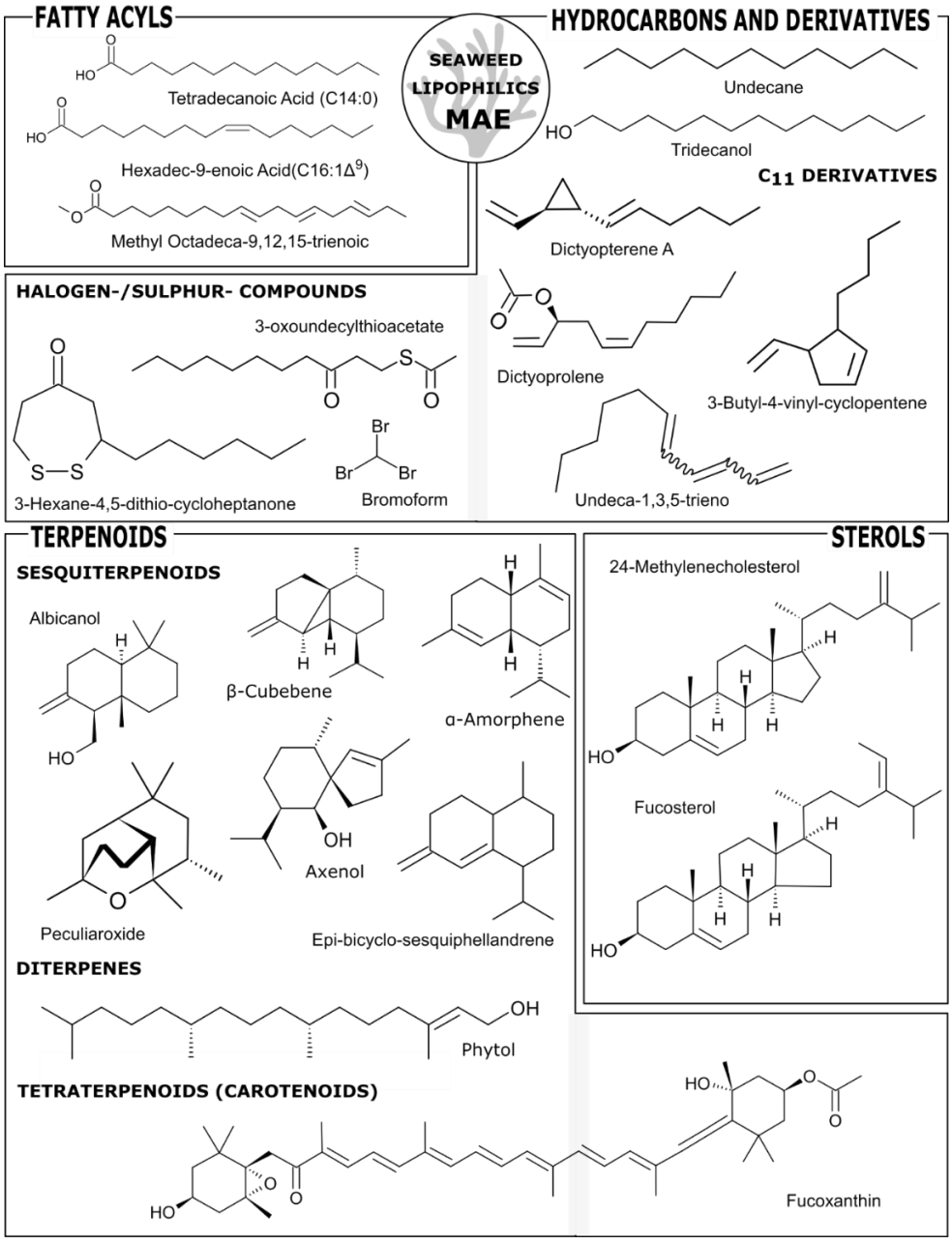


Figure 11 - Chemical structure of selected lipophilic compounds from seaweeds extracted by MAE.

Essential oils

Essential oils are typically mixtures of mono and sesquiterpenoids, with lower amounts of phenylpropanoids, fatty acids, hydrocarbons and derivatives. Seaweed essential oils contain uncommon C11-derived metabolites, as well as sulfur- and halogenated compounds [253]. C11 derivatives from macroalgae show interesting bioactivities, including sex pheromone and predator repellency [33], and for some species are the major essential oil components [254].

Essential oil MAE is, like in non-MAE, a hydrodistillation (HD), and therefore temperature is expected to stabilize at about 100ºC. L/S ratio, power and time are variables that may have some specificity upon MWh. All studies on MAE of seaweeds essential oils (Table IX) used one of two distinct MAE setups: a multimode, long, low power irradiation (4h at 40W) *vs.* a focused microwave-assisted HD (FMAHD, for 10min at 180W). None of the studies reported the optimization of experimental conditions, making impossible to analyze the effect MAE key parameters. The composition of the obtained essential oils shows that using focused microwaves a very significant increase in the contents of sesquiterpenes [32,255] and C11 hydrocarbons [32,34] is observed. On the other hand, the long, weak irradiation seemed to allow the extraction of rather heavy molecules – hitherto poorly hydrodistilled – such as long-chain fatty acids [256,257].

Essential oils from *Cystoseira sedoides* [255] and *Dictyopteris membranacea* [32] were also obtained by FMAHD, by HD and supercritical fluid extraction (SFE). In both species, the differences between FMAHD and both HD and SFE extracts are common: a) SFE yields the highest amount of essential oil (1.18- to 2.95-fold than those of FMAHD's), due to its solvating-like nature – however, it extracts a more diverse set of molecules, including non-volatile lipophilics; b) similar yields were obtained with FMAHD and HD; c) the composition of the oils was significantly different between the three methods, but surprisingly more between FMAHD and the other two – which is not intuitive given the similarity of FMAHD and HD; d) the nature of microwave heating seems to aid the release of moderately low-volatility compounds, while keeping the non-volatile out of the extract – thus leading to a selective enrichment of FMAHD extracted essential oil in sesquiterpenes and medium-size hydrocarbons (like the C11 metabolites). Indeed, in *Dictyopteris membranacea*, FMAHD extracted 17 sesquiterpenes, and none was detected on the same extract produced by HD or SFE [32].

Carotenoids

Carotenoids are colored tetraterpenoids, which due to their pigmentation and biological properties can be used for several applications, such as in e.g. the nutraceutical industry [252]. Due to their structural features carotenoids undergo degradation easily, and therefore mild extraction conditions are essential [258]. Seaweeds, are recognized as attractive and rich sources of carotenoids [252], and therefore either for better analytical extraction, or for an industrial-processing oriented insight, the extraction of carotenoids from these sources is deemed important to be optimized, notably using MAE. Furthermore, as carotenoids thermal resistance varies significantly between compounds [259], MAE optimization might require a compound oriented approach. To the best of our knowledge, MAE has only been used once in carotenoid extraction from seaweeds [260]. However, a number of studies concerning the MAE of carotenoids from microalgae (Table IX) allow some discussion to be held on the response of algal carotenoid extraction to different MAE parameters. Seaweed carotenoids MAE wasn't also compared to conventional extractions yet; however, a study with the microalgae *Cylindrotheca closterium* and *Dunaliella tertiolecta* suggested that a reduction of 10-20-fold in time could be achieved, compared to the conventional procedures [261], additionally, it was also verified that *Arthrospira maxima* carotenoids were more selectively extracted by MAE than by UAE [262].

Fucoxanthin was extracted by MAE from the seaweeds *Laminaria japonica, Undaria pinnatifida* and *Sargassum fusiforme* [260], with different solvents, using both dry and fresh samples. Temperature, L/S ratio, power, time of exposure and solvent were optimized based on fucoxanthin quantification by High Performance Liquid Chromatography (HPLC), showing that temperature increase led to overall yield increase, but selectivity for carotenoids decreased above 50ºC. Additionally, short extraction times (5-10 min) proved to be beneficial in fucoxanthin recovery and preservation, and higher L/S improve extraction yields.

Due to the nature of MWh, and the need of heat in carotenoid extraction, MAE of these compounds requires more polar solvents than those usually applied in conventional procedures – so that dielectric heating can occur. This was observed in seaweed fucoxanthin MAE [260], and a similar trend was obtained in the extraction of the microalgae *Arthrospira maxima* [262]. Effectively, pure ethanol resulted in the higher yields, while the traditionally used, less polar solvents (e.g. acetone, hexane) failed to extract carotenoids under MWh.

Table IX - The lipophilics extracted by MAE from seaweeds. Table's data on composition of the extracts has been simplified (for compound diversity refer to the original articles). In brackets, after each class of extractive, the most abundant compound of that fraction is given.

SPECIES (BIB SOURCE)	MAE BRIEF DESCRIPTION						EXTRACT CHARACTERISTICS
	Mode; vessel	Solvent	L/S (ml/g)	Power (W)	Time (min)	T (ºC)	
ESSENTIAL OILS							
Cystoseira sedoides [255]	FMAHD	Water	900:1[1]	180	10	n.d.[3]	48.5% FAD (C16:0) 36.7% STP (peculiaroxide) 10.5% HCD (C_{17})
Diclyopteris membranacea [32,34]							58,8% STP (albicanol) 13.3% C_{11}-HCDs (dictyopterene A) 8.5% SuC (3-hexyl-4,5-dithiacycloheptanone)
			1750:1[1]	125			61.6% C_{11}-HCDs 7.4% Bromoform
Undaria pinnatifida [256,263]		Water	10:1	40	240		FAD (C14:0), HCD (C6-C9 alcohols); Others; Antibacterial effect.
Enteromorpha linza [263–265]							54,58% FAD (C16:0) 13% HCD (C_{13}-OH) 26,16% Others; Antibacterial effect.
Laminaria japonica (dry) *Pyropia tenera (dry)* [263]							Antibacterial effect.
Laminaria japonica [263,265]			12:1				89.66% FAD Antibacterial effect.
CAROTENOIDS							
Laminaria japonica *Undaria pinnatifida*, *Sargassum fusiforme* [260]	MM; OV	Ethanol	15:1	300	10	60	Fucoxanthin
STEROLS							
Undaria pinnatifida *Sargassum fusiforme* [266]	MM; OV[2]	1.5 M ethanolic KOH	20:1	500	20	70	24-Methylenecholesterol Fucosterol, Phytol

1-Relative to grams of pre-extract material; 2-Simultaneous extraction and saponification; 3 - Temperature in HD, given enough time, stabilizes at ca. 100ºC
FMAHD: Focused Microwave-Assisted Hydrodistillation; FAD: Fatty acids and Derivatives; STP: Sesquiterpenes; HCD: Hydrocarbons and Derivatives; SuC: Sulfur-compounds.

However, too much polarity may revert the trend and decrease success of extraction: methanol or ethanol 80% v/v resulted in similar yields to those of ethanol, but selectivity for carotenoids decreased [260]. As a final note, MWh was also used to degrade carotenoids from microalgae into valuable volatile components (β-cyclocitral, α-ionone and β-ionone), and only 2 minutes at 90ºC in average were needed to reach total conversion [267].

Sterols

Phytosterols are of major nutraceutical interest, for their potential health effects, and macroalgae are known to contain some of these bioactive sterols that, among other properties, reduce cholesterol absorption and allegedly present anti-cancer activity [268,269]. MAE can be used to improve the extraction yield of phytol, fucosterol and 24-methylenecholesterol from *Undaria pinnatifida* and *Sargassum fusiforme* by 2 to 3.5 times, even recovering those in steryl ester forms if ethanolic KOH (1.5M) is used as extraction media [266]. Because sterols are not very thermolabile, power and temperature were maximized, and only time was limited according to the onset of phytol degradation after 30 minutes of irradiation

II.b.2.3. Phenolic compounds

Macroalgae are reported as valuable sources of phenolic compounds, including simple phenols, flavonoids and halogenated or sulfated derivatives, and phlorotannins, exclusive from brown seaweeds, with interesting structural and biological properties [252]. Unfortunately, studies reporting MAE of phenolic compounds from seaweeds have only performed a general characterization of the extracts, based in global spectrophotometric assays, e.g. Folin-Ciocalteau (for TPC determination) or DPPH radical scavenging activity assay (for antioxidant activity determination), often non-specific for phenolic components, leading in most cases to their overestimation; yet, general MAE conditions are presented in Table X.

Antioxidants phenolic compounds are prone to thermal degradation, and high temperatures and/or long extraction time were reported to decrease TPC regardless of the extraction technique [270]. The fast-heating capacity of MAE make it adequate for such delicate extractives – as shown in the extraction of antioxidants from *Enteromorpha prolifera*, where higher TPC, with shorter extraction time and less solvent used compared to other techniques was obtained [271]. Nonetheless, the triad power-time-temperature had a

major effect in extraction yields, due to the degradation of phenolic compounds upon exposure to high temperatures for prolonged periods.

Table X – Extraction conditions of MAE of antioxidant components (phenolic fraction) from seaweeds.

SPECIES (Bib source)	MAE CONDITIONS						EXTRACT CHARACTERISTICS
	Mode/ Vessel	Solvent	L/S (ml/g)	Power (W)	Time (min)	T (ºC)	
Caulerpa racemose [272]	MM; OV	EtOH 60%	40:1	200	40	50	TPC[1] = 4.43 mg GAE / g extract
Saccharina japonica [273]	n.d.	EtOH 55%	8:1	400	25	60	TPC = 0.644 PGE / g DW
Ecklonia radiata [92]	MM; OV	Enzymatic		n.d.	5	50	TPC = 85.4 mg PGE / g extract
Sargassum muticum [274]	MM; OV	- [2]		600	5x 7	100	TPC[2] = 22 mg GAE / g extract
		EtOH 96%	n.d.	400	2x 10	n.d.	TPC = ca 30 mg GAE / g extract
		EtOH 50%	n.d.	400	3x 10	n.d.	TPC = ca 30 mg GAE / g extract
Polysiphonia sp. + Ulva sp. + Cladophora sp.[275]	MM; CV	Water	3:1	1000	30	25	TPC = ca. 2.74 mg GAE / g DW
Chaetomorpha sp. [184]	MM; OV	Acetone 25%	20:1	300	8	n.d.	TPC = 0.98 mg TAE / g DW
Enteromorpha prolifera [271]	SM; OV	EtOH 40%	25:1	500	3x 25	n.d.	TPC = 112.97 mg GAE / g extract
Enteromorpha linza [265]	n.d.; OV (MWHD)	Water	10:1	40	240	n.d.	TPC = 21.40 mg GAE / g extract
Undaria pinnatifida [256]							TPC = 65.36 mg GAE / g extract
Monostroma nitidum [189]	MM; OV	EtOH 10-30%	67:1	800	15 x 10s 30s intervals	80	Ferrous ion chelating = ca 38% Radical Scavenging (DPPH) = ca 23%
Porphyra dentata [276]							Ferrous ion chelating = ca 50% Radical Scavenging (DPPH) = ca 60%

1 – Correlated DPPH-scavenging activity and. reducing power; IR spectroscopy indicative of phenolics.

2 - Microwave hidrodiffusion an.d. gravity (MHG) (87% natural moisture) followed by Microwave Hydrodistillation (added water to replace lost moisture, 4 cycles); Cummulative TPC presented, cycle-wise TPC discussed in the text..

MM-Multimode; SM-Single-mode; CV-Closed-vessel; OV-Open-vessel; TPC-Total Phenolic Content; n.d.= non-described; GAE-Gallic acid equivalents; TAE-Tannic Acid Equivalents; DW= Dry weight

MAE temperature promoting maximum antioxidant activities of seaweed extracts at relatively low values: 25 to 40ºC. Additionally, irradiation time was utterly important, since it similarly conditions both solvation and degradation. The extraction of *Caulerpa racemosa* antioxidants [272] at different irradiation periods (5min to1hour) led to a yield increase up to 30 minutes tending then to a plateau. The absence of degradation in this study is probably due to the low MW-power (200W). In other studies, with both power and time optimization, it is clear that these factors follow the parabolic shape of increasing yield to a given value and then starting to decay – as in the extractions of *Chaetomorpha sp.* [184] and

Enteromorpha prolifera [271], where at the higher powers tested, a clear time-limit arouse, past which irradiation became deleterious.

The studies involving the MAE of phenolic compounds from seaweeds used water or binary aqueous systems as extraction solvent. Traditionally, organic aqueous mixtures were more effective in the extraction of phenolic compounds than organic solvents alone [75,77]. Additionally, water leads in MWh to a very fast heating rate and has an acceptable maximum temperature (100ºC) for the stability of most compounds. Although plain water has been used for seaweed phenolics MAE (see Table X), some studies showed that the increase of ethanol content (in the range of 10-50%) improved extraction performance in terms of TPC [271] and DPPH radical scavenging activity [276]. However, other studies showed that higher ethanol contents (60-96%) might be also efficient, namely for extraction of phenolics from *Caulerpa racemosa* [272] and *Sargassum muticum* [274].

The efficiency of MAE of phenolic compounds from *Sargassum muticum* was studied using a solvent-free hydrodiffusion and gravity extraction step followed by subsequent cycles where water was added to compensate moisture loss [274], and comparing the results with autohydrolysis or enzyme assisted extraction. Although this approach produced extracts with lower TPC than autohydrolysis or hot-water extraction, it was superior to the use of ethanol-MAE, thus reinforcing the superiority of water in phenolic compounds MAE. Also, it was shown that a 17.5-fold increase in yield was obtained when 5 cycles of extraction were performed, compared to one-cycle. The overestimation of TPC in autohydrolysis extracts from co-extraction and degradation of polysaccharides might be responsible for the apparent diminished performance of MAE.

Microwave-assisted enzymatic extraction (MAEE) of phenolic compounds from *Ecklonia radiata* was compared with the corresponding (EAE) and conventional extraction [92]. At a medium temperature (50ºC), the extracts obtained by EAE and MAEE presented significantly ($p<0.05$) higher extraction yields, TPC and antioxidant activities (from 1.3 to 3-fold, depending on the parameter) than those obtained with conventional extractions; more importantly, MAEE allowed that to occur in 12.5% of the time.

II.b.2.4. Other MAE applications in seaweeds

Proteins are among the most exploited seaweed bioactive components in the last years, particularly the phycobiliproteins (PBP) pigments. Although to the best of our knowledge

MAE of PBPs in seaweed hasn't been reported, its success applied to microalgae clearly demonstrates its potential [277]. Indeed, temperature control was shown to be critical in the recovery of PBPs, and a thorough study of the effect of temperature on each class of these proteins (phycocyanin, allophycocyanin and phycoerythrin) was performed. While MAE of PBPs required different temperatures (for different periods) to preserve each of the classes, the authors successfully exploited such procedure's potential as an actual advantage, leading to sequential, selective extraction (and thus, pre-separation) of the PBPs.

MWh has also been applied in macroalgae valorization in areas distinct from extraction, yet with technical insights that might be relevant for bioactive components extraction from seaweed, as matrix and extractives' properties are rather similar. These include, for example, MWh preparative digestion in order to obtain macroalgae hydrolysates ready for alcoholic fermentation and, thus, valorize algal biomass for the energy/fuel sector, which was applied on *Ulva* spp. [278] and *Ascophylum nodosum* [279]. MWh was studied as a method for effective disruption of algal cells (mostly microalgae), extraction of their oils and simultaneous transesterification to produce biodiesel [280–282].

In microwave-assisted convectional drying of the *Gelidium sesquipedale* for agar-industry, processing time was substantially reduced without significant loss in final-product's properties [283,284], and microwave-assisted freeze drying, a recently reported method, claimed to be an effective strategy to further reduce moisture content when compared to the traditional ones in *Saccharina latissimi* [285].

Additionally, microwave-assisted digestion (MAD, either acid or alkaline) was extensively used in analytical chemistry [286], providing very efficient and short time digestion of organic matter, suited for high-throughput analytical platforms, which have been applied in heavy-metal [287,288] and pesticides [289,290] contamination analysis in seaweeds.

MAD and MAE have also been used in the quantification of iodine present in seaweeds, mostly as volatile organic iodine compounds (considered as a 'functional ingredient' from a nutritional perspective) with substantial reduction in processing times [252,291].

II.b.3 Concluding remarks on seaweed MAE

Microwave-assisted extraction is an emerging green extraction technique in which the disruptive innovation resides essentially in the use of dielectric heating instead of convection-based heating. Consequently, temperature build up occurs faster, rather homogeneously and in a chemically-selective mechanism. Because water is the main constituent in many biological samples, and the cleanest of the solvents, it is also one of the best dielectric media for heat generation by microwave irradiation. Water containing samples can be heated from inside, without solvent, and independently on the configuration of the system (organic, water based or no solvent, open or closed vessel, disperse or focused microwaves), heating can be controlled very precisely as microwave power output can be accurately monitored and energy input in the sample thus modified in real-time.

The recognized potential of MAE adverts mainly from two premises: its potential for solvent usage downscaling or total elimination and its equally excellent extraction performance (both yield- and product-quality-wise) in a much shortened amount of time.

This section provided evidence regarding MAE's suitability in the extraction of bioactive compounds from diverse biological matrices with emphasis on seaweed. Seaweed bioprospecting is an emerging area of R&D with fantastic promises of societal and economic impact. The merge between the need to search marine algae biomass for bioactive compounds and the need to start performing these studies in a greener approach, both at lab- and industrial-scale, resulted in the efforts described in this section in MAE implementation. Noticeably, phenolic compounds, lipophilics and polysaccharides are compounds already present in the industry and for whom an improvement of processing technology is greatly encouraged. MAE was shown to contribute to this improvement with a possibility to speed up extraction processes drastically. Also, future studies should be made to understand the microwave-associated effects of MAE on the extracts, both for an intelligent design of extraction setups and for an evaluation of MAE-exclusive potentialities – for instance, sequential extractions with high selectivity using a single extractive unit have been shown promising under MWh.

Future themes on MAE include, but are not limited to, the further development of continuous-MAE, solvent-free MAE, alternative solvents MAE (ionic liquids or natural deep eutectic solvents, for example), or enzyme-MAE. All these variants contribute, somehow, to the feasibility of MAEs introduction in industrial setups (although it is already present, it is

far from fully explored). Also, scale-up of MAE apparatuses is likely to involve some R&D before optimal extraction is achieved, as MAE is a size-dependent heating approach (limited by the wavelength of the microwaves). With research, it is expected that the drawbacks of MAE are understood and ultimately fixed or compensated by technological alternatives. By then, MAE holds great potential to help revolutionize the industrial processing paradigm.

II.c - Analysis of complex mixtures

Crude macroalgae polar extracts are complex mixtures, where not only phenolic components but also contaminant polysaccharides, proteins and other polar metabolites co-exist in abundant diversity. While a detailed analysis of the structural diversity in the extract is hard at this point, an initial characterization by spectrophotometric assays, such as Total Phenolic Content (TPC) by Folin-Ciocalteu reaction, or Total Phlorotannins Content (TPtC) by the 2,4-dimethoxybenzaldehyde (DMBA) assay, is frequently employed, mostly due to the simplicity, low cost, rapid execution and potential for comparison with other works. However, the lack of specificity and the sensitivity to interferences leads to poor reproducibility/reliability, greatly discouraging the usage of these techniques as sole descriptors of the extractives' identity; nonetheless, due to their spread use, an initial discussion is held on this topic in section II.c.1

More reliable techniques for the analysis of crude extracts have been reported. 1H NMR, FT-IR and UV spectroscopy have been extensively used, mostly as confirmative techniques, used to improve the reliability on spectrophotometric measures – i.e., confirm phenolic components' presence in the extracts through more specific (yet rather uninformative when applied to crude extracts) spectral signals characteristic of these compounds, thus clarifying the extent to which TPC and antioxidant activity are biased by contaminants. A brief discussion on these spectral "clues" is held on section II.c.2 Interestingly, 1H NMR has been reported as a good method to quantify certain phenolics in complex mixtures, and this application is also exposed in this section.

II.c.1. Spectrophotometric assays

The spectrophotometric characterization of the phenolic rich extracts from macroalgae involves mostly the estimation of TPC and total phlorotannins content TPtC, although some authors have also estimated the content of other specific families, such as flavonoids or anthocyanins [79,292,293]. The most common used method to determine the TPC in macroalgae extracts is the known Folin-Ciocalteu method, firstly established as Folin-Denis method [294] and then modified by Folin and Ciocalteu [295] and later by Singleton and Rossi [296]. Notwithstanding, some authors have used other methods [297], namely the Prussian Blue assay [298]. These assays are based on redox reactions, in which the phenolate ions are oxidized and the $Fe(CN)_6^{3-}$ ion (in Prussian Blue assay), or phosphotungstic-

phosphomolybdic (in Folin-Ciocalteu assay) are reduced, forming colored products. Other free hydroxyl groups can participate in that reactions, therefore these assays only give an estimation of the amount of total phenolic compounds present in the extract. The lack of specificity of the Folin-Ciocalteu method has been described as its major limitation [299], with the possible contribution of non-phenolic compounds leading to an overestimation of the results. However, considering its use for comparative purposes, the main problem concerns in the lack of a standardized methodology. In fact, from the vast range of studies regarding the TPC of macroalgae extracts there is a lack of consensus in what concerns the volume and concentration of extracts and reagents [49,99,292,300]. The incubation time has being also quite divergent, with some authors chosing 20 [49] or 30 minutes [184,301] and others extending up the incubation time to 2 hours [292,300]. Additionally, no consensus is found in the standard used for the calibration curves, which makes even more difficult the comparison between different studies. Most of the authors have expressed the TPC of macroalgae extracts as gallic acid [49,75,274,300–302] or phloroglucinol equivalents [95,303,304]. However, the use of other standards have been also adopted in other studies, such as catechol [85,292] or tannic acid [184]. As mentioned above there is no specificity of Folin-Ciocalteu method for phenolic compounds still less for phlorotannins. Therefore, inaccurately, some authors have used Folin-Ciocalteu method to estimate the TPtC (expressed as phloroglucinol equivalents) [77,78,81,273]. Actually, the comparison of Folin-Ciocalteu and Prussian-Blue methods to DMBA assay, a colorimetric method described as specific for 1,3- and 1,3,5- substituted phenols, showed a great overestimation of the first two methods [297,305]. The reaction of 2,4-dimethoxybenzaldehyde and phlorotannins is based on an electrophilic attack by the aldehyde at acidic conditions, forming a pink colored chromophore. Actually, in the last years several authors have accomplished the TPtC estimation by this assay [80,86,95] Consensus is found in the standard used, namely phloroglucinol, despite being suggested that the TPtC is underestimated when this standard is used [297].

II.c.2. Direct spectroscopic analysis of the crude extract

Crude extracts are often too complex to be analyzed by high-resolution techniques. However, some spectroscopic techniques allow a rough estimation of both qualitative and quantitative composition, through the presence of “diagnostic” signals. For instance, it is

widely known that phenolic compounds absorb radiation in the UV-region of the spectra (with maximums between 260 and 330nm); which is often used as an indicator for the presence of this family of compounds. However, molecular absorptivity of the derivatives of simple phenolic compounds is quite variable, and full spectra are highly influenced by media pH [306].

Also, due to the high absorbance of proteins and nucleic acids in the same region, this technique is more relevant when working with standard compounds, or as detector in chromatographic equipments, as discussed in the next section. A workaround for the confirmation of UV absorbance being the product of phenolic moieties has been to evaluate the existence of a bathochromic shift of absorption maxima towards longer wavelengths, as a result of phenol ionization to phenolate upon the addition of NaOH [307]. The method has been used in the study of seaweed phenolic extracts from *Fucus spiralis*, where a shift from the maxima 270nm peak (with a shoulder at 285nm) of 16nm was registered upon the addition of two drops of NaOH 2M directly in the spectrophotometer cuvette [308]; this value was reproduced in the analysis of *Sargassum siliquastrum* extractives [309].

Fourier-Transform Infrared spectroscopy (FT-IR) has been a common method explored in the characterization of phenolic extracts. The presence of phenolic groups in seaweed has been assessed by the simultaneous occurrence of bands absorbing in the regions correspondent to hydroxyl groups (3,300 to 3,500 cm-1) and aromatic rings (1,200 to 1,700 cm-1 as well as 2850-3000 cm-1) [308–311]

Nuclear magnetic resonance (NMR) allows a more detailed (albeit still very unspecific) analysis of complex mixtures. Indeed, proton NMR (1H NMR) has been used to confirm the presence of phenolic constituents [93] as well as to detect [312] and quantify [313,314] phlorotannins. The protons from phenolic units have characteristic chemical shifts that can be used to detect the presence of phenolic moieties. Also, thanks to the resolution of 1H NMR, not only phenolics but also carbohydrates can be detected, allowing a qualitative measure of the extract's purity. In an experimental setup for the optimization of green extraction processes for *Sargassum muticum*'s bioactive phenolics, Anaëlle et al. [93] managed to track the relative proportions of phenolic compounds (measured in the 5.5 to 6.5 ppm range) and of mannitol, a common co-extractive in phenolic extracts, by the signal obtained in HR-MAS at 3.6-3.9 ppm (the region of polyols). Other studies with brown

seaweed have also used 1H NMR chemical shifts in the range of 5.5 to 6.5 ppm to evaluate phenolic compounds presence and their relative quantity [312,315]

Parys et. al [314] have used another NMR approach to quantify phenolic compounds. More specifically, resonances in the region of 6.0 - 6.3 ppm (range of typical resonances from phlorotannins' aromatic protons) were integrated and quantified against an internal standard (trimesic acid). However, this first study obtained highly over-estimated values for phenolic compounds concentration (at least compared to Folin-Ciocalteu calculated values, which are already assumed to be oversized). Nonetheless, Jégou et al. [313] have used 1H HR-MAS to detect phloroglucinol in *Cystoseira tamariscifolia'*s unprocessed biomass, and managed to develop a method for the quantification of phloroglucinol with 94.2% accuracy – unlike the previous study, one single resonance (that of the three C-H bonded protons in phloroglucinol) at 6.02ppm, was used. Simultaneously, values up to 30x higher with FC assay (thus, much overestimated) were obtained. The authors claim that quantitative NMR can potentially be optimized to most compounds, becoming a practical, reliable method to assess the concentration of a metabolite in the complex mixture of a crude extract.

13C NMR has also been used for the assessment of phlorotannins-rich extracts' purity [297,316]. In both studies, the attribution of given spectral traits to phlorotannins was made by the comparison with published spectra of isolated compounds [66], on which common chemical shift resonance regions at 95-107, 123-134, and 143-164 ppm were identified.

II.d - Primary fractionation of macroalgae polar crude extracts

In most cases, the study of phenolic compounds from seaweed involves separation and purification steps, for the fractionation and/or isolation of the compounds of interest, and the subsequent analysis for structural characterization, by spectroscopic techniques. Primary fractionation of the extracts precedes most analytical studies, resulting in the separation of the extracts into fractions according to one or more characteristics of the compounds in each one – molecular weight, charge, chemical affinities, solubility, among others. Such processing is intended to reduce complexity, allowing further high resolution chromatographic separation steps, but also to prevent damages to the columns and remaining components of the instruments. Thus, studies of both approaches were included in this section. From 85 of a total of 99 peer-reviewed articles analyzed, data on solubility-based separations (liquid-liquid extraction and solutes precipitacion), adsorption-based separation (including Solid-Phase Extraction, SPE), particle-size-based separation (Molecular-Weigh Cut-Off Dialysis, MWCOD, and ultrafiltration, UF) and molecular-charge-based separation (capillary electrophoresis, CE) were gathered, and are discussed below.

II.d.1. Solubility-based separation

Liquid-liquid extraction (LLE) is by far the most common method employed in first place to phenolic crude extracts. The extraction of seaweed phenolics is often conservative, i.e., along with the phenolic components, other molecules get co-extracted, such as sugars, proteins and some medium-polarity lipophilics (e.g., carotenoids). For that reason, the partition of these distinct classes in carefully chosen solvents, given their relative differences in solubility, allows to obtain polarity-segregated fractions. LLE was essentially reported for the isolation of phlorotannins, which besides resulting from the higher amount of studies with phlorotannins comparing with those of miscellaneous phenolics, is also a result of the broad range of polarities that miscellaneous phenolics present. This variability in polarity makes solubility-based separations quite ineffective in preserving this entire class of compounds while discarding the others. Phenolic compounds solubility is, indeed, highly variable, depending on the degree of hydroxylation, the molecular weight, the presence of different functional and/or alkylic groups or the protonation of the molecule at the solvent's pH [317]. Thus, a linear behaviour along the eluotropic series of polarity of solvents is not

observed, since different intermolecular forces co-occur to solvate, or not, the solutes. Phenolic acids, for example, are not extremely soluble in water, but slight alkalinisation promotes an increase in solubility, since the carboxylic group ionizes; flavonoids, due to their large size, are generally more soluble in methanol or ethanol than in water; however, because their predominant form in many biological matrices is glycosylated, their recovery might be higher in aqueous mixtures [318–320].

In fact, only one study used LLE in the processing of an extract for whole-phenolics analysis. The crude methanol 60% (aqueous) extract of *Himanthalia elongata* was partitioned using ethyl acetate (EA), a medium polarity solvent often used for PCs. Effectively, meta/para-hydroxybenzaldehyde, phloroglucinol, gallic acid, kaempferol, cirsimaritin, gallic acid 4-O-glucoside and carnosic acid were detected in the EA fraction. A note on the recovery of miscellaneous phenolics is now required: first, one must account that the fact that phenolics partition into the organic extract (to some extent), while other contaminants don't (such as sugars), is what makes relatively apolar solvents suitable for this kind of purification – at the expense of losing a significant amount of phenolic extractives in the aqueous residues; second, one must consider the fact that biological matrices are much more concentrated in hydrophilic substances (sugars and proteins) than in phenolic secondary metabolites – and for that reason, crude alcoholic/aqueous extracts, even though ideal for phenolic compounds solvation, will readily lose these components when fresh solvent (one that can solvate the phenolics) is added in large volumes (far from saturation). Once again, external factors affect the behaviour of phenolic substances in solution, with an impact in their laboratorial processing: the concentration of more hydrophilic contaminants, and the compromise that the researcher needs to establish between quantitative or qualitative recovery.

Furthermore, a lot of experimental data exists on the fractionation of phlorotannins (e.g., [62,311,321–324]), allowing an empirical model to be drawn. The majority of LLE were initiated by removing the hydrophobic components extracted, using hexane, chloroform, dichloromethane, petroleum ether or a combination of these. The remaining hydrophilic fraction (from the solvent of extraction) was usually treated with ethyl acetate (where phlorotannins get partitioned), and further washed with butanol. Nonetheless, different approaches have gathered interesting results as well. For instance, *Cystoseira abies-marina* phlorotannins have been successfully analysed by simply defatting the extract with

dichloromethane [57], while the opposite approach, i.e., partitioning the phlorotannins-enriched fraction in a one-step addition of ethyl acetate, was equally sufficient for further processing in other studies [325,326]. Tierney et. al [76] actually obtained a phlorotannins fraction with high potential for food industry by simply washing the ethanolic extract with water, which was frequently replaced (top layer decanted); similar approaches have also been reported [49,327].

Finally, Leyton et al. [71] have used methanol to precipitate carbohydrates, while Glombitza et. al [62] have used petroleum ether to precipitate the high-molecular-weight fraction of an acetylated phlorotannins extract previously partitioned into ethyl acetate.

II.d.2. Adsorption-based separation

Adsorption based separations are an emerging technology due to its simplicity, potential for scale-up and higher specificity compared to other primary fractionation techniques [328]. Separation of compounds is achieved by mixing the solubilized extract with a solid matrix, to which the target compounds and the unwanted contaminants have distinct affinities. Thus, specific compounds can be recovered by separating the solid and the liquid phases (e.g. by reduced pressure filtration), and processing the one known to contain the desired compounds (i.e., further processing the supernatant, if the target metabolites are not adsorbed, or eluting the analytes from the matrix, if they do adsorb).

In the case of seaweed phenolics, three matrices have been reported to adsorb phlorotannins. Diaion® HP-20 is a hydrophobic, synthetic resin, widely used to adsorb compounds with hydrophobic moieties. This matrix has been used to enrich an ethanolic extract (aqueous, 70%) in phlorotannins, by placing it on top of a filled column and washing it with water [329]. In this case, salts and hydrophilic compounds (such as sugars and proteins) are eluted, while phlorotannins and other apolar compounds (readily removed by previous partition with an hydrophobic solvent) get adsorbed and released only after rinsing with ethanol 60%. Another relatively apolar matrix is polyvinilpyrrolidone (PVP), with which the sulphated phlorotannins of *Pleurophycus gardneri* have been purified [61]. In this work, a suspension of PVP particles was mixed with an acidified phlorotannins fraction, where the pH of 5.5 allowed the solubilization of the sulphated molecules (target), while keeping them protonated and thus less polar – i.e., more adsorbed. Consequently, after

washing with water, the recovery of the sulphated phlorotannins was achieved by elution with aqueous NH_3, increasing pH from 8 to 11. The deprotonation of the acidic sulphate moieties leads to the desorption of the molecules.

Several works have proposed cellulose as an adsorbent [56,80,297,330–332]. This polymer has revealed high affinity for phlorotannins, allowing the removal of residual lipophilics and salts without a significant loss of the target analytes. One approach to this adsorption setup is to evaporate a methanolic solution of the crude extract after agitation with cellulose (often, microcrystalline cellulose – 'Avicel' – was used), and further rinse it with toluene, discarding pigments, and with aqueous acetone (66-70%), recovering the phlorotannins [56,80,297,330]. Another approach is that of Glombitza et al. [331,332], who performed the removal of lipophilics prior to the adsorption step, and used cellulose as a media to wash off salts and other contaminant solutes, which can be done by solubilizing the sample in methanol 50%, thus solvating the contaminants as well, and treating the extract with cellulose, posteriorly removed by filtration or centrifugation; the remaining liquid can be discarded, and phlorotannins recovered by re-washing the cellulose with methanol:acetone 2:1. Cellulose can be a very interesting matrix for further research, as it has the potential to be industrially used, in a sustainable valorization of industrial by-products, without compromising the applicability of the extracts for the food industry, given its biocompability. Although recovery of more than 75% of the weight of the crude extract was reported [332], suggesting a reduced loss of analyte, optimizations of adsorption methods are still required to increase specificity and reproducibility.

Despite the ambiguous classification of solid phase extraction (SPE) as a type of chromatography or not, such purification steps have been included in this section. In this method, the adsorbent is immobilized in a cartridge, allowing the sequential elution of compounds to occur with gradient solvents; however, the size and nature of the cartridge make it more suitable to binary separations, i.e., it is typically used to receive an extract and split it into two polarity- and/or charge-opposed fractions. For this reason, it has been considered an extractive step rather than a chromatographic one. Alike chromatography, however, SPE can be used in either normal or reversed phase. Reversed-phase, octadecyl-derived (C18) cartridges are the main choice in seaweed phenolics SPE [55,70,75,333–335]. Several commercial products exist, and most are adapted for the acceleration of elution with vacuum/reduced pressure systems.

The simple phenols and phenolic acids of *Padina gymnospora* have been purified by a Sep-Pak C-18 Cartridge (Water Associates, Millipore™, Billerica, MA, USA), after resuspension in water, thorough washing and elution of adsorbed phenolics with 30% ethanol [335]. Also, a method for the extraction and purification of these classes of bioactive phenols was suggested by Onofrejová et al. [302] consisting of a pressurized liquid extraction followed by a SPE. In their article, the authors demonstrate how a special mixed mode (reverse-phase/ion-exchange) SPE cartridge (Oasis MCX, 60mg) allowed the concentration and purification of a diverse set of phenolic substances by means of pH manipulation of the eluent. More noticeably, the method allowed the downstream analysis of the fractions by HPLC-ESI-MS in the sub-nanogram scale [302].

Phlorotannins have been processed by RP-SPE several times [55,70,75,333,334]. Although C18 solid phases were always used, the application of different elution schema allows different processes to be drawn: distribution of phlorotannins between the washing fractions and the posterior eluted ones will depend on the initial solvent of resuspension, since the phase of application of the sample to the cartridge will define what molecules get eliminated in the run-through and to what extent the phlorotannins will adsorb in the octadecyl matrix. For instance, RP-SPE of previously defatted phlorotannins extracts can eliminate the hydrophilic contaminants by resuspension of the extract and washing of the column with water, eluting minerals, proteins and sugars in these fractions, while phlorotannins stay retained due to the hydrophobic interactions with C18 [55,333]; posteriorly, their recovery can be done by eluting the cartridge with 50% [333] or 100% [55] methanol, which is apolar enough to desorb these compounds. Pantidos et al. [70] actually improved the process by acidifying the solvents (both the water in the resuspension/washing phase, and the organic solvent – in that case, 80% acetonitrile) with 0.1% formic acid, protonating the phlorotannins which despite improving their solubility in water, also improves their retention in hydrophobic media by reducing the polarity in acidic hydroxyl groups. Another way to use C18 cartridges has been described by Nwosu et al [75], which used SPE to remove both salts/minerals and lipophilics. Thus, 50% acetonitrile acidified by 0.2% formic acid was used to carry the extract into the cartridge, allowing the minerals, salts and other hydrophilics with no apolar moieties to flow in the run through with little retention. The remaining water-soluble molecules that were retained were the phenolic

components, which were washed with water, until no more residue was obtained. This way, the hydrophobic metabolites remained adsorbed.

The application of normal-phase SPE has only been reported once for seaweed phenolics [336]. After several purification steps (including LLE and column chromatography) without sufficient removal of polar contaminants for phlorotannins ESI-MS analysis, *Ascophyllum nodosum*'s extract was ressuspended in methanol:chloroform, and passed through a silica-filled cartridge [336]. Elution was carried out first by passing the same solvent, and afterwards by washing with methanol. Phlorotannins with enough purity to obtain good signal peaks in ESI-MS were obtained in the first fraction (methanol:chloroform), probably because the polar contaminants had little solubility in this solvent mixture, and became adsorbed to the silica, while the hydrophobicity of phlorotannins was sufficient to maintain them solvated and repeled from such a polar matrix. Even though NP-SPE was only applied once, the abundant reports of normal-phase chromatographies suggest this phase can be successfully employed in a cartridge-scale, adsorption-based fractionation of seaweed crude extracts.

II.d.3. Other primary separation methods

Other separations of complex extracts have been performed prior to preparative or analytical chromatographies by means of Ultrafiltration (UF) [78,308], Molecular-Weight Cut-Off Dialysis (MWCOD) [49,76,337], a combination of these two [315] and Capillary Electrophoresis (CE) [338]. UF and MWCOD are techniques that segregate compounds by their molecular weight (MW) with less instrumental requirements, time, expertise or investment than the alternatives. Actually, with UF/MWCOD, a clear-cut separation of the molecules around a defined limit of MW can be achieved, allowing an easy separation of discrete fractions over a wide range of MWs with a few combinations of membranes/filters.

Isolation of the Low-Molecular Weight Phlorotannins (LMWP) from *Fucus vesiculosus, Fucus serratus, Himanthalia elongata,* and *Cystoseira nodicaulis* was carried out using membrane dyalisis with a MWCO of 3.5kDa (meaning a maximum degree of polymerization – DP - slightly above 25) [49]. The study of phlorotannin profiles in seaweed has often been reported in terms of the number of phloroglucinol units (PGU). In fact, as DP gets higher, the number of isomers grows drastically, preventing common MS approaches of distinguishing them. Phlorotannins with DP between 2 and 10 are what is often reported as low molecular weight; however, by separating the phlorotannins pool at the 3.5kDa cut-off, phlorotannins of 16 PGU could be analyzed by the chromatographic platform [49]. Nonetheless, most of the antioxidant activity remained in the >3.5kDa fraction. This is because although LMW phlorotannins are much more described in literature, the HMW phlorotannins are estimated to actually be much more abundant in seaweed biomass. The same rationale – of separating ranges of molecular weights for a more efficient analysis – was used by Tierney et al. [76], but the >3.5kDa fraction was further dialyzed with a 100kDa cut-off membrane, therefore producing the fractions of 3.5-100kDa and >100kDa. Spectrophotometric measures suggested that the 3.5-100kDa was the richest in phenolic compounds, which is in agreement with the expected distribution of phlorotannins molecular weight. The fraction higher than 100kDa is thought to retain most polysaccharides, contributing to such enrichment; on the other hand, while LMWP are dialysed into the <3.5kDa fraction, so is one of the major contaminants in seaweed phenolic extractives – mannitol -, which leads to the lower values of TPC and antioxidant activity of this fraction. Nonetheless, by applying further purification (namely, reversed-phase chromatography), the autors managed to obtain fractions containing phlorotannins up to 25PGU, as analyzed by

mass spectrometry. This study reinforced the notion that besides the <3.5kDa fraction containing many different phlorotannins to be analyzed, the non-analyzable fraction of 3.5-100kDa actually corresponds to a rich, biologically potential extract.

Similarly, UF has been used to generate MW-dependent fractions, alone or in combination with MWCOD. The separation of *Fucus spiralis* phenolic extract into three fractions (F1<1kDa<F2<3kDa<F3) using a series of filters also demonstrated that functional assay results (namely ACE-inhibitor activity) are improved in the fraction of >3kDa [308]. The lower MW fractions probably contained phenolics as well (as suggested by their FT-IR spectra), but the higher concentration of small organic and inorganic contaminants might explain their lower activities. Other reports of similar studies, illustrating the inclusion of UF in the fractionation of phlorotannins can be found in [78,315].

Capillary electrophoresis (CE) of miscelaneous phenolics has been developed by Truus et al. [338], successfully separating catechin and gallic acid in the Fucus vesiculosus extract. The whole profile could be obtained in 1100 seconds. This method is an alternative to chromatographic setups, and has been highly appreciated for its rapidity, small scale and low solvent waste. It has also been suggested as a good method to analyze polar extractives of plant and seaweed matrices, because of its capacity to separate sugars and phenols [339]. Despite not being a preparative purification method, the very small usage of sample and the very short time to obtain results make CE an excellent candidate for routine analysis of extracts along the process of purification to monitor the qualitative and quantitative changes in between steps.

II.e - Isolation of phlorotannins and other SPCs

Seaweed phenolic acids, flavonoids and other phenolic components ubiquitous in vegetal matrices have been isolated and readily identified by NMR and/or MS, by comparison of their fingerprints rather than by de novo structure elucidation – possible due to the vast research background concerning plant phenolics, which made available a large amount of spectral references to compare samples with. Thus, this type of systematic analysis on methods and interpretation for the spectroscopic data of seaweed phenolic extractives is essentially directed to phlorotannins, whose structural characterization is still a challenging task for researchers.

Crude extracts obtained with water, alcohols or mixtures of both (the most commonly used solvents) are often highly concentrated in polysacharides, as well as in proteins. Phenolic components are secondary metabolites present in biomass at much lower concentrations than those primary constituents. Furthermore, less polar contaminants such as carotenoids are often found to partition into alcoholic solvents easily. This chemical diversity poses an obstacle, as phenolic components need to be isolated prior to most analytical approaches. While more modern techniques – based on chromatographic-mass-spectrometric platforms – allow the simultaneous separation and identification of organic compounds, such analysis can only become routine once enough data on structural diversity and mass spectra have been gathered for individual compounds (see scheme in Figure 12).

The characterization of phenolic constituents by spectrocopic analysis of pure compounds is highly dependent on the success of preparative chromatographic separations, from which isolated compounds can be recovered. The methods for this fractionation are analyzed in this section.

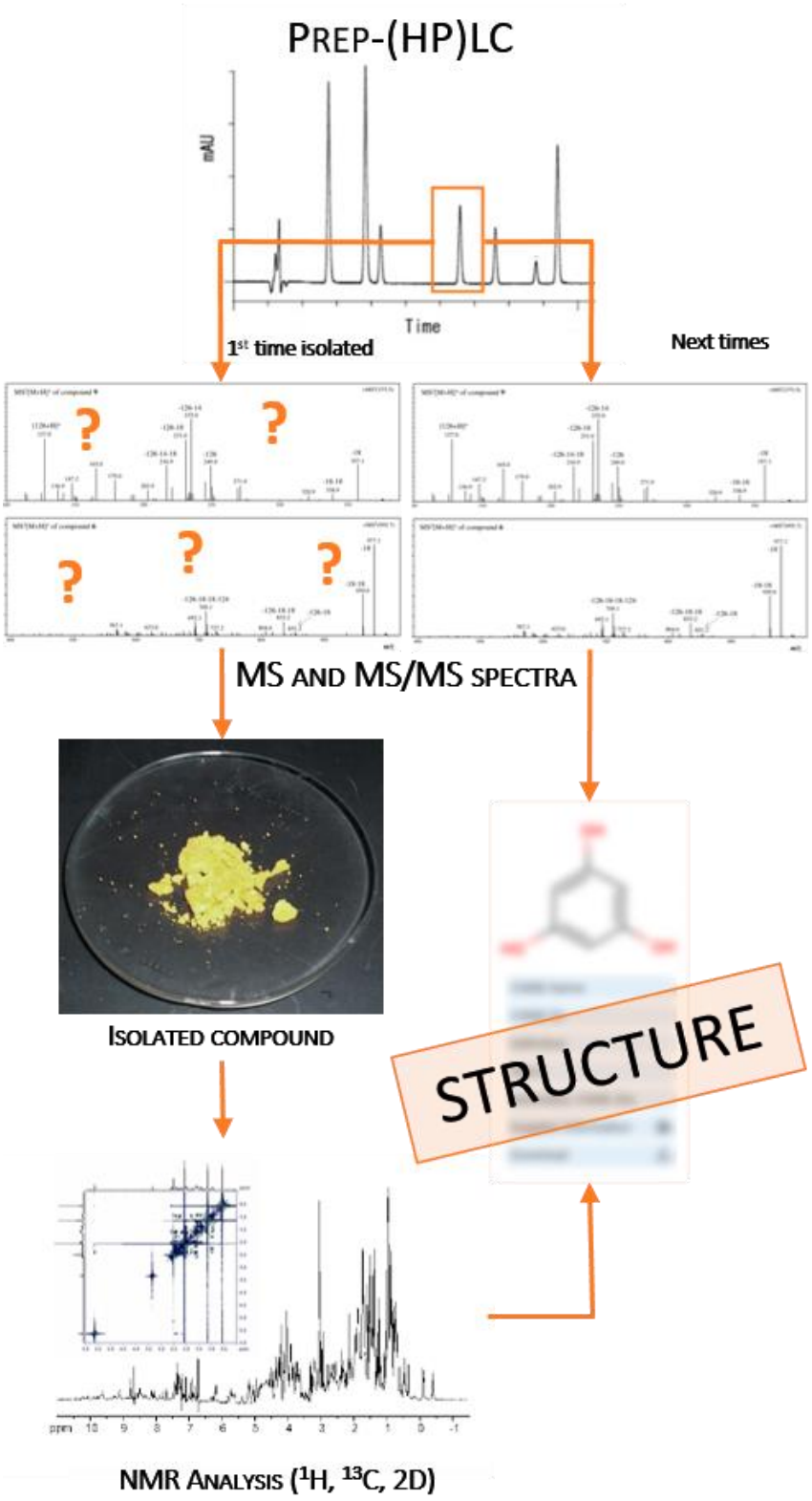


Figure 12 –Typical scenario in natural products identification from extracts of biomass. A chromatographic peak is detected and the corresponding fraction isolated, but direct identification of the structure of the compound from its mass spectra can only be achieved once a prior study using NMR spectroscopy has been conducted.

II.e.1. Column chromatography

In spite of the reduced capacity for handling complex samples in low quantities, limiting its applicability in fractionation for structural characterizations, preparative column chromatography (CC, also noted as LC by some authors) is still a very useful technique to remove impurities that would both difficult posterior analysis and potentially damage the HPLC equipment or to narrow the range of compounds to a sharper variation of a given physical-chemical trait (polarity, molecular weight, etc). In seaweed phenolic compounds analytical processing pipelines, CC has been used in normal phase (more often), reverse phase and size-exclusion separation modes. Table XI lists CC application to seaweed extracts.

Normal phase CC (NP-CC) has been the most used of all stationary phases (see table). Effectively, silica gel fillings for column packing are one the most widespread and accessible materials, justifying the routine application of often multiple NP-CC to the same extract, allowing successive adjustments of eluent and therefore of separative power. This type of stationary phase has been often employed to separate the ethyl acetate fraction after liquid liquid extraction [52,326,340–343], since this fraction might still contain metabolites soluble in ethyl acetate, that are likely to be more rapidly eluted than phlorotannins, which despite the bulky structure present many hydroxyl groups on their surface. Thus, with a very simple and inexpensive combination of methodologies, some extracts might be practically narrowed to the phlorotannin fraction. In other cases, NP-CC-originated fractions were further fractionated by RP-CC [52,340,344] or SEC-CC [326], providing bidimensional resolution of the components from the initial extracts, resulting in isolated compounds. Silica gel is thought to interact with phlorotannins and other phenolic components by hydrogen bonding between hydroxyl groups of both molecular structures [54]. Thus, it is logic that more hydroxylated compounds get retained longer, and that increasing polarity of the eluent results in the sequential elution of compounds. Depending on the range of polarities of the metabolites on the extract, more or less accentuated gradients of polarity in the eluent can be used – an overview of the mobile phases used in NP-CC is displayed at Table XI.

Table XI – Compilation of column chromatography systems reported in seaweed polar extractives fractionation.

Compounds	Stationary phase	Mobile phase[a]	Ref
Normal-Phase			
Phlorotannins	Silica gel	TCM:MetOH 9:1	[64]
		TCM to TCM:MetOH 4:1	[65]
		Hex to Hex:EtOAc 1:1 to DCM to DCM:MetOH 1:1	[323]
		DCM:MetOH 6:1-1:6	[342]
		TCM:MetOH 99:1 to MetOH	[311]
		TCM:Hex 1:1-4:1, TCM-AcetO 49:1-4:1, MetOH	[58,324,332, 345,346]
		EtOAc–MetOH 50:1-5:1	[52,321,340, 344]
		DCM, MetOH	[347]
		TCM:MetOH 1:1	[343]
		TCM:MetOH 100:1 to 1:1	[326]
		EtOAc:MetOH 50:1−5:1	[52]
		EtOAc, MetOH, Water	[315]
		TCM:MetOH:Water 80:18:2-50:49:1	[348]
		Hex:EtOAc 1:1	[52,340]
	Diatomaceous earth	Hex, DCM , EtOEt, MetOH	[322]
Miscellaneous	Silica gel	EtOAc:MetOH 1:0 to 0:1	[40]
Phenolic acids	Silica gel	Hex–TCM 1:1, TCM:MetOH 1:1	[37]
Flavones	Silica gel	TCM:MetOH 3:2	[349]
Aurones	Silica gel	Hex:TCM:MetOH 1:0:0-0:1:0-0:0:1	[43]
Reversed-Phase			
Phlorotannins	Octadecyl (unspecified)	MetOH (aq.) 10-100%	[350]
		MetOH (aq.) 20-100%	[52,321,340]
	Cosmosil 75C 18 -OPN	MetOH (aq.) 0-100%	[329]
	LiChroprep RP-18	MetOH (aq.) 20-100%	[344,351]
Size-Exclusion			
Phlorotannins	Sephadex LH-20	MetOH	[321,340,343 ,348,350–353]
		MetOH (aq.) 80%	[326]
		MetOH (aq.) 60-100%, AcetO (aq.) 70%	[354]
		TCM:MetOH 2:1–1:1–0:1	[341]
		EtOH (aq.) 50-80%, AcetO (aq.) 50-80%	[75]
		EtOH (aq.) 50-0%, AcetO (aq.) 0-70%	[70]
		MetOH:EtOAc (7:3)	[325]

[a]Chloroform – TCM; Dichloromethane – DCM; Methanol – MetOH; Ethanol – EtOH; Ethyl acetate – EtOAc; Diethyl ether – EtOEt; Hexane – Hex; Acetone – AcetO.

Reversed-Phase CC (RP-CC) is not as common as NP-CC. While silica gel is widely available, RP stationary phases are more specific products that are not so ubiquitous. Besides, RP bench applications have been popularized as SPE cartridges, and high resolution RP is nowadays a common configuration of HPLC equipments. Thus, the use of either RP-SPE or RP-HPLC combined with NP-CC provides two dimensions of separation that in many cases replace the need for column approaches. Nonetheless, some studies have reported the use of RP-CC of phlorotannins [52,321,329,340,341,344,350,351], mostly with octadecyl-derivatized stationary phases. This matrix is the most widespread RP phase, since it provides excellent resolution for a wide variety of organic compounds while using aqueous and low toxicity polar solvents for elution (instead of the hydrophobic ones associated to NP-CC, which are costy, toxic and polluting). Effectively, aqueous methanol has been the only mobile phase used for phlorotannins RP-CC (see table), using gradients of decreasing polarity (by increasing methanol percentage in water).

At last, size exclusion chromatography (SEC-CC) using Sephadex LH-20 has been repeatedly used in the fractionation of seaweed polar extracts [70,75,321,325,326,340,341,343,348,350–354]. The separation of compounds in Sephadex LH-20 is promoted by the metabolites' molecular size, but also by its solubility in the eluent. This gel-filtration media is a reticulated network of dextran derivatized with hydroxypropyl groups, such that both hydrophilic and lipophilic regions exist in the surface of the mesh. Along elution, an extra dimension of separation is introduced by the interactions of the analytes with the media, so typical gradient elutions allow an augmented separation. Effectively, SEC-CC has been used both in the begining of purification procedures, to separate polyphenolic compounds from simpler, low molecular weight polar metabolites, and in the final steps of fractionation, rendering HPLC-suitable fractions or even isolated compouds.

II.e.2. High-Performance Liquid Chromatography

HPLC is a chromatographic technique in which the stationary phase is composed of solid particles of such reduced dimensions that surface area and consequently retention time are greatly improved; silica, coated silica or polymers are examples of materials packed into a HPLC column, and the mobile phase can be any suitable liquid [355]. The increased compaction of the column creates a need to pressurize solvent pumping, which led to the High-Pressure alternative designation of the initials HPLC. The successful use of HPLC

involves the choice of an appropriate column, mobile phases and detector [355]. Apart from the different columns and mobile phases, photodiode array or simple UV detector have been widely used to detected and/or quantify phenolic compounds from macroalgae upon chromatographic separation (Table XII).

Normal-Phase HPLC (NP-HPLC) was more commonly used for phlorotannin isolation in the past. In fact, many of the studies of Glombitza and coleagues (see table XII, under Normal Phase), where a vast amount of phlorotannins were firstly isolated and described, achieved their purification using NP-HPLC [58,62–65,324,345,356,357]. Adding to these results, there are strong theoretical considerations supporting the use of NPs in phlorotannin separation, suggesting this type of column might be equally useful to isolate these molecules as the more modern C18-based RPs. The use of proper elution conditions, and the previous acetylation of the phlorotannins, might be key factors for their separation. Interestingly, the various reports of NP-HPLC describe a phenomena of elution according to degree of polymerization, suggesting that bigger molecules, for presenting more hydroxyl groups on their surface (or acetyloxy- groups, which also provide nuclei for hydrogen bonding), become retained longer in the silica-based columns [54]. In fact, elution with increasing concentration of ethanol in chloroform, albeit to low ethanol final percentages (2-3% v/v), was the primary choice of eluent regardless of the phlorotannin type. This is because interaction with silica, and sequential disruption of this interaction by ethanol, is not dependent on (or sufficiently sensitive to) the variations in polarity among a given degree of polymerization. Figure 13 is a chromatogram from the separation of a fuhalol-enriched fraction from the algae *Sargassum spinuligerum* [324]. This chromatogram has superscript roman numbers that translate de degree of polimerization, perfectly illustrating the elution from the smallest to the largest. In this paper, the authors also point to the fact that the more abundant phlorotannins are consistently of an even number of PGUs. Because the chloroform-ethanol system has been used, a given DP is represented by a relatively well-defined peak, although different isomers are being co-eluted.

Separation of a given DP fraction in the same column has been done by changing the eluent (re-injecting the collected fraction of the chloroform-ethanol system and eluting it with chloroform-hexane, decreasing concentration of the latter) [62,65]. This way, a more ‘fine tunable’, slow separation of the compounds exists, and the overall low polarity of the eluent during the entire chromatographic separation ensures the solvent is not disruptive for

the silica-phlorotannin interactions, increasing resolution of isomers. The brominated phlorotannins of *Cystophora congesta* [65] and the trihydroxyphlorethols of *Carpophyllum angustifollium* [62] were separated this way. Noteworthy, this resolution is particularly amazing since NP-HPLC of halogenated phlorotannins has been shown to be difficult due to very high retention, while in this case, isomers where only the position of the halogen differed could be resolved [65].

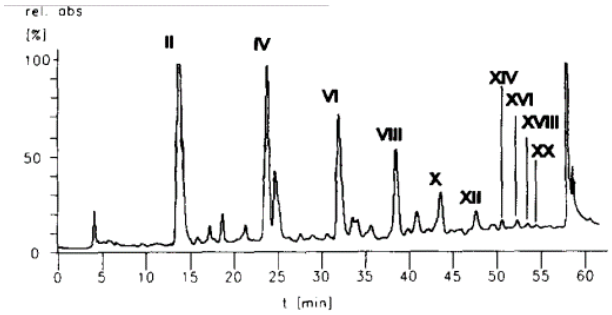


Figure 13 – NP-HPLC chromatogram of the separation of Fuhalols And Deshydroxyfuhalols from *Sargassum spinuligerum*. Roman numbers represent degree of polimerization of the compounds. From [324].

Reversed-phase (RP) HPLC columns have been the most used in macroalgae phenolic rich extracts [52,61,73,326,329,341,342,347,353,358–362]. These are non-polar stationary phases, while the mobile phase is polar, being the less polar compounds retained on the column longer than polar ones. Octadecyl (“C18” or “ODS”) has been the most used stationary phase, with a particle size of 5µm. Several commercially available C18 columns, with varying internal diameters and lenghts, have been shown to separate phlorotannins (see table). Elution of these molecules from reversed phases has been performed using either water, methanol or acetonitrile in different combinations. Water to methanol gradients have been used with success [326,360,362], which is an advantage since methanol is relatively easy to evaporate, and is much cheaper than acetonitrile. However, UV detection is made difficult when methanol is used. Nonetheless, acetonitrile might provide greater resolution at lower percentages, and might be adequate to use in columns of lower maximum pressures [355].

More interestingly, although elution with non-pH-adjusted solvents has been successfully achieved, the adition of trifluoroacetic acid [358,361], of formic acid [52,329,342,359] or of acetic acid [73] has been reported in accordance to general RP-HPLC standard practice.

Table XII – Preparative HPLC-UV conditions for the isolation of seaweed phenolic components (mostly phlorotannins).

Column (a/b/c)[a]	**Mobile phase[b]**	**Flow-rate (ml/min)**	**Detection (nm)**	***Species*** **Outcome [Ref]**
Normal Phase				
LiChrosorb Si-60 (250/16/7) (250/8/5)	A: TCM B: EtOH B:0.5-3.0%	-	275	*Sargassum Spinuligerum* Isol. of 20 fuhalols and deshydroxufuhalols [324]
	A: TCM B: EtOH B:0-2.5%	-	275	*Sargassum Spinuligerum* Sep. of pseudofuhalols [346] Isol. of 14 new (hydroxy)phlorethols and [(des)hydroxy]fuhalols [345]
				Sargassum Spinuligerum And Cystophora Torulosa. Isol. of 9 new fucophlorethols and 8 known ones from NP-CC fractions [58]
				Cystophora Retroflexa Isol. of 18 phlorethols and fucophlorethols (to isomer lever) [356] Isol. of 12 halogenated phlorethols and fucophlorethols (partially co-eluted) [63]
LiChrosorb Si-60 (250/16/7)	TCM-Hex (+ 0.3% MetOH) TCM-MetOH (higher MW)	-	-	*Cystophora Congesta* Isol. of 11 brominated and non-halogenated phlorotannins [65]
LiChrosorb Si-60 (250/8/5)	TCM-Hex TCM-MetOH (higher MW).	-	250-275	*Carpophyllum Angustifolium* Isol. of 3 trihidroxyphlorethols (2 isomers) [62] Sep. of NP-CC fractions into 4 trihidroxyphlorethols. [357]
LiChrosorb Si-60 (250/7.5/10) Partisil 10 (500/5/10)	A: TCM B: EtOH B:0.3-2.0%	-	275	*Eisenia Arborea.* Sep. of extract into 17 fractions, containing a total of 21 compounds (19 new eckols). [64]
Reverse-Phase				
µBondapak C18 (300/7.8/10)	36% ACN	1.0	245	*Ecklonia cava* Isolation of dieckol (Rt ~ 6 min) from NP-CC fraction [347]
Luna-C18 (-)	A: 0.1% formic acid (aq.) B: 0.1% formic acid in ACN 20 - 100% B	3.0	245	*Ecklonia stolonifera* Isolation of 2-phloroeckol, eckol, phlorofucofuroeckol B and bieckol from previous RP-HPLC fractions.[52]
Shim-pack PREP-ODS (250/20/5)	A: 0.1% formic acid (aq.) B: 0.1% formic acid in ACN 20 - 100% B	7.0	245	*Ecklonia stolonifera* Separation of SEC-HPLC fraction into 6 subfractions. SF1,SF4,SF5,SF6 further purified by RP-HPLC. [52,342]
Develosil ODS5 (-)	A: 0.1% TFA (aq.) B: 0.1% TFA in ACN 0 - 30% B	-	-	*Eisenia arborea* Isolation of active fraction at TR 130-150min; Repetition with 47% methanol 0.1% TFA for isolation of phlorofucofuroeckol B at tR=41min [358]
C18 (150/4.5/5)	18% ACN (aq)	0.7	-	*Ecklonia maxima*

	(+ 0.1% formic acid)			Isolation of eckol, 7-phloroecckol and 2-phloroeckol [359]
J'sphere ODS-H80 (150/20/4)	MetOH (aq., increasing % V/V)	0.8	230	*Ecklonia cava* Purification of dieckol with purity>95% from SEC-CC fraction [326] [341]
Alltima C18 column (250/10/5)	MetOH 30 - 100%	4.0	290 nm	*Ecklonia cava* Purification of dieckol and phlorofucofuroeckol A from SEC-CC fraction. [353]
Mightysil RP-18 GP II (250/10/5)	MetOH 40% (1.0% formic acid aqueous)	0.5	DAD 190-500 (rec=204 -208)	*Ecklonia kurome* Separation of dieckol, 974-B, 974-A, phlorofucofuroeckol B and phlorofucofuroeckol A from RP-CC fraction. [329]
LiChrosorb RP-18 (250/8/5)	0.015 M K_2SO_4	-	235 nm.	*Pleurophycus gardneri* Isolation of sulphated phlorotanin from PVP adsorbate of crude extract. [61]
Phenomenex aqua C18 (250/10/5)	(A) 1% acetic acid (B) 1% acetic acid in ACN B: 1-100%	2.5	-	*Fucus vesiculosus* 10 fractions. Repetition with isocratic A:B 90:10 isolated six subfractions, from which 3 fucophlorethols were isolated. [73]
C18 (250/10/-)	30 to 100 % MetOH	1.0	290	*Ecklonia cava* Isolation of 7-phloroeckol and eckol from ethyl ether fraction of crude extract. [360] Isolation of dieckol, phlorotannin 974B, phlorotannin 974A, and phlorofucofuroeckol-A from ethyl ether fraction of crude extract. [362]
C18 (50/2.1/1.8)	A: 0.5% TFA (aq.) B: 0.5% TFA in ACN B: +4%/min	1.0	280	*Sargassum wightii, S. tenerrimum and Turbinaria conoides* Isolation of a phlorotannins-enriched fraction from crude extract. [361]
Size-Exclusion				
Asahipak GS-310 (500/20/13)	MetOH	5.0	245	*Ecklonia stolonifera* Separation of NP-CC fraction in 5 subfractions; SF4 further purified by RP-HPLC. [52,342]
Superose 6 colum (300/10/13)	100 mM sodium acetate buffer (pH 6.3) 20 mM EDTA, 250 mM NaCl	0.25	210	*Padina. gymnospora* Recovery of 2-[1′-Oxo-hexadecyl]-1,3,5-trihydroxybenzene (a phloroglucinol derivative) from RP-CC fraction. [335]

[a] letters a, b and c are for lenght, in millimeters, internal diameter, in millimeters, and particle size, in micrometers.

[b]- Chloroform – TCM; Methanol – MetOH; Ethanol – EtOH; Hexane – Hex; Acetonitrile – ACN; Trifluoroacetic acid - TFA

No study was found to compare the performance of RP-HPLC with and without acid for the same conditions. When acid is not used for the sole purpose of column maintenance, its effect on retention times should be adressed and optimized.

Finally, Size Exclusion Chromatography (SEC) columns suitable for HPLC conditions have been used in the preparative separation of different molecular weight fractions from *Ecklonia stolonifera* extract [52,342], as well as to confirm the molecular size of an isolated metabolite from *Padina gymnospora* using two standards [335]. Isocratic elution was performed in both cases, using either 100% methanol or 100 mM sodium acetate buffer (pH 6.3, 20 mM EDTA, 250 mM NaCl), since fractionation was to occur only by molecular size, and no chemical interactions were to be differentially explored.

II.e.3. Other preparative chromatographies

Besides the more common column approaches to liquid chromatography, other chromatographies have been used, with different specificities, in the analysis of phenolic components from seaweed. For instance, thin-layer chromatography (TLC) was widely used as a complementary technique to other chromatographies, either as a method to track the evolution of the extracts' composition or as a preparative separation of a given class of compounds by scraping the silica after elution and recovering the compounds by CC. TLC is a very common method in organic chemistry, and even in the articles that reported using it, no detailed description of the conditions was performed – only the ubiquitous use of Silica gel plates. Revelation of the chromatograms and elution are standard procedures that can be easily found in the literature (see [363,364]), and are therefore not covered in this section.

One study accomplished the separation of phlorotannins by Centrifugal-Partition Chromatography (CPC) [53]. This type of chromatography uses liquid stationary and mobile phases, the latter maintained in the "column" – a rotor – by centrifugal force from rotation. As the mobile phase – the sample, immiscible with the stationary phase – is injected through an online series of rotors, each passage of mobile phase will carry the compounds with less affinity for the stationary phase, and sequentially lose those with higher affinities in the first rotors. By using a two-phase system of n-hexane:EtOAc:methanol:water (2:7:3:7, v/v), bioactive phlorotannins of *Ecklonia cava* have been isolated (purity > 90%), which represents an improvement in the operational ease with which purified fractions of the phenolic extracts of seaweed can be obtained.

II.f. High-performance liquid chromatography-tandem-mass spectrometry

HPLC and its higher-performance variants (Ultra-high performance liquid chromatography - UHPLC) are the most powerful tools for analytical chemists and biochemists to separate complex mixtures of biomolecules and/or secondary metabolites rapidly, efficiently and with a low amount of sample. The use of HPLC for fraction obtainment has been described in section II.e.2. Analytical U/HPLC is very similar to those previously reported, especially regarding the stationary and mobile phases. The major operacional difference that can be found is the column, in terms of dimensions, i.e., length, diameter and internal particle size. In the case of analytical-scale HPLC, the low amount of sample needed to reach the detector allows very small volumes of extract to be analyzed, allowing studies of chemical composition even when biomass availability is limited. Liquid chromatography is often coupled to UV-Vis detectors - mostly photodiode-array (PDA) - as this type of detection is instrumentally simple, widely applicable to a large set of molecules and can be used for quantification (from the beer-lambert's law, given linearity). However, UV-Vis detectors fail to provide unambiguous identifications for each peak, as the different extractives being separated are often similar, and their response to radiation in this range of wavelenghts indistinguishable. Alternatively, the use of mass spectrometers directly coupled to U/HPLC instruments has revolutionized the capacity to analyze complex extracts in a relatively short period of time, with a low amount of compounds. Nonetheless, it is important to note that UV-Vis detectors are often present, even if MS is present as well, since it helps in the interpretation of the chromatograms and in the real-time tracking of the elution.

U/HPLC-coupled MS instruments are more commonly of the electrospray ionization (ESI) type. This is understandable since this ionization method is adapted to receive a liquid stream of sample and continuously deliver it ionized onto the analyzer. Interesting considerations can be drawn by analyzing the LC-MS configurations used for SPCs characterization (summarized in Table XIII). First, the already identified trend in seaweed phenolics research of an augmented attention towards phlorotannins is maintained. However, some other phenolic components have been successfully separated and identified using HPLC: the 60% methanol extract of *Himanthalia elongata*, for instance, has been screened for antioxidant activity and the most active sub-fraction after CC eluted with ethyl acetate was subjected to RP-HPLC [40]. A triple quadrupole was used to perform percursor ion fragmentation (usually, the pseudomolecular ion, $[M-H]^-$) and neutral losses scan

allowed the identification of 8 phenolic components of distinct classes (hydroxybenzoic acid derivatives, flavonols, flavones, phenolic terpenes and others). Apigenin and gallic acid have also been separated and identified from red seaweed extracts (*Gracillaria birdiae* and *G. cornea*) by a linear methanol-to-aqueous formic acid 0.1% (w/v) gradient in a C18 stationary phase [365].

Table XIII - HPLC-MS conditions for the on-line analysis of SPCs during chromatographic separation.

Species [Ref]	Outcome	Configuration	MS mode	HPLC Column	Mobile phase[a]
Fucus vesiculosus [54]	Detection of phlorotanins.	NP-HPLC-ESI-QQQ	(-) FULL SCAN	LiChrospher Si 60 250 × 4 mm, 5 µm LiChrospher Si 60 guard cartridge 4 × 4 mm, 5 µm	A: 2% water, 2% AcAc, 14% MetOH (in DCM) B: 2% water, 2% AcAc (in MetOH) %B: 0-87.8(50min)-0(80min) 1.0 mL/min
Sargassum muticum, Sargassum vulgare, Hypnea spinella, Porphyra sp., Undaria pinnatifida, Chondrus crispus, Halopytis incurvus [41]	Sep. and ident. of isoflavones	NP-HPLC-ESI-QQQ	(-) MRM	Zorbax SB-CN 100 × 2.1 mm, 3.5 µm	A: 0.2% AcAc (aq) B: ACN %B: 30-80(6min)-30(10min) 0.4 mL/min
Fucus distichus, Saccharina latissima, Saccharina groenlandica, Alaria marginata, Porphyra fallax, Ulva lactuca, [366]	Sep. and ident. of phlorotannins (DP-level)	SEC-HPLC-ESI-IT-TOF	(+) / (-) FULL SCAN	Develosil Diol 250 × 4.6 mm, 5 µm	A: 0.2% AcAc (in ACN) B: 0.2% AcAc, 3% water (in MetOH) %B: 0-100(40min)-0(50min) 0.8 mL/min
Ecklonia stolonifera [367]	Detection of phlorotanins.	RP-HPLC-ESI-IT	(+) FULL SCAN	Hypersil Gold C-18 250 × 4.6 mm, 5µm Gemini C-18 guard column 30 × 4.6 mm, 5µm	A: 0.1% FA (aq) B: 0.1% FA (in ACN) %B: 13-60(48min)-13(60min) 1.0 mL/min
Ascophyllum nodosum, Laminaria digitata [334]	Detection of phlorotannins	RP-HPLC-ESI-IT	(-) FULL SCAN	Nova-Pak C18 -	A: Formate:methanol 95:5 v/v B: Methanol %B: 0-100(30min) 1.0 mL/min
Porphyra tenera, Undaria pinnatifida [302]	Sep., ident. and quant. of misc. phenolics	RP-HPLC-ESI-Q	(-) SIM	Zorbax SB-C18 150 × 4.6 mm, 3.5 µm	A: 0.2% AcAc in water B: ACN %B: 4-30(10min)-4(15min), 1.1 mL/min
Macrocystis pyrifera [71]	Sep. and tentative ident. of phlorotannins	RP-HPLC-ESI-IT/n	(+) / (-) FULL SCAN	LunaC18 150 × 4.6 mm, 5 µm	A: 1% v/v formic acid (aq) B: ACN %B: 5-100(90min) 1.0 mL/min

Himanthalia elongata [40]	Sep. and ident. of misc. phenolics	RP-HPLC-ESI-QQQ	(-) FULL SCAN	Atlantis C-18 250 × 4.6 mm, 5 µm C-18 guard cartridge 4.0 × 3.0 mm, -	A: 0.25% aqueous AcAc B: ACN/water (80/20) 0.25% AcAc. %B: 10(20min)-20(30min) 1.0 mL/min
Ecklonia stolonifera [368]	Sep. and tentative ident. of phlorotannins	RP-HPLC-ESI-IT/n	(+) FULL SCAN	Capcell Pak C18 UG120 2.0 × 150 mm, -	A: 1% FA, 5% MetOH (aq) B: 1% FA, 70% MetOH (aq) %B: 0-100(60min) 0.2 mL/min
Ecklonia bicyclis, Ecklonia kurome, Ecklonia arborea, Ecklonia cava [369]	Sep. and ident. of phlorotannins (DP-level)	RP-HPLC-ESI-Q	(-) FULL SCAN	Inertsil ODS-3 250 × 4.6 mm, 4 µm	A: water B: MetOH %B:20-100(40min) -
Gracilaria birdiae, Gracilaria cornea [365]	Detection and tentative ident. of misc. phenolics	RP-HPLC-ESI-?	(+) / (-) MRM	Supelcosil LC-18 250 x 5 mm, -	A: MetOH B: 0.1% formic acid (aq) %B: 50 (isocratic) 0.4 mL/min
Ascophyllum nodosum [70]	Sep. and ident. of phlorotannins (DP-level)	RP-HPLC-ESI-IT/n	(+) / (-) FULL SCAN	Synergi Hydro C18 w/polar end-capping 2.0 × 150 mm, -	A: 5% ACN (0.1% formic acid) B: 40% ACN (0.1% formic acid) %B: 0-100(30min) 0.2 mL/min
Porphyra dentata [370]	Sep. and ident. of phlorotannins	RP-HPLC-ESI-IT	(+) FULL SCAN	Luna C18 150 × 2 mm, 3 µm	A: 0.05% TFA in ACN B: 0.05% TFA (aq.) %B: 15-100(50min) 0.2 mL/min
Cystoseira nodicaulis, Cystoseira tamariscifolia, Cystoseira usneoides, Fucus spiralis [56]	Sep. and ident. of phlorotannins	RP-HPLC-ESI-IT/n	(+) / (-) FULL SCAN	Luna C18 250 × 4.6 mm, 5 µm	A: 1% formic acid in water B: ACN %B: 0-80(40)-0(52) 1.0 mL/min
Ascophyllum nodosum [75]	Sep. and ident. of phlorotannins (DP-level)	RP-HPLC-ESI-IT/n	(+) / (-) FULL SCAN	Synergi Hydro C18 with polar end capping 150 x 2 mm, -	A: Water B: ACN %B: 5-100(30min) 0.2 mL/min
Ascophyllum nodosum [371]	Sep. and tentative ident. of phlorotannins	RP-HPLC-ESI-IT/n	(-) FULL SCAN	Zorbax SB C18 100 x 2.1 mm, 1.8 µm	A: 0.1% FA (aq) B: 0.1% FA (in ACN) %B: 10-70(50)-10(65) 2.0 mL/min.

Sargassum muticum [86]	Detection of phlorotanins.	RP-HPLC-ESI-IT/n	(+) / (-) FULL SCAN	Zorbax Eclipse XDB-C18 150 x 4.6 mm, 5 µm	(A) 0.1% FA (aq) (B) ACN %B: 2-20(35min) 0.2 mL/min
Palmaria spp., Porphyra spp., Himanthalia elongata, Laminaria ochroleuca, Undaria pinnatifida [372]	Sep. and detection of phlorotannins	RP-HPLC-ESI-TOF	(-) FULL SCAN	Mediterranean Sea18 150 × 4 mm, 3 µm	A: 1% AcAc (aq) B: 1% AcAc, 32% ACN (aq) %B: 0-100(25min) 0.5 mL/min.
Ulva intestinales, Porphyra umbilicalis, Palmaria palmata, Fucus sechees, Himanthalia elongata, Laminaria japonica, Saccorhiza polyschides, Ascophyllum nodosum, Fucus vesiculosus, Laminaria digitata, Undaria pinnatifida [373]	Sep. and detection of phlorotannins	RP-HPLC-ESI-QqTOF	(-) FULL SCAN	Luna C18 150 × 4.6 mm, 3 µm	A) 0.1% FA, 5% ACN (aq) (B) 0.1% FA, 5% water (in ACN) %B: 40-100(65min)-40(67min) 0.5 mL/min
Fucus vesiculosus [337]	Sep. and ident. of phlorotannins (DP-level)	RP-UPLC-ESI-QQQ	(-) MRM	HSS PFP 100 x 2.1 mm, 1.8 µm	A: 0.1% FA (aq) B: 0.1% FA in ACN %B: 0.5-90(28min)-0.5(30min) -
Ascophyllum nodosum, Pelvetia canaliculata, Fucus spiralis [336]	Sep. and ident. of phlorotannins (DP-level)	RP-UPLC-ESI-QQQ	(-) MRM	HSS PFP 100 x 2.1 mm, 1.8 µm	A: 0.1% FA (aq) B: 0.1% FA in ACN %B: 0.5-90(28min)-0.5(30min) 0.5 mL/min
Ecklonia cava [322]	Sep. and detection of phlorotannins	RP-UPLC-ESI-?	(+) FULL SCAN	Bridged Ethyl Hybrid (BEH) C18 100 x 2.1 mm, 1.7 µm	A: 0.1% FA (aq) B: 0.1% FA in ACN %B: 10–45(5min) 0.4 mL/min

Fucus serratus, Fucus vesiculosus, Himanthalia elongata, Cystoseira nodicaulis [49]	Sep. and ident. of phlorotannins (DP-level)	RP-UPLC-ESI-QQQ	(-) MRM	HSS PFP 100 x 2.1 mm, 1.8 µm	A: 0.1% FA (aq) B: 0.1% FA in ACN %B: 0.5-90(28min)-0.5(30min) 0.5 mL/min
Sargassum pallidum [374]	Sep. and ident. of phlorotannins (DP-level)	RP-UPLC-ESI-QQQ	(-) FULL SCAN	Bridged Ethyl Hybrid (BEH) C18 100 x 2.1 mm, 1.7 µm	A: methanol B: water %B: 30-100(10 min)-30(20min) 0.3 mL/min
Pelvetia canaliculata, Fucus spiralis, Fucus vesiculosus, Ascophyllum nodosum, Saccharina longicruris [55]	Sep. and ident. of phlorotannins (DP-level)	HILIC-UPLC-ESI-Orbitrap	(-) FULL SCAN	UPLC W BEH Amide 100 x 2.1 mm, 1.7µm	A: 10.0mM ammonium acetate pH 9.0 B: ACN. %B: 95-65(16min)-95(21min) 0.4 mL/min
Cystoseira abies-marina [375]	Sep. [35 spots] and ident. of phlorotannins (DP-level)	HILICxRP-2D-HPLC-ESI-IT	(-) FULL SCAN	D1 precolumn Lichrospher diol-5 Lichrospher diol-5 150 × 1.0 mm, 5 µm D2 C18 precolumn Partially porous C18 50 × 4.6 mm, 2.7 µm	D1 A: ACN/AcAc (98:2, v/v) B: methanol/water/AcAc (95:3:2, v/v/v) %B: 3-35(70min) 15 µL/min D2 A: 0.1% FA (aq) B: ACN %B: 0-90(1min)-0(1.01min)-repeat 3 mL min-1
Sargassum muticum [95]	Sep. [73+ spots] and tentative ident. of phlorotannins (Class-level)	HILICxRP-2D-HPLC-ESI-IT	(-) FULL SCAN	D1 Lichrospher diol-5 150 × 1.0 mm, 5 µm D2 C18 precolumn Ascentis Express C18 50 × 4.6 mm, 2.7 µm	D1 A: 2% AcAc (in ACN) B: 2% AcAc, 3% water (in MetOH) %B: 0-25(85min) 15 µL/min D2 A: 0.1% FA (aq) B: ACN %B: 0-90(0.9min)-0(1.3min) 3 mL/min

Cystoseira abies-marina [57]	Sep. [52+ spots] and ident. of phlorotannins (DP-level)	HILICxRP-2D-HPLC-ESI-IT	(-) FULL SCAN	D1 precolumn Lichrospher diol-5 Lichrospher diol-5 150 × 1.0 mm, 5 µm D2 precolumn C18 Ascentis Express C18 50 ×4.6 mm, 2.7 µm OR Kinetex pentafluorophenyl (PFP) 50 × 4.6 mm, 2.6 µm	D1 A: 2% AcAc (in ACN) B: 2% AcAc, 3% water (in MetOH) %B: 0-25(85min) 15 µL/min D2 A: 0.1% FA (aq) B: ACN %B: 0-90(0.9min)-0(1.3min) 3 mL/min

[a] DCM – Dichloromethane; MetOH – Methanol; CAN: Acetonitrile; AcAc – Acetic Acid; FA: Formic Acid;

Onofrejová et al. [302] developed and validated a RP-HPLC-ESI-Q method to separate phenolic acids and aldehydes. Using the spectrometer at single ion monitoring mode (SIM), it was possible to obtain Limit of Detection (LOD) and Limit of Quantification (LOQ) values at sub-nanogram scales. NP-HPLC of several seaweed species was carried out using a Zorbax SB-CN column (amide-derivatized stationary phase) [41]. By applying fragmentation voltages selected for individual compounds, along with Multiple Reaction Monitoring (MRM) data acquisition mode, 8 isoflavones (daidzin, genistin, ononin, daidzein, sissotrin, genistein, formononetin and biochanin A) were detected for the first time in seaweed, even at the fentogram per injection scale.

RP-HPLC has been the most frequently employed method for phlorotannin separation prior to electrospray ionization (see table XIII). The reverse-phases became popular for increased resolution of many organic compounds, but also for the shift in mobile phase composition, from non-polar to polar solvents – often water or aqueous mixtures. [375] The most common RP stationary phase is C18/ODS, which is composed of packed silica-bonded octadecyl chains. These chains provide an hydrophobic moiety that retains the more hydrophobic compounds. Most of the RP-U/HPLC applications in table XIII have been performed under this type of stationary phase, as well as most non-MS-coupled preparative HPLC of phlorotannins. Another common matrix used in RP chromatography of phlorotannins is pentafluorophenyl (PFP). This phase has been hypothesized to provide a basis for efficient separation of phlorotannins since its mechanism of retention is highly propense to interaction with these polyphenols: five atoms of fluor attached to a phenyl ring originate an electron-deprived aromatic moiety which is likely to interact with the phenolic moieties, and differentially retain structural isomers [57]. In fact, Macherey-Nagel GmbH & Co. KG has publicized their PFP bonded phases as an alternative to C18 for the separation of phenolic isomers – achieving perfect resolution of meta- and orto-cresol, and of the various isomers of dimethylated- or dihalogenated- phenols [376]. In fact, the studies that used PFP-bonded phases achieved consistently good resolution of phlorotannin peaks [49,57,336,337]. Montero et al. [57] actually compared C18 and PFP separation under the same experimental conditions. While the profiles obtained were both of good resolution, and qualitative differences were obtained – showing the techniques to be complementary rather than alternative -, C18 still performed better than PFP (see Figure 14).

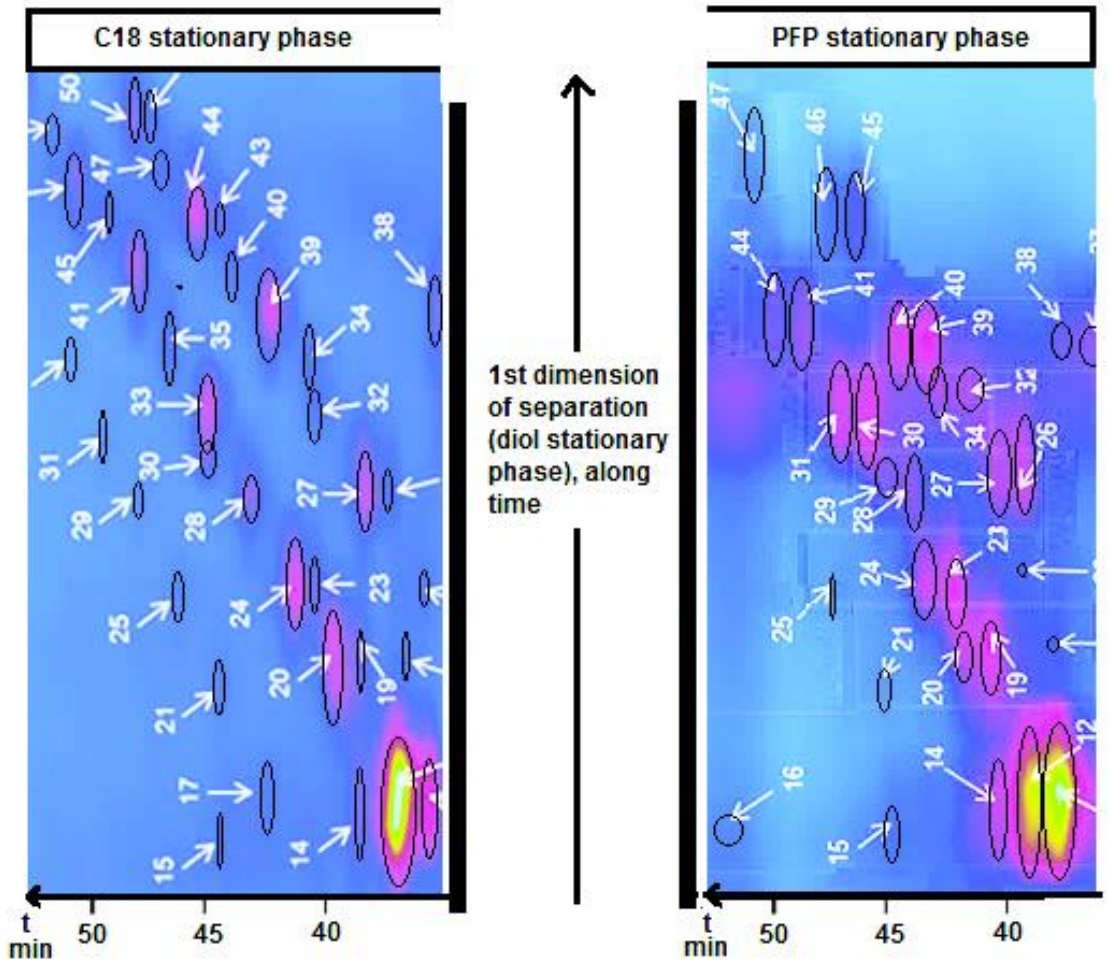


Figure 14 - Bidimensional chromatograms (parts of) of the separation of a phlorotannins extract from *Cystoseira abies-marina*. The C18 phase provided clearer spots than the PFP, but both allowed isolation of more than 50 spots. Adapted from [57].

Despite the vast majority of studies employing RP, some authors claim NP could perform separation of phlorotannins, because the abundant hydroxyl groups could provide a basis for differential elution [41,54]. Once a chromatographic setup has been optimized, NP and RP should in theory perform similarly (with inversed elution order of compounds); however, the poor diversity of stationary phases and other operational conditions for NP-HPLC – for instance, the need for less polar, organic solvents – make it unatractive and inadequate for ESI ionization. Also, past experience with NP separations of phlorotannins have shown that medium and high DP phlorotannins could not be resolved properly (see section II.e). For that reason, even though NP-HPLC could in theory be used, it was only

reported once in the MS analysis of phlorotannins, and effectively, the LMW fraction of *Fucus vesiculosus* phlorotannins was well resolved [54].

Hidrophilic interaction (HILIC) stationary phases have been used, solely or prior to C18 separation in a two-dimensional apparattus [55,57,95,375]. Similar to NP, HILIC columns interact with compounds of high polarity, but the elution systems used are of mixed polarity, such that gradients lead to internal partition of the solvent in the column, and of the compounds in the solvent phases [55]. Nonetheless, HILIC as well as NP are uneffective to separate high-MW components, probably due to their high retention [55]. Yet, HILIC-UPLC (using an amide-functionalized stationary colums) has managed to separate phlorotannins with DP up to 49 [55]. Also, HILIC-RP-2D-HPLC has been developed to characterize phlorotannins from *Cystoseira abies-marina* [57,375] and of *Sargassum muticum* [95]. The resolution in the 2D separation was largely improved compared to HILIC or RP alone (up to 73 compounds separated in one run [95]). Negative mode ESI followed by an ion trap allowed the identification of the majority, and in the case of *S. muticum*, the subclass of phlorotannins was tentatively assigned [95]. With an upgrade in mass spectrometrical determination of phlorotannins, 2D-HPLC configurations might be very useful to distinguish isomers (several resolved peaks with the same DP were found in both species [57,95,375]). Finally, size-exclusion HPLC (SEC-HPLC) was carried out with a Develosil Diol 250mm column on brown seaweed extracts, followed by ion trap-time-of-flight hybrid MS, allowing the separation and identification by degree of polimerization of several phlorotannins (Figure 15) [366].

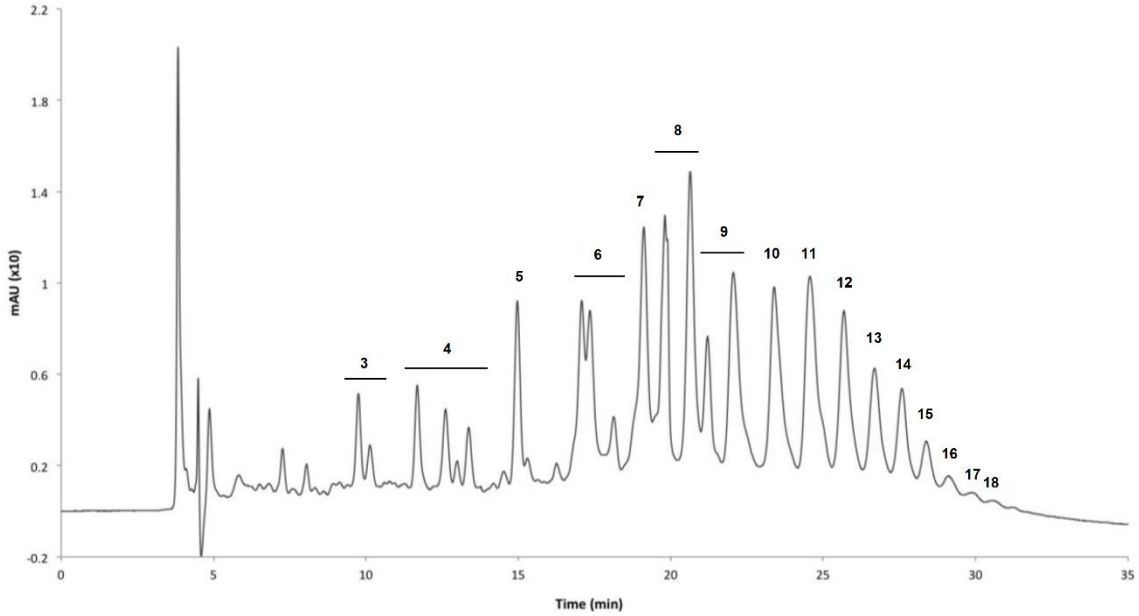


Figure 15 – Chromatogram of *Fucus distichus* phlorotannins separated by degree of polimerization (numbers above the peaks) in a Develosil Diol column, using acetonitrile and 97:3 methanol:water. Detection at 254nm. Adapted from [366].

II.f.1. Phlorotannins Mass Spectrometry

Phlorotannins are formed by largely uncharacterized biosynthetic pathways, for which no restriction regarding bonding sites and macromolecular structure are known. Thus, for compounds with more than two phloroglucinol units (PGUs), several possible structures arise, and their number increases drastically with the degree of polymerization (DP). Several phlorotannins have been described for a given DP, and their abundance in seaweed, ecophysiological roles and bioactivities have been shown to differ. Phlorotannins need to be identified to isomer-level, and that is largely achieved by NMR spectroscopy techniques. Unfortunately, NMR requires pure samples, at the miligram scale to be performed. An illustration of the ambiguity of most phlorotannin designators is present at Figure 16.

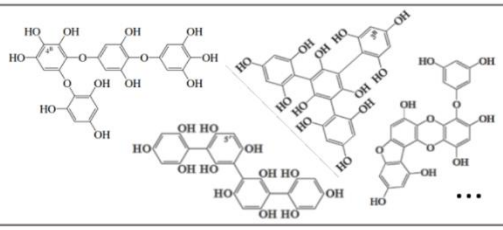
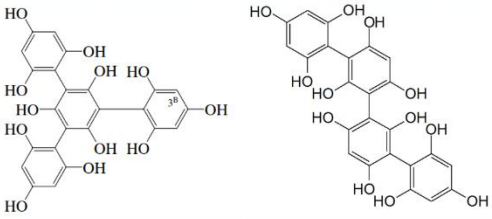
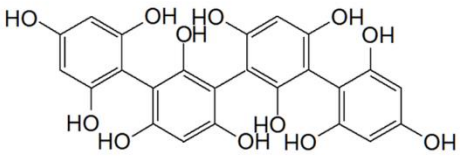
Level	Structural information	Example	Possible structures
I	Degree of polymerization (DP)	**Phloroglucinol-tetramer** DP=4	
II	DP and Bonding Scheme	**Tetralfucol** DP=4 All C-C bonded	
III	Unique structure	**Tetrafucol A** DP=4 All C-C bonded Linear chain of PGUs	

Figure 16 - Schematic representation of the three levels of structural detail in phlorotannin identification.

Mass spectrometry (coupled to HPLC) is an alternative method to reliably identify compounds in complex mixtures. As already mentioned, a major goal of this thesis was to develop a framework of interpretation of phlorotannins MS, mostly from ESI sources. Along this thesis, it has become rather evident that such a step would facilitate a plethora of research and valorization steps to be accomplished. For that, all the relevant papers on phlorotannins were collected by performing an exhaustive bibliographic search routine[1]. Ionization on the ESI chamber is dependent of several factors, among which the pH of the media and the voltage applied. Depending on these, positive or negative ions will be predominantly formed and selected to enter the analyzer. Interestingly, while more is known about positive ionization (i.e., protonation, easily aided by weak acids), negative ionization (loss of acidic

[1] All papers resulting from the search query (Seaweed* OR Macroalg* OR "Brown alg*") AND (Phlorotannin* OR Phloroglucinol OR Phenol* OR Polyphenol* OR Polyphloroglucin*) AND (MS OR NMR OR "mass spec*" OR "nuclear magnetic resonance") in Web of Science (31/07/2017) were retrieved and analyzed, and results were intercalated with the last version of the Dictionary of Marine Natural Products [398].

protons and negative charge build-up), which is actually more easy to obtain in many different types of molecules, remains less mastered [377]. For instance, formic and acetic acids have often been used in phlorotannin elution and negative mode ionization [40,54,302,371–373], although acids have been known to promote positive mode ionization (through protonation); even if the intention is to improve chromatographic separation, its presence does not supress the deprotonation at ESI source. Thus, experimental optimization is the safest way to achieve successful ionization.

Phlorotannins contain numerous hydroxyl groups, which are somewhat acidic. To obtain quasimolecular ions, phlorotannin eluates are often ionized in negative mode ($[M-H]^-$). Polyphenol anions have been reported to be more stable than their counterpart cations, which undergo fragmentation rather easily [378]. However, several articles on phlorotannin positive ionization by ESI have been published [322,367,368,370], as well as on both modes simultaneously [56,70,71,75,86,365,366]. Ideally, both should be performed, since factors other than the target compounds can have an influence on ionization performance (traces of salts, for instance). MS fragmentations can occur in both modes: quasimolecular ions of phlorotannins will undergo neutral fragment losses.

Different modes of spectra acquisition have been reported. Typical mass spectrometry involves the detection of the molecular ion and of its products; by selecting a percurson ion, tandem mass spectrometry (MS^n) can reveal further details of that ion's own structure. This type of mass spectra acquisition is often used with full scan of the range of *m/z*, and the limitation is usually the upper limit of the instrument. Thus, when full scan mode is used, one might consider the voltage manipulation of the spray, the capillar and the orifice-skimmer-lens path in such way to promote the desired level of fragmentation and/or multiple charge accumulation. For instance, phlorotannins of increasing DP have been detected due to the occurrence of di- and trivalent ionization, that cuts *m/z* values by a factor of 2 or 3, [49,54,55,57,95,374,375]. However, other acquisition approaches have been useful for certain purposes. Besides SIM (referred above), multiple reaction monitoring (MRM) is a method of mass analysis that allows the researcher to choose a pair of parent-product ion known to be specific of a given compound. When multiple compounds co-elute (for example, in the case of same-DP isomers of phlorotannins), this strategy allows the quantification of each of them separately, should a specific fragment be known for the target analyte. MRM studies of phlorotannins can be found in table XIII [41,49,336,337,365].

However, major difficulties have been identified in the application of MS to phlorotannins, since their isomerization by multiple combinations of PGUs relative position has little impact on mass spectra. Thus, while MS can easily provide a profile of DPs in a phlorotannins mixture, it often fails to attribute a chemical structure to the detected molecules. Besides, MS of isolated phlorotannins has been performed almost exclusively on their peracetylated derivatives [59,62–65,73,324,345,356,357,379,380], since high-energy ionization techniques were the only available at the time of their structural description, and such ionization was impracticable in the native compounds due to degradation. Thus, most MS data supported by NMR-confirmed identification is of Electron Impact (EI) or Fast-Atom Bombardment (FAB) of acetylated phlorotannins, and have little value for the development of phlorotannins HPLC-MS.

Evidently, for a framework to be developed regarding mass spectra patterns, diagnostic fragments and standard methodologies, literature needs to be sufficient for secure relations to be established. Table XIV shows the data retrieved on the monomers and dimmers of phloroglucinol, including that of halogenated derivatives. Noticeably, despite the attractiveness of these molecules and the abundant references to them in biological extracts studies of seaweed, a scarcity of spectral information has been reported with sufficient detail and background validation to allow a definitive theory to be developed. For instance, most mass spectra in the table correspond to those obtained during the first (!) description of the compound [62–65,381–385]. This is a problem for several reasons: MS had been performed, in such cases, with the intention to confirm molecular mass more than structure (which was invariably determined by NMR); most data was obtained by EI and FAB, which reduces the ability to infer ESI-MS useful information; most data was obtained for the peracetyl-derivatives of the compounds, which might influence the mass spectra drastically; in most cases, relative abundances of the fragment ions were not reported; for most compounds, only one report could be found, which is not enough to assure reproducibility and to assess variability associated to operational specificities. Mass spectrometry data of higher molecular weight phlorotannins has been found to be equally impaired [324,356,357].

At this point, it might be of more interest to analyze and discuss the reasons impeding the successful application of mass spectrometry to phlorotannins (resulting in the lack of data compared to what could be expected). Partly, the topics covered along this thesis (the

different processing steps), as a whole, contribute to the lack of phlorotannin-specific information: most extracts of seaweed are studied as a complex mixture, using spectrophotometric approaches and *in vitro* bioactivity assays, due to the lack of consensual suggestions regarding extraction and separation of seaweed phenolic compounds. However, a specific part of the processing pipeline might be to blame for the difficulty in advancing phlorotannin MS to the point of becoming mainstream practice in seaweed research – the co-extraction of carbohydrates. Mass spectrometry by ESI is an extremely sensitive technique, which implicates that complex mixtures lose resolution by the overlap of signals from different molecules co-eluting. Thus, studying a compound class to the detail of relative abundance of fragments demands an extract enriched in the target analyte.

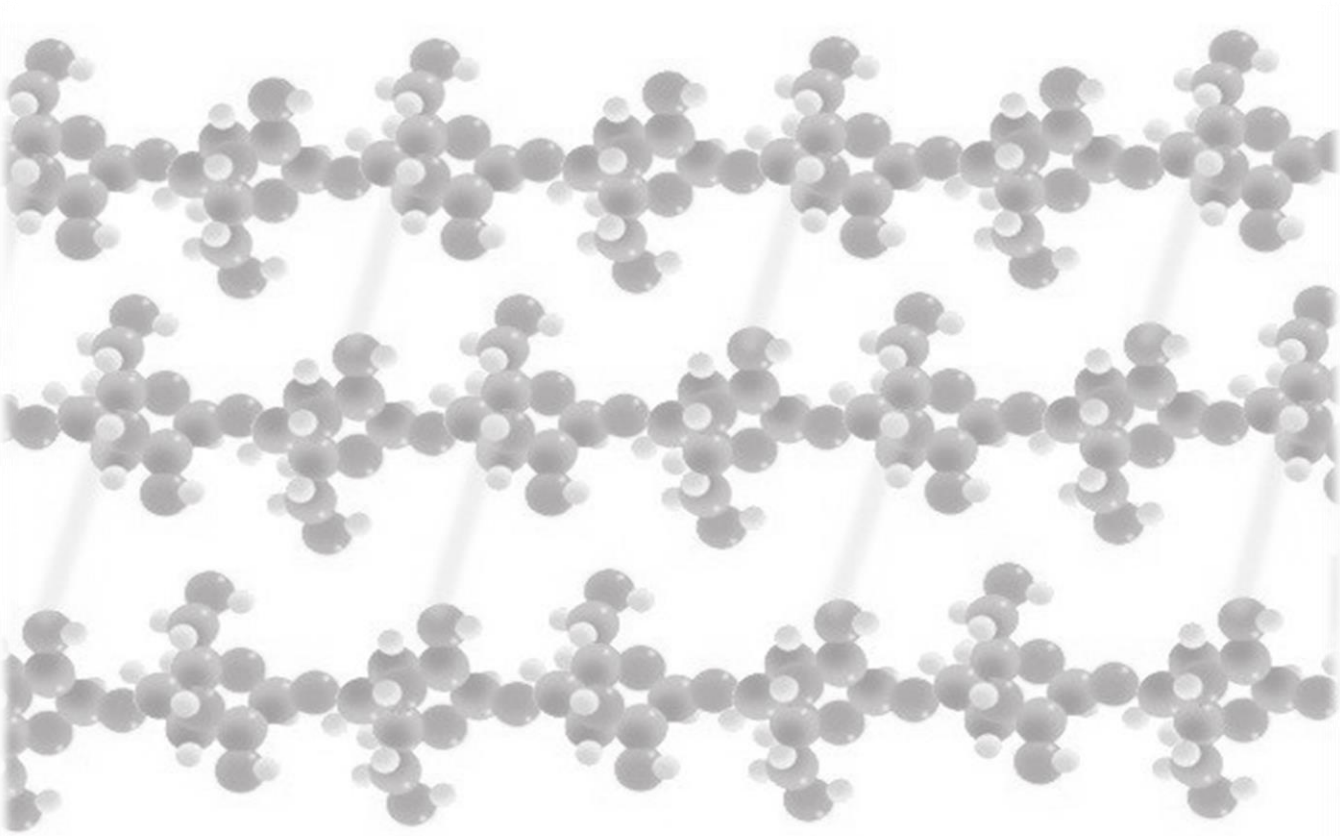
In phlorotannin research, a gap between the first studies (those of Glombitza and colleages [59,62–65,73,324,345,356,357,379–385]) describing this class of phenolic compounds, with an emphasis on their chemistry and structure, and the sudden growth in publication number of the last years, where most studies with brown algal antioxidants rushes to the conclusion that phlorotannins are present, responsible for the activities detected and therefore that the extract is promising for the biotechnological industry exists. However, the lack of background literature to support extraction, separation, purification and identification of these molecules perpetuates the absence of meaningful discussion about these very same topics. In the few studies performing LC-ESI-MS/MS, a lot of data on fragmentation patterns is reported, yet with very little interpretation performed. While to date a fragmentation fingerprint is still not assignable to a chemical entity, this type of studies illustrate the potential that this technique holds, once enough data is gathered simultaneously to NMR structural elucidation.

Phlorotannin research and development is then paced by two different conditions: the rigorous implementation of a structure characterization routine in studies concerning the valorization of this class of metabolites, and the development of more efficient extraction and separation procedures, that allow the application of high-throughput techniques in phlorotannin enriched extracts, such as mass spectrometry. To complement the theoretical contribute of this thesis' first sections, where a selection on bibliography has been compiled and analyzed under the criteria of proper molecular identification, a final work was developed to address the enrichment problem, presented in part III.

Table XIV – Compiled MS data on the phlorotannins with DP=1 and DP=2.

DP	Class	Compound *IUPAC name*	Formula	Mass spectrometry	Ref
1	Phloroglucinol	**Phloroglucinol** *1,3,5-trihydroxybenzene*	$C_6H_6O_3$	***Native*** EI: 126 ([M]$^+$, 100), 111 (9), 97 (20), 85 (30), 69 (41), 52 (26) ESI: 125.05 ([M-H]$^-$, 100), 97.0 (-), 80.0 (-)	[40,323,359]
	Phloroglucinol (H)	**2-Bromo-phloroglucinol** *2-bromo-1,3,5-trihydroxybenzene*	$C_6H_6BrO_3$	***Native*** EI: 206/204 ([M]$^+$, 100/91), 188/186 (4.7/5.3), 177/175 (4.2/4.0), 97 (19), 85 (11), 79 (11), 78 (14), 69 (88) ***Tri-Ac*** EI: 332/330 ([M]$^+$, -/-), 290/288 (-/-), 248/246 (-/-), 206/204 (-/-)	[64,386]
		2-Chloro-phloroglucinol *2-chloro-1,3,5-trihydroxybenzene*	$C_6H_6ClO_3$	***Tri-Ac*** EI: 288/286 ([M]$^+$, -/-), 246/244 (-/-), 204/202 (-/-), 162/160 (-/-)	[62]
		2-Iodo-phloroglucinol *2-iodo-1,3,5-trihydroxybenzene*	$C_6H_6IO_3$	***Tri-Ac*** EI: 378(-), 336(-), 294(-), 252(-), 210(-), 168(-), 126(-)	[64]
2	Fucol	**Difucol** *2,2',4,4',6,6'-Hexahydroxybiphenyl*	$C_{12}H_{10}O_6$	***Hexa-Met*** EI: 334 (100), 181 (9.5) ***Hex-Ac*** EI: 502 ([M]+, -), 460 (-), 418 (-), 376 (-), 334 (-), 292 (-), 250 (-)	[381,382]
		Difucol-4,4'-disulphate (dipotassium salt) *2,2',6,6'-Tetrahydroxybiphenyl-4,4'-disulphate*	$C_{12}H_{10}S_2O_{12}$	***Native*** FAB: 485 ([M]$^+$, 25), 447 (100),409 (15), 367 (30), 351 (40), 329 (50), 281 (35), 243 (100), 227 (35), 205 (15) ***Tetra-Ac*** FAB: 615 ([M]$^+$, 70), 599 (17), 577 (15), 573 (40), 557 (5), 535/531 (20), 497 (25), 493 (15), 481 (4), 469 (12), 455 (20), 289 (100), 273 (31), 255 (31), 249 (56), 243 (51), 230 (31), 215 (51)	[61,387]
	Fucol (H)	**3-Chloro-difucol** **3[A4]-Chloro-difucol** *3-Chloro-2,2',4,4',6,6'-biphenylhexol*	$C_{12}H_9ClO_6$	***Hex-Ac*** FAB: 559 ([M+Na]$^+$, -), 537 ([M+H]$^+$, -), 495 (-), 453 (-), 411 (-), 369 (-), 327 (-), 285 (-) EI: 538/536 ([M]$^+$, -/-), 496/494 (-/-), 454/452 (-/-), 412/410 (-/-), 370/368 (-/-), 328/326 (-/-), 286/284 (-/-)	[62]

Fuhalol	**Bifuhalol** *5-(2,4,6-Trihydroxyphenoxy)-1,2,3-benzenetriol*	$C_{12}H_{10}O_7$	***Native*** ESI: 265 ([M-H]$^-$, -), 247 (-), 141 (-), 139 (-), 125 (-), 123 (-), 111 (-) ***Hex-Ac*** EI: 518 (-), 476 (-), 434 (-), 392 (-), 350(-), 308 (-), 266 (-), 142 (-), 126 (-)	[383,384]
Fuhalol (H)	**3'-Chloro-bifuhalol** **3[A]-Chloro-bifuhalol** *5-(3-chloro-2,4,6-Trihydroxyphenoxy)-benzene-1,2,3-triol*	$C_{12}H_9ClO_7$	***Hex-Ac*** FAB: 591 ([M+K]$^+$, -) 575 ([M+Na]$^+$, -), 553 ([M+H]$^+$, -), 511 (-), 469 (-), 427 (-), 385 (-), 343 (-), 301 (-) EI: 554/552 (-/-), 512/510 (-/-), 470/468 (-/-), 428/426 (-/-), 386/384 (-/-), 344/342 (-/-), 302/300 (-/-)	[62]
Phlorethol	**Diphlorethol** *2-(3,5-dihydroxyphenoxy)benzene-1,3,5-triol*	$C_{12}H_{10}O_6$	***Native*** ESI: 251 ([M+H]$^{+,}$ -), 233 (-), 139 (-), 123 (-), 109 (-), 93 (-) ***Penta-Ac*** EI: 460 ([M]$^+$, -), 418 (-), 376 (-), 334 (-), 292 (-), 250 (-), 142 (-), 126 (-), 110 (-)	[383,385]
Phlorethol (H)	**3-Bromo-diphlorethol** **3[A]-Bromo-diphlorethol** **3[A1]-Bromo-diphlorethol** *2-(3,5-Dihydroxyphenoxy)-3-bromo-1,5-benzenediol*	$C_{12}H_9BrO_6$	***Penta-Ac*** EI: 540/538 ([M]+, 3/3), 498/496 (21/20), 456/454 (40/40), 414/412 (65/65), 372/370 (45/44), 330/328 (28/29), 418 (1), 376 (1), 334 (3), 292 (6), 250 (2), 248 (12), 231 (9), 69 (18), 43 (100)	[63][65]
	2'-Bromo-diphlorethol **2[D]-Bromo-diphlorethol** **2[D']-Bromo-diphlorethol** *2-(2'-bromo-3',5'-Dihydroxyphenoxy)-1,3,5-benzenetriol*	$C_{12}H_9BrO_6$	***Pent-Ac*** EI: 540/538 ([M]+, 3/3), 498/496 (21/20), 456/454 (40/40), 414/412 (65/65), 372/370 (45/44), 330/328 (28/29), 418 (1), 376 (1), 334 (3), 292 (6), 250 (2), 248 (12), 231 (9), 69 (18), 43 (100)	[63][65]
	4'-Bromo-diphlorethol **4[D]-Bromo-diphlorethol** **4[D']-Bromo-diphlorethol** *2-(4-bromo-3,5-Dihydroxyphenoxy)-1,3,5-benzenetriol*	$C_{12}H_9BrO_6$	***Pent-Ac*** EI: 540/538 ([M]+, 3/3), 498/496 (21/20), 456/454 (40/40), 414/412 (65/65), 372/370 (45/44), 330/328 (28/29), 418 (1), 376 (1), 334 (3), 292 (6), 250 (2), 248 (12), 231 (9), 69 (18), 43 (100)	[63][65]
	4'-Chloro-diphlorethol **4[D]-Chloro-diphlorethol** **4[D']-Chloro-diphlorethol** *2-(4-bromo-3,5-Dihydroxyphenoxy)-1,3,5-benzenetriol*	$C_{12}H_9ClO_6$	***Pent-Ac*** EI: 496/494 (-/-), 454/452 (-/-), 412/410 (-/-), 370/368 (-/-), 328/326 (-/-), 286/284 (-/-)	[63]
	2'-Iodo-diphlorethol **2[D']-Iodo-diphlorethol** *2'-iodo-2-(3,5-Dihydroxyphenoxy)-1,3,5-benzenetriol*	$C_{12}H_9IO_6$	***Pent-Ac*** EI: 586(-), 544(-), 502(-), 460(-), 418(-), 376(-)	[62]



PART III – TOWARDS AN ADSORPTION METHOD FOR POLYSACCHARIDES REMOVAL FROM SEAWEED PHENOLIC EXTRACTS

III.a. Overview

As covered in the part II, the primary fractionation of phenolic extracts is a crucial step in the processing of these added value resources, wether for enhancing the resolution of analytical platforms during the bioprospecting, or for enhancing the purity (and thus, functionality) of the compounds of interest for future applications. Furthermore, methods that can be scaled up are being given preference, since the time and money invested in R&D leads to more direct applicability in industrial environments. Actually, a major problem in the field of bioprospecting and bio-based product development is exactly the gap between lab-scale, knowledge-generating methodologies, and industrial-scale, market-oriented ones. For a method to be scaled up and to be "market-oriented", several requirements must be met, among which: the increased volume of processing must be compatible with the technology, in order to allow large scale production; the infrastructures, supplies and energy required for the scaled-up method must be cost-effective and preferentiably adaptable to existing infrastructures; the impact of the technology in the final product must be compatible with market's requirements and standards; the technology must be environmentally and socially sustainable. The purification of biomolecules for industrial bioproducts is an example of a processual step that faces many challenges concerning the compliance with the above criteria. For example, the upscalling of chromatographic processes for the separation of biomolecules is limited by the cost at industrial scales. In the same way, liquid-liquid extractions and precipitations, although simple from an instrumental perspective, require large volumes of solvents, which not only increase the costs but also damage the environment and are nowadays discouraged.

Despite the clear trend of recognition of the potential of seaweed phenolic compounds (and particularly phlorotannins), and even though several papers of increasing detail have started to appear regarding the structural characterization of these metabolites by LC-MS^n (as seen in previous sections), the laboratory analysis of seaweed phenolics is still very limited due to the quality of the phenolic extracts. Along this thesis, in the different sections already covered, several obstacles have been identified regarding the analysis of the phenolic metabolites of seaweeds, with none of the solutions currently proposed being consensual. From our experience and after a careful analysis of the literature, we consider that one of the limitating problems in seaweed phenolic extractives characterization is the co-extraction of polysaccharides and phenolic compounds. In seaweeds, polysaccharides concentration

largely surpasses that of phenolics [22], and this prevents the quantitative and selective recovery of the latter, the performance of functional analyses directly adressable to phenolic components, the application of seaweed extractives into certain types of chromatographic apparatus and the proper analysis of extracts by MS.

Adsorption-based methods to recover specific compounds from solution are thought to be a viable strategy for this type of separative intervention [388]. Effectively, some strategies of adsorption solve most of the above mentioned problems: the extract is contacted with the adsorbent in the original solvent, and desorption of target molecules can be done with relatively low volumes of fresh solvent, which is cost-effective due to solvent savings and energy savings associated with the concentration of the extractives; besides, existing infrastructures require little or no upgrades, as adsorption requires mostly agitation and, eventually, temperature control. More interestingly, biological matrices with high biocompability and low price have been found to serve as adsorbents, with tunable properties for different specificity requirements [389]. Adsorption-based separations of phenolic components from seaweed have been described before in the literature (see section II.d.2). Among these reports, the use of microcrystalline cellulose (Avicel) captured our attention based on the potential this material has, in theory, to differentially interact with polysaccharides and phenolic compounds.

Cellulose is a natural polymer of condensed β(1-4) linked D-anhydroglucopyranose units (Figure 17a). Its applications in industry are endless, due to its multiple physical-chemical properties: insoluble in water, macroscopical fibers held together by hydrogen bonds with high tensile strengh, maintenance of a crystalline structure in solution under harsh environmental conditions, among others. Cellulose molecules are relatively inert chains of variable dimensions, presenting hydroxyl (-OH) groups, hydrogen atoms and oxygen atoms in the ring-structure and in between monomers (Figure 17a). These groups establish intramolecular and inter-fibrilar hydrogen bonds (Figure 17b), as well as with organic compounds in solution; furthermore, in the crystalline form, inter-fibrilar planes (Figure 17c and 17d) semiparallel to the rings are relatively hydrophobic regions, contributing to cellulose's potential to adsorb less polar compounds. Besides, cellulose-based materials can be prepared with different structural organizations, i.e., micro- and nano-scaled crystals and/or fibers (with varying amounts of amorphous portions), which possibilitates a highly tunable biomaterial to be produced.

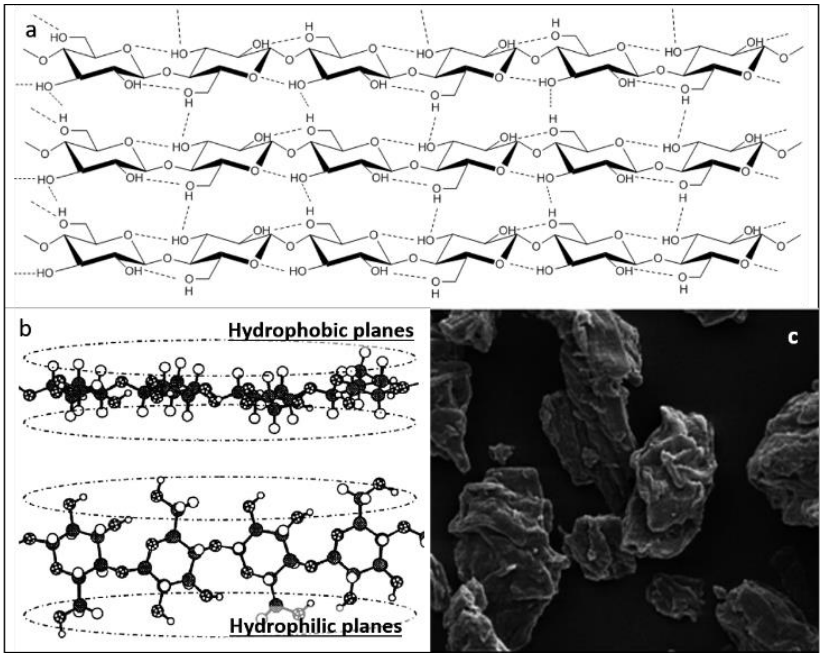


Figure 17 - (a) Cellulose fibers (chains of β1->4 linked residues of D-anhydroglucopyranose), held together by hydrogen bonds. (b) the periphery of the residues is hydrophilic, while the planes parallel to the ring form hydrophobic "pockets" between chains. (c) Quasi-spherical cellulose particles (Avicel PH-101) micrographed by SEM (1000x). Image adapted from [390–392]

However, the partition of compounds between the extracts and this cellulosic adsorbent has been assumed with little experimental support. Besides, no optimization of the experimental conditions (mass of adsorbent, concentration of the extract, solvents composition, solid-to-liquid ratio, temperature, pH, ionic strenght, etc.) has been performed in previous studies. Some studies, however, suggest that cellulose might be a good adsorbent for seaweed phenolic compounds separation from polysaccharides [393–396]. For instance, several studies regarding the adsorption of polysaccharides to cellulose exist [393,394], since it is a natural phenomena involved in the cohesion of cell wall macromolecular structure. Even though vegetal cell wall polysaccharides differ from those of seaweed, the polyol nature of polysaccharides is probably what regulates the adsorption between both. Despite not being natural phenols, some studies observed the adsorption of aromatic dyes on cellulose, with the purpose of water treatment [395,396], suggesting that the phenolic moiety might indeed adsorb onto the cellulose stucture, at least to some extent, probably by hydrophobic interaction at the interfibrilar planes, and maybe stabilized by hydrogen bonding of the OH groups. Although a detailed characterization of the molecules involved

was not performed, Wu et al. [328] showed that apple pomace (a polysaccharide rich residue of apple processing) was effective in the adsorption of epigallocatechin gallate. Costa et. al [397], on the other hand, showed that phenolic acids in aqueous solutions had low adsorption in cellulose, although the authors suggested that this might be due to the hydration effect on the ionized forms of the molecules, preventing non-ionic adsorbents from successfully establishing intermolecular interactions.

III.b. Objectives

In this work, the main goal was to **study the adsorption and desorption of phenolic compounds and polysaccharides from seaweed extracts in cellulose,** with the purpose of separating them. For that, model solutions and an extract from *Laminaria ochroleuca* were used as samples; the methodologies to be applied in the adsorption experiments were preliminarily addressed; and different adsorption experiments were performed, in order to assess different questions regarding this separation process.

III.c. Materials and methods

III.c.1. Biomass, reagents and instruments

Dry, shredded *Laminaria ochroleuca* was collected in Mindelo, in March 2013 by AlgaPlus (Portugal). Fucoidan standard, sodium alginic acid, ascorbic acid, glacial acetic acid, gallic acid, phloroglucinol, quercetin-dihydrate and Avicel PH-101 were acquired from Sigma-Aldrich. Vanillin, catechin and hesperidin were supplied by Alfa-aesar. Solvents used were from various suppliers, of analytical or HPLC-grade. FT-IR analysis were conducted on a Spectrum BX FT-IR Spectrophotometer (Perkin Elmer), and UV-Vis spectra recorded using a UV-1700 Pharma-Spec Spectrometer (SHIMADZU).

III.c.2. Extraction of *Laminaria ochroleuca'*s polar fraction

The polar metabolites of *L. ochroleuca* were extracted by Solid-Liquid Extraction (SLE): 1.015 g of seaweed material (relative humidity 8.3%) were mixed in with 20.00 mL of the extraction solvent (water:methanol:acetone:acetic acid:ascorbic acid 59:20:20:1:0.2 (V/V/V/V/W)), in the dark, under magnetic stirring. After one hour, the solids were separated by centrifugation and re-extracted with 20.00 mL of fresh solvent, a procedure that was performed 3 times (total volume of extract of 80.00 mL). The four fractions were pooled and filtrated under reduced pressure to remove any remaining insoluble material. The filtrate was concentrated using a rotary evaporator under reduced pressure at 40ºC, until organic solvents were removed. The remaining liquid was the aqueous portion, and was removed by lyophilization. The dry extract was accurately weighed and stored under nitrogen atmospher, on a desicattor chamber, until usage. Prior to the adsorption experiments, this extract (from now on refered to as LOE) was ressuspended in water:methanol (1:1) (final concentration of 2.34 mg crude extract/mL).

III.c.3. Adsorption method optimization

An optimization of the protocol of adsorption was made by tackling three different phases of the process: the adsorption matrix used, the strategy of separation employed and the analytical methods that best suited the characteristics of our samples. This pipeline is schematized in Figure 18.

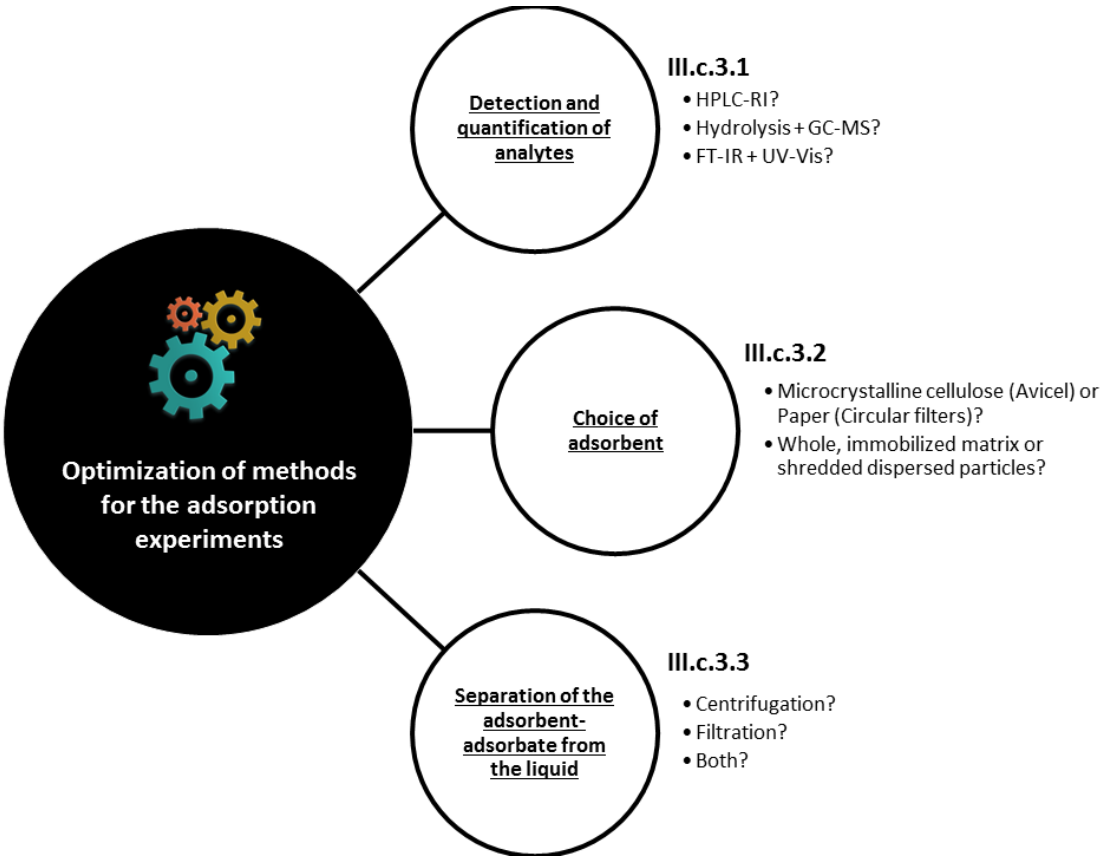


Figure 18 - Rationale for the optimization experiments performed.

III.c.3.1. Detection and quantification of the analytes

Given that experiments with both polysacharides and phenolic compounds of different classes were to be performed, an analytical method – or a combination of analytical methods – was tentatively created to quantify both sugars and phenolics.

Methanol standard solutions of phloroglucinol, catechin, gallic acid and glucose were prepared, to produce a mixture with the respective final concentrations of 0.003 mg/mL, 0.003 mg/mL, 0.003 mg/mL and 0.2 mg/mL. The mixture was then subjected to HPLC (Jasco, Japan) separation on a Prevail™ Carbohydrate ES column (HiChrom) in isocratic

mode using acetonitrile:water 7:3. A refractive index (RI) detector was used to monitor compounds elution. Glucose was used under the assumption that a chromatographic method to quantify the polysacharides alone or simultaneously to the phenolics would require hydrolysis of the polymer into its monosacharide constituents.

GC-MS after hydrolysis and sylilation of the polysacharides-phenolics mixture was then considered. To test the protocol of hydrolysis, 1.000 mg ± 0.001 mg of fucoidan was weighed on a microbalance, and 1 mL of H_2SO_4 at 97% was used to resuspend it. Water was added to dilute the acid to 72 %, and this mixture was incubated at 30ºC for 1 h. At the end of incubation, water was added to the reaction tube to dilute the acid to 4 %, and the tube was transferred to an oil bath at 120ºC where it was left to stand for 40 additional minutes. This procedure was repeated for 50 µg of hesperidin, and for a mixture of 1 mg fucoidan + 50 µg catechin + 50 µg gallic acid + 50 µg phloroglucinol + 50 µg hesperidin. The rationale for these solutions' compositions is discussed in the corresponding section of results and discussion.

Trimethylsililation was performed on gallic acid, quercetin, catechin and phloroglucinol. For that, standard solutions of each compound were prepared in pyridine, at a concentration between 0.5 and 1.5 mg/mL. Adequate volumes of each standard were transferred into separate sovirel tubes, such that 50 µg of each compound were carried. These volumes were completed with pyridine for a final volume of 250 µL, and 250 µL of N,O-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylchlorosilane were added to each reaction tube, further put at 70ºC for 30 min.

A mixture of the four trimethylsililated phenolic compounds was prepared by transferring 50 µL of each reaction product to a vial. A volume of 0.8 µL was then injected on a Trace Gas Chromatograph 2000 Series, equipped with a Thermo Scientific DSQ II single-quadrupole mass spectrometer and a DB-1 column (30 m×0.25 mm i.d.; 0.25 mm film thickness) and the following temperature program was used: initial temperature, 80 °C for 5 min, temperature rate, 4 °C/min up to 260 °C and maintained for 5 minutes, 2 °C/min up to 285 °C, maintained for 10 min.

Given the lack of positive results from the above mentioned methods, FT-IR and UV-Vis spectra were acquired after evaporating the organic solvents fraction of the solutions, and diluting the remaining aqueous fraction to a known volume. This way, comparison between fractions was possible, albeit in a less specific and quantitative way. FT-IR data

were obtained after background spectra and baseline correction (integrated functions of the software Spectrum). The UV spectra were registered using quartz cuvettes and water as blank. The mathematical treatment of UV-Vis data is described below, as pertinent.

III.c.3.2. Cellulose filters *vs.* Avicel

Several experiments were designed to test the performance of cellulose filters (CFs) against that of Avicel. Filter's cellulose differs from Avicel in both its crystalline fraction and surface area. Thus, its adsorption properties might be drastically different, and worthy experimenting.

A first approach to CFs adsorption was to design an apparatus where circular filters could be piled with one millimeter of distance, and the entire apparatus submerged in a solution of alginic acid, with agitation (Figure 19). Three ratios of alginic acid to filter-paper's surface area (mg/cm^2) were tested: 0.03, 0.1 and 0.53. The percentage of alginic acid adsorbed was determined by determining the concentration before and after adsorption in the remaining liquid, by dry weight calculation.

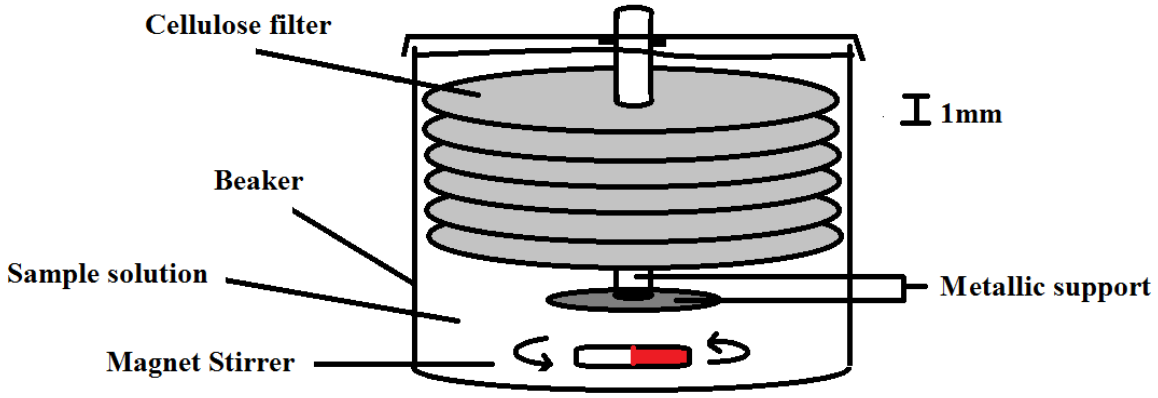


Figure 19 – Schematic representation of the adsorption apparatus prepared to test the use of whole circular filter papers as adsorbent. The number of filters and the size scale of the different elements in the figure are not representative of reality.

Because the absolute values of adsorption were very low even at the lowest ratio of 0.03, a different approach was used: filter paper was triturated until a fibrous cotton-like material was obtained to be used as adsorbent and fucoidan was used instead of alginic acid

to also evaluate the effect of sulphate groups in the adsorption. An adsorption experiment to compare Avicel and triturated CFs (tCFs) was performed by putting 20 mg of Avicel or tCFs in 15 mL falcon tubes with 200 µg of fucoidan[2] (blanks without fucoidan were also prepared). 2.5 mL of distilled water were added to each of the 4 tubes, which were then agittated for 1.5h. At the end of this period, the tubes were centrifuged, the liquid removed and passed through a 0.22µm syringe filter. A piece of the pelleted solids was held with the help of tweezers and the remaining liquid was blown away under an intense nitrogen stream, so that residual solutes were minimized and the adsorbed ones were retained. A drop of each liquid, or a piece of each solid, were analyzed by FT-IR as described above.

III.c.3.3. Separation of adsorbent in suspension from the liquid phase

The separative method to be used in the adsorption experiments was previously determined by testing centrifugation and filtration on a suspension in water of Avicel PH-101 Microcystalline cellulose (particle size ~ 50 µm) (further refered to as "Avicel"). For that, a 0.8% (W/V) suspension of Avicel was centrifuged at 17000xg for 20min in a Heraeus Megafuge 16-R bench centrifuge (ThermoScientific); alternatively, filtration under reduced pression using 16-40 µm pore DURAN® filter funnels was performed, to compare results.

[2] Alginic acid was initially used in the different experiments, but later fucoidan was used to cover different types of seaweed polysaccharides (alginic acid is an anionic polysaccharide, and fucoidan contains sulphate groups).

III.c.4. Adsorption experiments

III.c.4.1. Seaweed polysaccharides-Avicel adsorption

A schematic overview of the adsorption experiments using seaweed polysaccharides is provided in Figure 20. Details are provided in text, below.

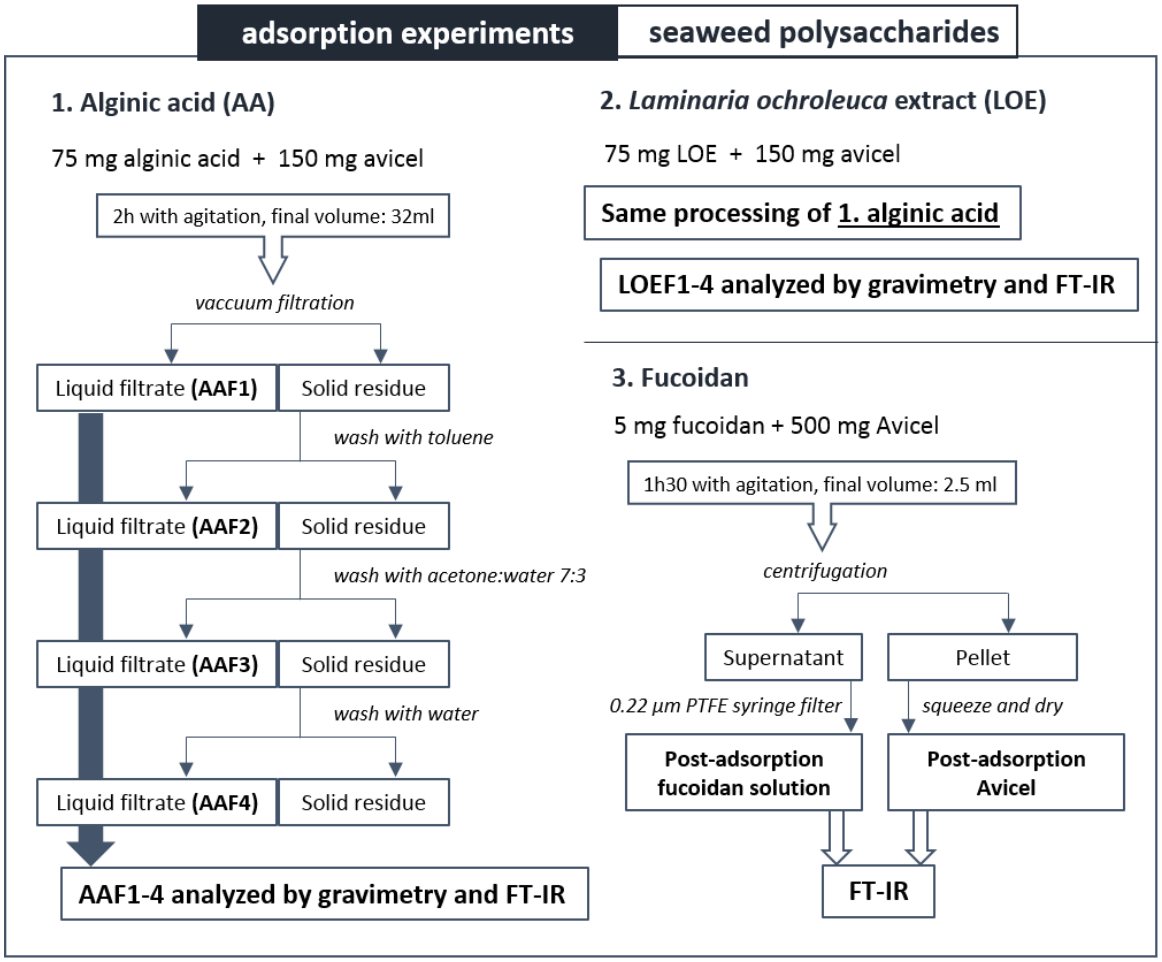


Figure 20 - Schematic overview of the adsorption experiments using seaweed polysaccharides.

An Alginic Acid (AA) standard solution was prepared at a concentration of 2.26 mg/mL using water to solubilize the powdered alginic acid until no opacity of the solution was observed, and completing the volume with methanol (final solvent composition ca. 1:1 (V/V)). A fucoidan standard solution was prepared using water as solvent to the final concentration of 10 mg/mL.

An aliquot (32 mL, 75 mg of AA) of the model solution of alginic acid was transferred to a 100 mL flat-bottom flask, and 150 mg of Avicel PH-101 were added. The mixture was agitated on a magnetic stirrer for 2 hours, after which it was filtrated under reduced pressure. The filtrate was collected (AAF1, see Figure 20). The adsorbent-adsorbate complex was maintained in the filtering unit, and the following solvents were passed through it slowly, in this order: 20 mL of toluene (AAF2); 100 mL of acetone:water 7:3 (AAF3) ; 500 mL of water (AAF4). The initial solutions and each fraction (AAF1-AAF4) were analyzed by gravimetry (dry weight after fixed volume correction) and FT-IR spectroscopy. The procedure was replicated with an aliquot of LOE (32mL, 75mg of extract), resulting in fractions LOEF1-LOEF4.

500µL of fucoidan standard solution were mixed with 500mg of Avicel and 2.0 mL of destilled water. Following 1h30min (with stirring), the bulk of the adsorbent was separated by centrifugation, and the supernatant was passed through a PTFE syringe filter (0.22 µm) to obtain an aliquot suitable for FT-IR analysis . At this point, a confirmation of a similar behavior of fucoidan-Avicel to that of alginic acid-Avicel was the goal, and therefore no washing experiments were done. On the other hand, FT-IR spectra of the avicel after adsorption and without any kind of elution was obtained to compare the decreased signals in the supernatant with the increased signals in Avicel.

III.c.4.2. Seaweed phenolic compouds-Avicel adsorption

A schematic overview of the adsorption experiments using seaweed phenolic compounds is provided in Figure 21. Details are provided in text, below.

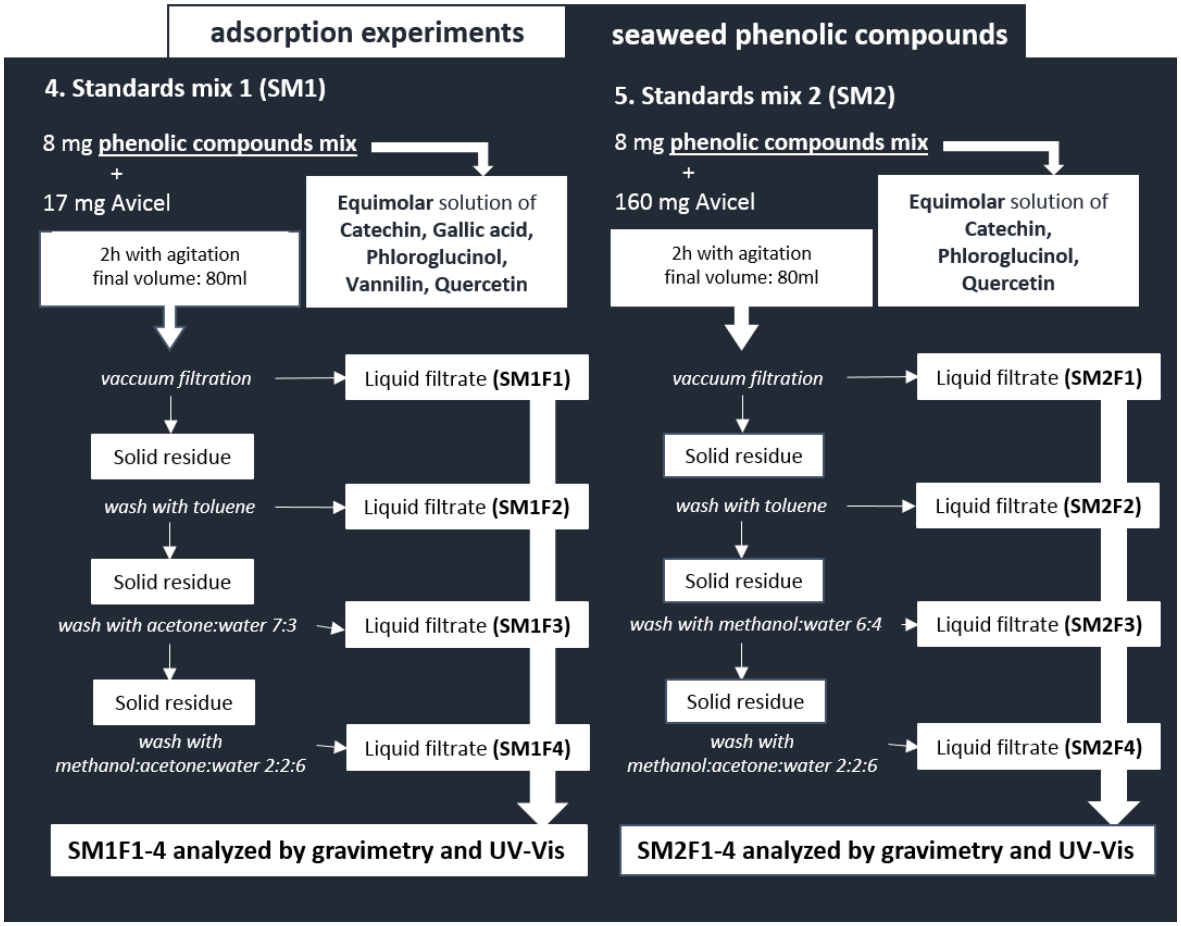


Figure 21 - Schematic overview of the adsorption experiments using seaweed phenolic compounds.

50.00 mL of standard solutions of catechin, gallic acid, phloroglucinol, vanillin and quercetin (structures in Figure 22) were prepared in volumetric flasks, at a concentration of ca. 1 mM in methanol (real concentrations in Table XV). From these solutions, 10.00 mL of each one were pipetted into a volumetric flask, and filled to 100,00 mL with methanol. This standards mix (SM1) was thus an approximately equimolar mixture of phenolic compounds at a final concentration of 0.11 mg/mL.

Table XV - Real concentrations of the standard solutions used to prepare SM.

Phenolic Compound	Concentration (mg/mL)/(mM)
Catechin	0.304/1.05
Gallic acid	0.17/1.0
Phloroglucinol	0.14/1.1
Vanillin	0.16/1.0
Quercetin	0.308/1.02

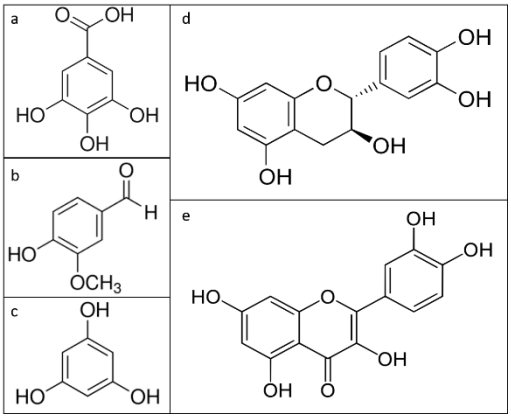


Figure 22 - Structures of the phenolic compounds used in the first adsorption experiment – a) gallic acid; b) vanillin; c) phloroglucinol; d) catechin; e) quercetin. Different molecular sizes and functional groups between compounds are likely to cause differential adsorption kinetics and affinities.

Approximately 17 mg of Avicel were added to 80.00 mL of SM (twice the mass of phenolics) and allowed to stand for 2 hours with agitation (Figure 21). Following this period, Avicel was filtered and the filtrate reserved for analysis (SM1F1). A wash with toluene (30 mL) and two additional washes of 100 mL each with, first, acetone:water 7:3 and second, methanol:acetone:water 2:2:6 were performed (fractions SM1F2, SM1F3 and SM1F4, respectively). An aliquot of each fraction and of the initial mixture were analyzed by both gravimetry and UV-Vis spectrophotometry (after evaporation of the organic phase and correction of the volume with water), attempting to distinguish the relative proportions of each phenolic compound by its absorption maxima. Due to the overlapping of absorption spectra between the 5 phenolics, and to the low absolute values of adsorption, a second experiment was done with an equimolar mixture of only catechin, phoroglucinol and quercetin (SM2), using 20x the mass of solutes in Avicel. A similar elution scheme was employed, resulting in fractions SM2F1-SM2F4, except for the eluent of SM2F3, which was replaced by methanol:water 60:40, to assess at what point would the phenolics begin to

quantitatively desorb. The extinction coefficients were determined in the range of 250-600nm for each compound. Mathematical modeling (multiple regression fitting) of the absorbance data was performed in R using the graphical interface R Studio.

III.d. Results and discussion

The adsorption of seaweed polysaccharides and phenolic compounds to cellulose for the fractionation of these classes was studied, using model solutions of alginic acid, fucoidan, different phenolic compounds and an extract from *Laminaria ochroleuca*. First, the methodologies to be used along the experiments were approached, namely the analytical methods that could provide information on the qualitative and quantitative composition of the different fractions obtained after the adsorption process (III.d.1.1.), the nature of the cellulosic matrix (III.d.2.2.) and the protocol for separation of the adsorbent from the liquid sample (III.d.3.3.). For this, gravimetric and spectroscopic/spectrophotometric (FT-IR/Uv-Vis) data were gathered. Afterwards, actual adsorption experiments with commercial standards of compounds naturally present in seaweed were performed. In these experiments, definite results of the composition of the samples after adsorption could not be obtained, as the analytical methods development was partially impaired. Nonetheless, a discussion on the performance of Avicel for adsorption of seaweed metabolites is held on the basis of FT-IR and UV-Vis spectra of the adsorbent and of the sample, before and after adsorption.

III.d.1. Adsorption method optimization

III.d.1.1. Detection and quantification of the analytes

The development of an analytical methodology to follow phenolic compounds and polysaccharides during the adsorption studies was essential to develop this separation strategy. Thus, we attempted to develop specific methods to individually analyze the components of each adsorption experiment. Likely, any method with the power to identify and quantify both the phenolic compounds and the polysaccharides, will require the analysis of the latter indirectly, by their hydrolysis products (monosacharides). Firstly, HPLC-RI was chosen, given the wide applicability of this detector for carbohydrates and phenolic components. The HPLC-RI chromatogram obtained for a standards mixture of glucose and phenolic compounds (phloroglucinol, gallic acid and catechin) is shown in Figure 23.

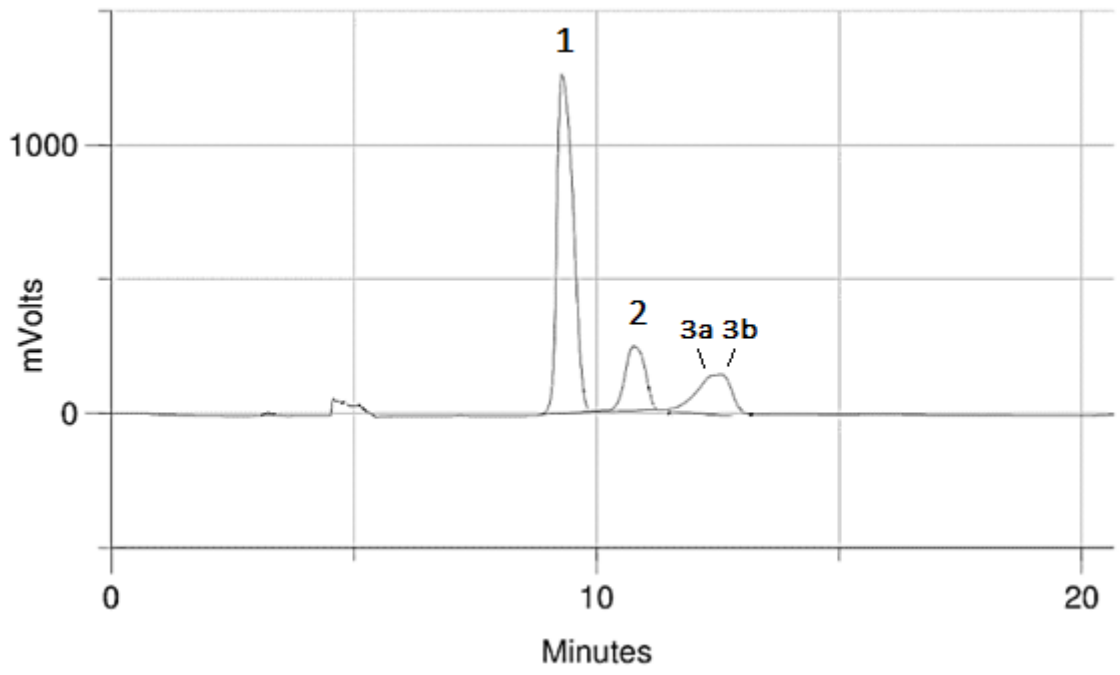


Figure 23 – HPLC-RI chromatogram of a standard solution of glucose (peak 1), phloroglucinol, catechin and gallic acid (mass ratio of 66:1:1:1) on a Prevail™ Carbohydrate ES (HiChrom) column. The order of elution of the phenolic compounds could not be determined.

The first peak, given the increased signal, as well as the faster elution, is attributable to glucose. The phenolic compounds were then detected at retention times between 10 and 13 min, and two of them are partially overlapped. Arguably, optimization of elution conditions could alter retention times and allow separation. Nonetheless, the fact that such optimization wouldn't be certain to work (RI detector's limitation of isocratic eluent lowers the possibilities to separate similar compounds), led us into attempting a different analytical approach: GC-MS.

In order to analyze our samples by GC-MS, which contain different amounts of phenolic compounds and polysaccharides, two steps prior to chromatography were necessary: hydrolysis of the samples to convert fucoidan into its monosaccharides, thus becoming detectable and quantifiable by GC-MS, and trimethylsilylation to volatilize the monosacharides and the phenolic compounds. Thus, three main questions were adressed in these preliminary assays: a) will it be possible to analyze the phenolic compounds by this method without losing them due to degradation in the hydrolysis process?; b) given the complexity of the samples (several different phenolic compounds and monosacharides), will it be possible to resolve the components in a chromatogram by GC-MS?; and c) is hesperidin

going to be hydrolyzed, giving rise to a peak for hesperitin-tri-TMS plus confounding the intensities of the peaks of L-Rhamnose and D-Glucose (the monosacharides in the glycoside moiety in hesperidin)?

To tackle these questions, different assays were prepared. A standard solution of fucoidan with phenolic compounds was subjected to hydrolysis in order to evaluate the degradation of the phenolic components, as well as the resolution in GC, but it couldn't be analyzed since trimethylsilylation requires total water removal and by the time of this experimental work, no freeze-drying equipment was available, and reduced pressure water removal led to the degradation of the solutions (as suggested by a very dark color development). Nonetheless, hydrolysis by itself did not seem to promote color development, which might indicate that this step doesn't totally impair the analysis of these mixtures – which was the main concern regarding the use of this method.

Separation of trimethylsilylated phenolics compounds (namely, of gallic acid, quercetin, catechin and phloroglucinol) without hydrolysis was tested by GC-MS (Figure 24). The goal was to evaluate if the chromatographic separation was successful, otherwise the attempts to evaporate the samples for derivatization were pointless. Quercetin was not found to elute, which might signal that the TMS-quercetin derivative becomes too heavy to be volatilized under the chromatographic conditions used. The remaining phenolics were successfully volatilized and separated.

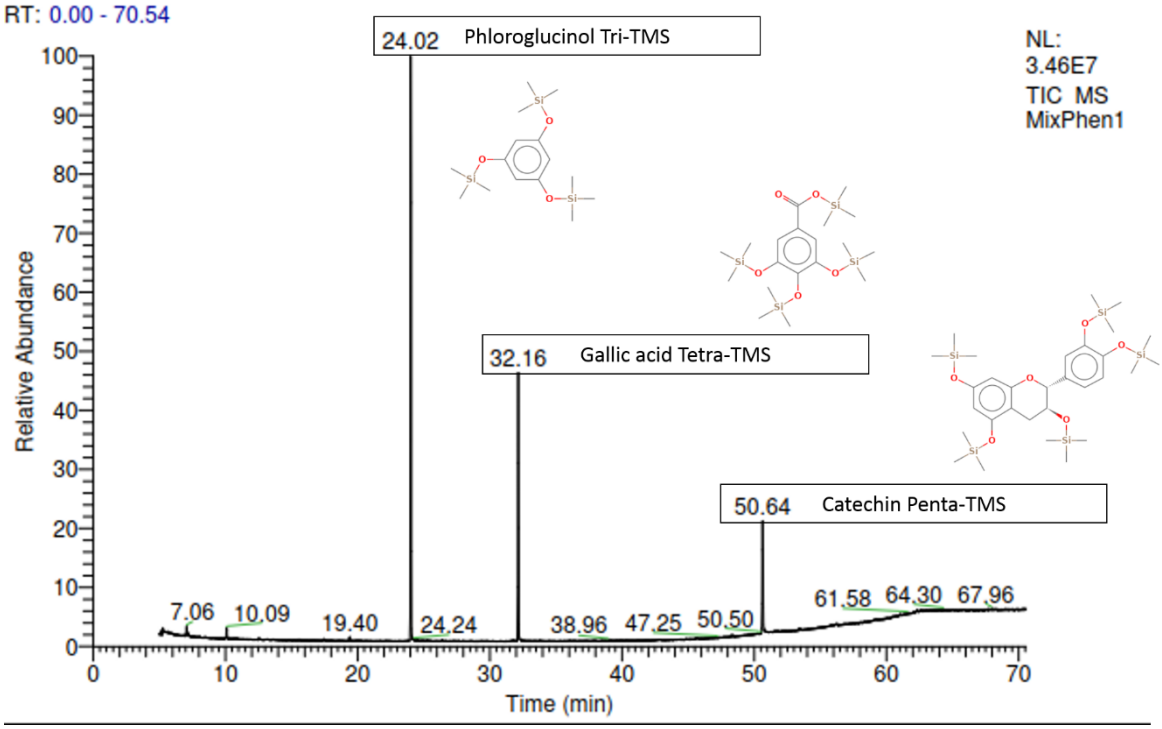


Figure 24 - Total ion chromatogram of a derivatized mixture of phloroglucinol, gallic acid, catechin and quercetin. Compounds were identified by their mass spectra fragmentation patterns, in comparison with spectral libraries.

Due to the lack of a methodology that successfully allowed the characterization of the method's outcomes, UV-Vis and FT-IR spectra were used to monitor the evolution of adsorption in a less specific, yet very practical way. Despite their limited resolution/specificity, these methods allowed the analysis of phenolic compounds (mostly by their UV absorbing property) and of polysaccharides (distinguishing features in FT-IR) during the adsorption experiments (and are discussed below, as relevant).

III.d.1.2. Cellulose filters *vs.* avicel

To assess the extent to which cellulose's crystaline form influenced the capacity to adsorb compounds, circular filter papers were mounted on a screw with 1mm distancing for adsorption of alginic acid. Different configurations were tested, in order to achieve three different alginic acid to filter paper's surface area ratios, in a manner that maintained proper agitation of the media and full submersion of the adsorbent. Previous studies using cellulose to adsorb seaweed extractives have used microcrystalline cellulose (Avicel) [56,80,297,330–332], which is a rather expensive material considering the industrial scales it could

potentially serve; if instead cheaper cellulosic materials were usable, it would improve the applicability of this method. The results of these adsorption tests are reported in table XVI, as the percentage of alginic acid removed from solution, determined by dry weight of an aliquot of the supernatant before and after the experiment.

Table XVI– Adsorption of alginic acid onto filter papers, mounted in a pile, at different ratios of alginic acid to surface area of paper.

Ratio*	% adsorbed
0.03	18.4
0.10	nd
0.53	6.7

*-alginic acid (mg) : useful surface area (cm^2)

These experiments illustrated the need for an efficient optimization of every factor affecting adsorption. An increase in surface area resulted, as expected, in an increase of the relative amount of alginic acid adsorbed. Yet, even at a very low ratio (0.03 mg/cm^2), only 18.4% of the alginic acid could be removed from the solution. Also, it is noteworthy that the visual inspection of the filters upon removal from the solution revealed a thick, gelly layer at the surface of the adsorbent. A similar phenomena occurred with avicel, under filtration, when the filter colmated (described above). This gelling property of seaweed polysaccharides, while extremely valuable for their valorization in industry, represents a challenge during seaweed processing (from extraction to characterization), as many mass-transfer phenomena get impaired by the gellification of the media.

Given the poor performance of whole CFs in adsorbing alginic acid, triturated CF (tCF)'s performance was compared to avicel, by using equal masses of each in the adsorption of fucoidan instead. For that, FT-IR spectra of the tCF residue and supernatant after contacting with a fucoidan standard solution were recorded (Figure 25). These results were compared to those obtained in the Avicel-Fucoidan adsorption experiment (section III.d.4.1).

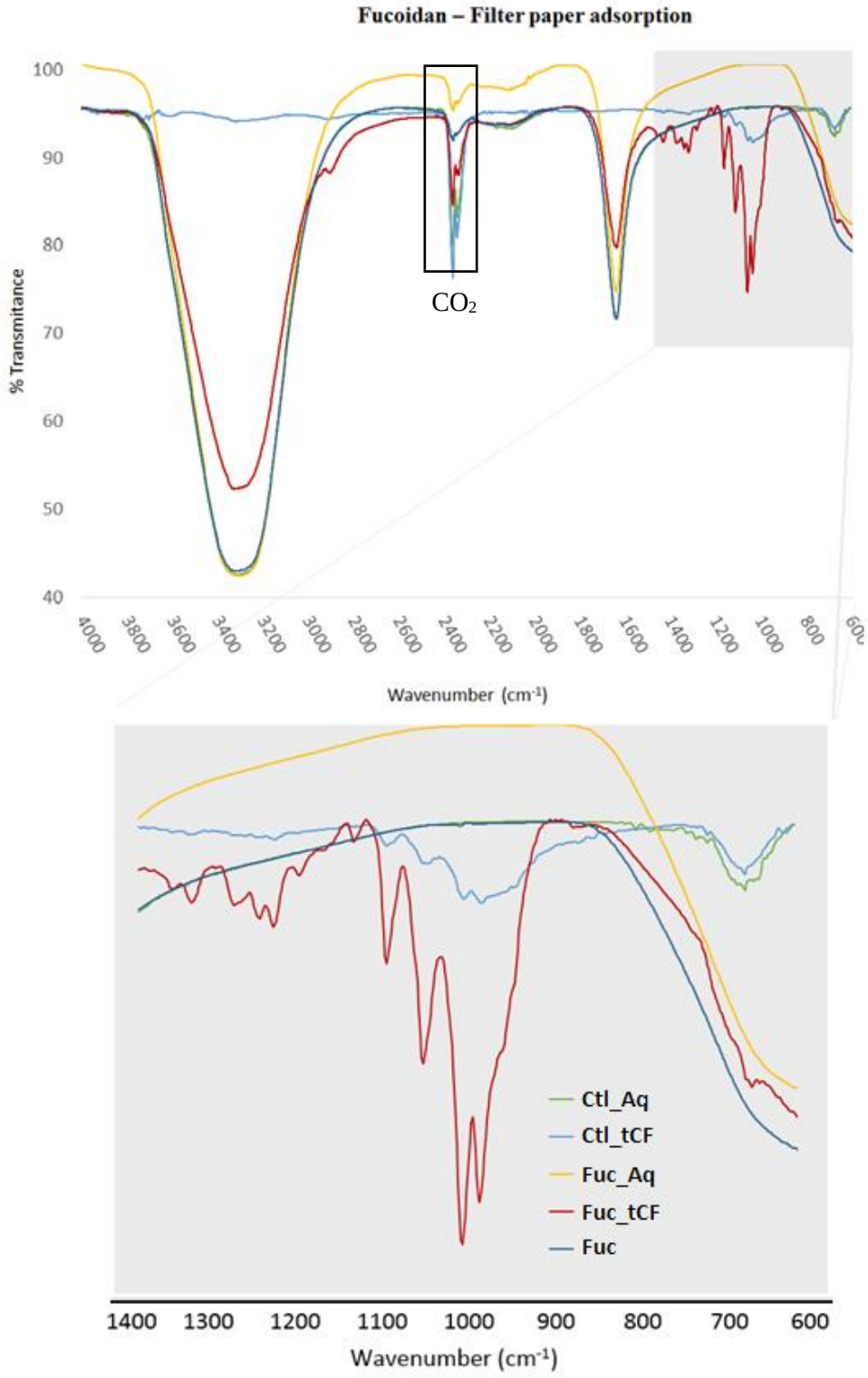


Figure 25 - FT-IR transmitance values spectra obtained for the aqueous phase of an avicel suspension (Ctl_Aq), triturated filter paper recovered from an aqueous suspension (Ctl_tCF), the aqueous phase of a fucoidan solution after contact with triturated filter paper (Fuc_Aq), triturated filter paper recovered from a fucoidan suspension (Fuc_Avi) and fucoidan aqueous standard (Fuc). Detail of the range 600-1400 cm^{-1} below the main graph.

A fucoidan-associated signal at 600-640 cm^{-1} is present in the standard solution and in the adsorption products (both the tCF residue and the supernatant). This suggests that although some fucoidan remained in solution, some was indeed adsorbed onto the cellulose matrix. This opens the possibility that unexpensive cellulose matrices are applicable to an adsorption protocol, perhaps even leading to different outcomes, since polysaccharide retention is comparable to that for Avicel (as seen below, in the fucoidan adsorption experiments), but phenolic compounds might interact differently with this type of cellulose (less crystaline).

III.d.1.3. Separation of adsorbent in suspension from the liquid phase

An initial obstacle to the execution of this workplan was identified related to the methodology of separation of the adsorbent (solid particles of Avicel) from the liquid containing the analytes. Thus, the performance of centrifugation and filtration in the separation of Avicel particles from the liquid matrix was tested, in order to determine the best method to employ in the adsorption experiments to recover the supernatant free of particles. In the case of Avicel-in-water suspensions, centrifugation managed to remove 99.8% of the suspended solids (as determined by dry weight) and filtration using a 16-40 µm pore DURAN® filter funnel under reduced pressure also removed most of them – 99.9%. Thus, filtration was chosen because it was much easier to perform, considering that to achieve 99.8% removal through centrifugation, 3 rounds of 20min at 17000*xg* were required.

However, when preliminary experiments using polysaccharides were initiated, a slight excess of the summed dry weights of the filtered solutions was obtained relative to the expected amount of total dissolved solids (the solutes carried by the standard solutions). In one of the trials, surprisingly, actual deposit of Avicel was observed after filtration, despite being absent in the tests conducted with only water, indicating that this pore size could be too large to filter smaller Avicel particles. One last attempt, with an alginic acid model solution, revealed the accumulation of a thick gel in the filtration funnel which resulted in the filter becoming colmated. Besides, the experiments using fucoidan required a miniaturization of the entire adsorption experiments – due to the higher costs of this compound -, which allowed a different separative approach to be used. Briefly, the use of 15 mL falcon tubes (with a pointing end) allowed a rapid centrifugation (15000 xg,5min) to pellet the adsorbent, and the liquid-phase could thus be removed by pipetting and passing it

through a 0.22 µm filter syringe. This method has worked with no apparent losses of solids or liquids.

III.d.2. Adsorption experiments

III.d.2.1. Seaweed polysaccharides-Avicel adsorption

The adsorption of alginic acid (a seaweed polysaccharide) and of a crude extract from *Laminaria ochroleuca* (LOE) onto Avicel were studied (a simplified scheme of the fractions collected is in Figure 26).

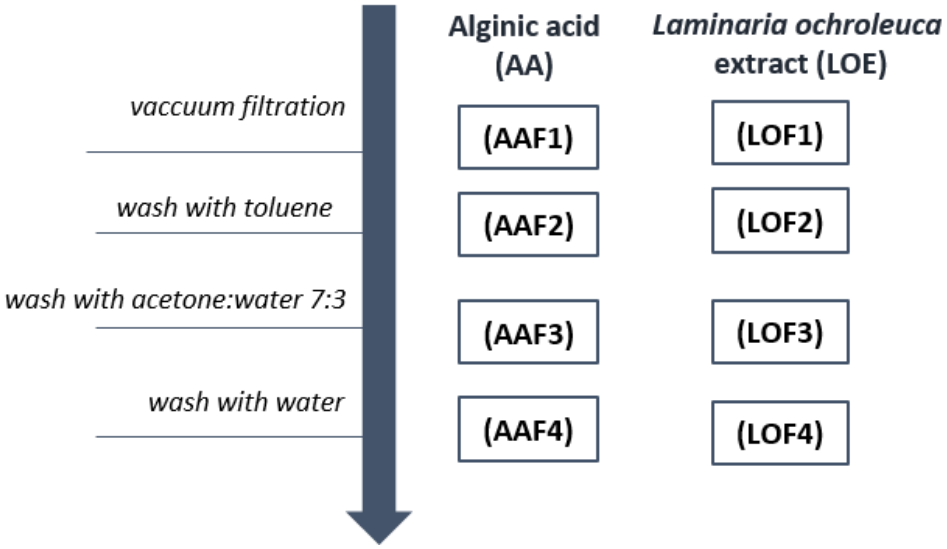


Figure 26 – Fractions collected after contacting a standard solution of alginic acid or a *Laminaria ochroleuca* extract with Avicel, and eluting the supernatant as well as washing with different solvents.

A known amout of solutes were contacted with twice their mass of Avicel, filtrated (recovering the supernatant, AAF1 and LOEF1), and sequentially eluted with toluene (AAF2 and LOEF2), acetone:water 7:3 (V/V) (AAF3 and LOEF3) and water (AAF4 and LOEF4). The solutes in the liquid fractions recovered were determined by gravimetry. Table XVII shows the massic distribution of total suspended solids along the experiment.

Table XVII – Percentage of solutes at each stage of the adsorption experiments, relative to initial mass.

Eluent	Fraction	Alginic acid 2.26 mg/mL		Fraction	LOE 2.34 mg/mL	
		Mass (mg)	%		Mass (mg)	%
-	Initial	72.3	100	Initial	74.7	100
-	AAF1	47.3	65.4	LOEF1	60.6	81.1
Toluene	AAF2	-	-	LOEF2	-	-
Acetone:Water 7:3	AAF3	1.9	2.7	LOEF3	2.3	3.1
Water	AAF4	23.8	32.8	LOEF4	16.3	21.7

The results obtained for the toluene fraction (AAF2 and LOEF2) were not possible to determinate, as the alginic acid standard was devoid of pigments and other medium polarity lipophilics contaminants, and the LOE, despite presenting color that was eluted with the toluene, was not possible to be analyzed because after evaporation no residue was recovered. Nonetheless, a very interesting distribution of the wheighs of total suspended solids recovered along the tested protocol was found. In the standard solution of alginic acid, around 35% of the initial mass was captured by Avicel, which is an interesting observation since only 2 mg of adsorbent per mg of alginic acid were used. Noticeably, at this ratio, it was possible to recover approximately twice the percentage of alginic acid than using whole filter papers, at the maximum surface area tested (as discussed previously – III.d.1.2). This coefficient of adsorption (of around 0.16 mg alginic acid/mg Avicel) is a good indicator that polysaccharide retention with phenolic compounds recovery is possible (Costa et al. [397] obtained values of adsorption of phenolic compounds on cellulose <0.01 mg compounds/mg adsorbent). Though, no such conclusions can be drawn based only on a single result. Even more interestingly, the elution with acetone:water 7:3, just as intended, was not sufficiently hidrophilic to quantitatively desorb the polysaccharide; however, upon passing water, the totallity of the remaining solute was recovered.

A similar result was obtained for LOE. The reduction in mass of LOEF1, low recovery in LOEF3 and remaining solids' recovery in LOEF4 were observed. Actually, the relative adsorption of a lower percentage in the crude extract that in the standard solution is a positive observation, since the crude extract is supposed to contain non-sugar materials as well. Besides, alginic acid might have a specially high affinity to cellulose (compared to the diverse polysaccharides from the extract), since it has highly polar groups (the carboxylic), potentially leading to the establishment of several hydrogen bonds with cellulose. The

observation that 2x the mass of extract in Avicel led to an adsorption phenomena qualitatively as expected, but quantitatively insuficient, led us to design the future experiments with increasing Avicel mass relative to solute.

FT-IR spectra were obtained for each of the four fractions analyzed. Prior to analysis, the organic fractions of the solvents were evaporated under reduced pressure and the final volumes adjusted, so that a drop of the aqueous solutions could be used at FT-IR's crystal. However, very noisy spectra were obtained, with no identifiable bands suitable for comparison or even asignment to bond types (Figure 27).

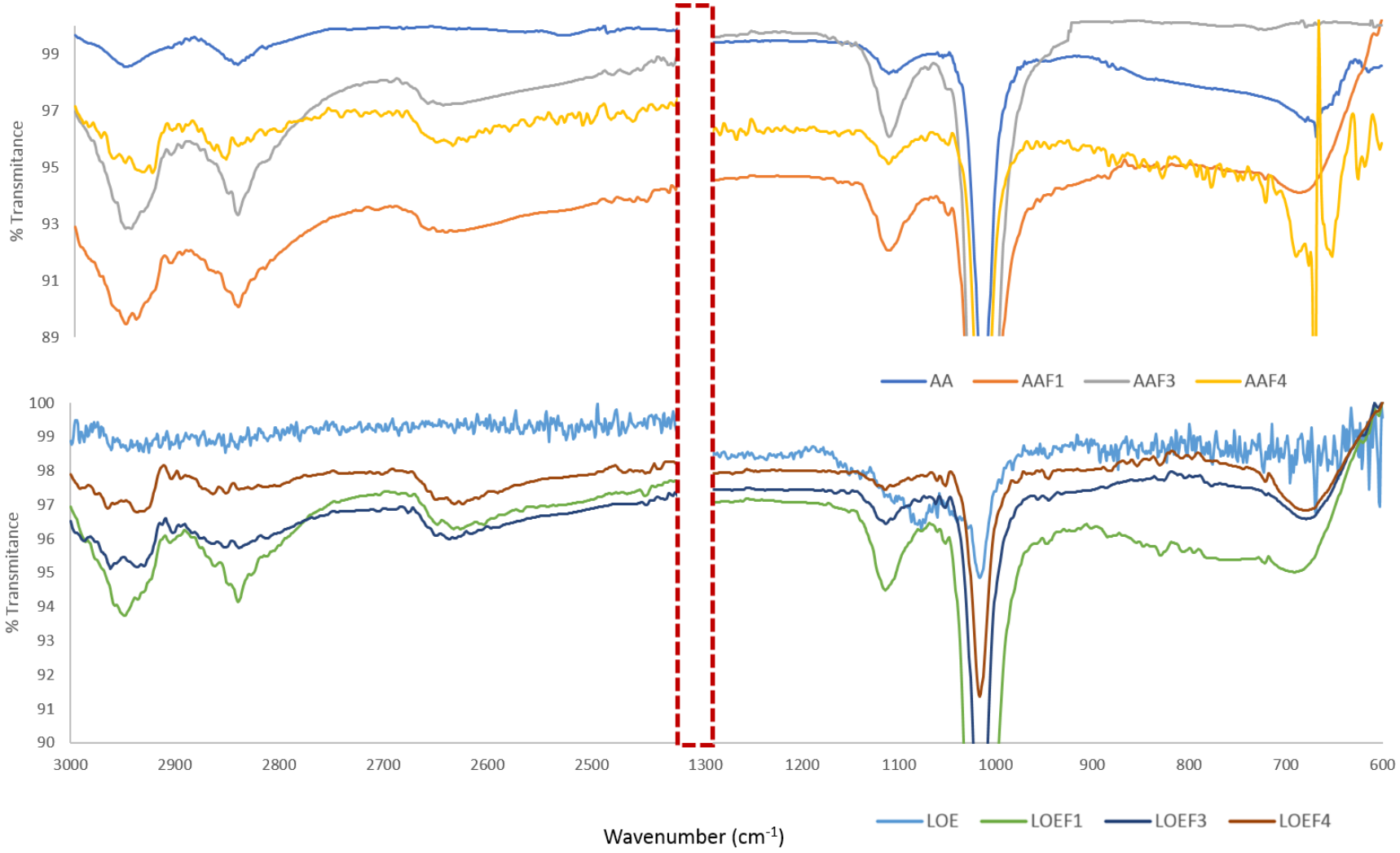


Figure 27 – FT-IR spectra of the 4 fractions obtained after the adsorption experiments with alginic acid and LOE.

Because alginic acid is a non-sulphated polysaccharide, and sulphated polysaccharides are common in many seaweed species, another experiment was performed using fucoidan commercially obtained. The different fractions analysed by FT-IR to characterize the adsorption of fucoidan in Avicel are those schematically represented in Figure 28, and their FT-IR spectra are shown in Figure 29.

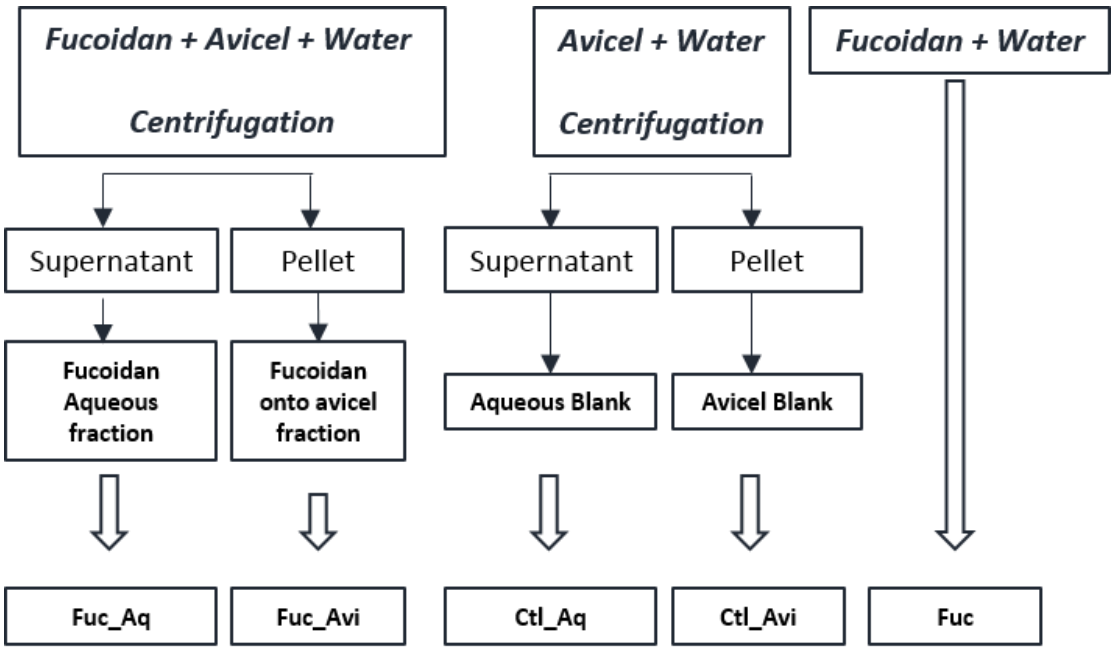


Figure 28 – Different fractions characterized by FT-IR spectroscopy after fucoidan-avicel adsorption experiments.

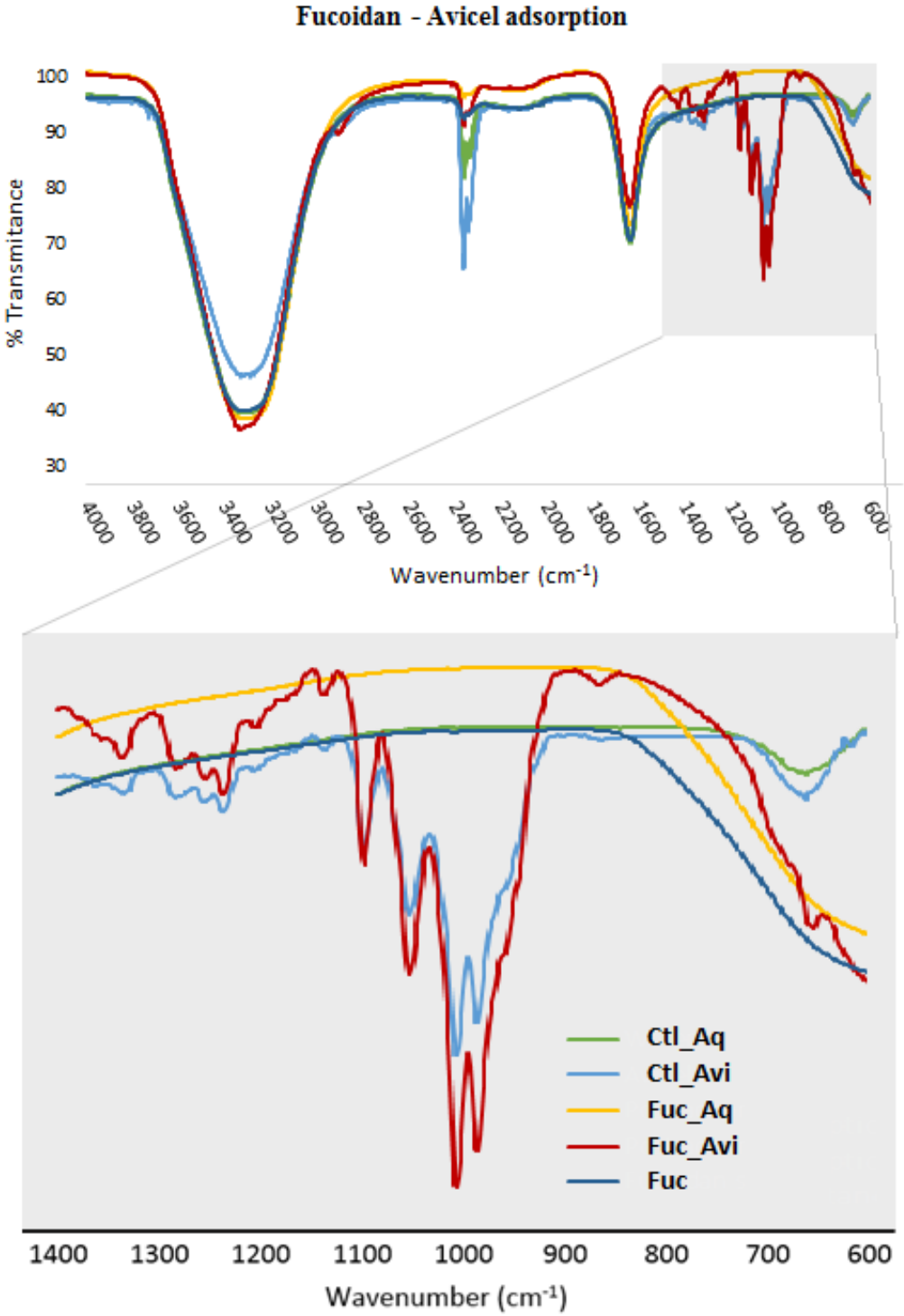


Figure 29 FT-IR transmitance values spectra obtained for the aqueous phase of an avicel suspension (Ctl_Aq), avicel recovered from an aqueous suspension (Ctl_Avi), the aqueous phase of a fucoidan solution after contact with avicel (Fuc_Aq), avicel recovered from a fucoidan suspension (Fuc_Avi) and fucoidan aqueous standard (Fuc). Detail of the range 600-1400 cm-1 below the main graph.

Fucoidan was shown to adsorb to avicel, as seen in FT-IR spectra (Figure 29). Two blanks (the aqueous and solid phases of an avicel suspension, Ctl_Aq and Ctl_Avi) were plotted to control the bands corresponding to these materials. A very discrete separation of the absorption bands associated to both carbohydrates was found: a "ramp" of 4 bands (approximately at 1033cm^{-1}, 1057 cm^{-1}, 1109 cm^{-1} and 1160 cm^{-1}) characteristic of avicel [21] was proeminent in the samples of avicel, prior and posterior to the adsorption, but absent in fucoidan standard (Fuc) or post-adsorption supernatant (Fuc_Aq). Thus, this region is characteristic to avicel. Fucoidan standard (Fuc), on the other hand, presented a large band at 600 cm^{-1}, absent in the blanks. Despite the fact that fucoidan supernatant after adsorption (Fuc_Aq) was still absorbing intensely in this region, indicating fucoidan was still present in solution, a very proeminent band of equal wavenumbers was present in avicel after adsorption (Fuc_Avi), indicating the presence of fucoidan in this solid. FT-IR is not a good method for quantitative analysis; therefore, no conclusions regarding the partition of fucoidan between the two phases – the adsorbent and the supernatant – could be retrieved.

III.d.2.2. Seaweed phenolic compounds-Avicel adsorption

The adsorption of different classes of phenolic compounds to cellulose (Avicel) was evaluated as represented in Figure 30.

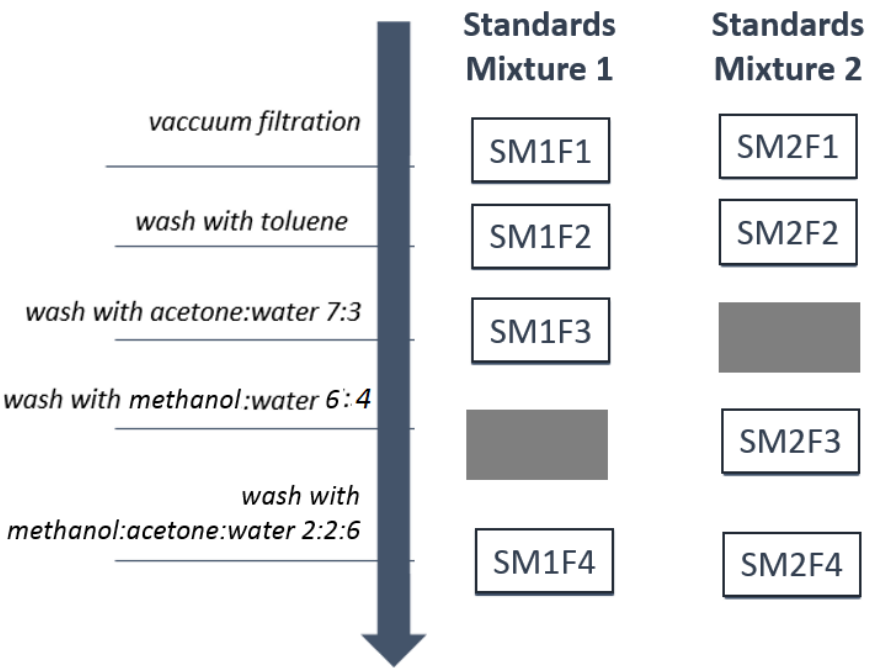


Figure 30 - Fractions collected after contacting two solutions of phenolic compounds (see Materials and methods for composition) with Avicel, and eluting the supernatant as well as washing with different solvents.

Specifically, the adsorption of a phenolic acid (gallic acid), a simple phenol (phloroglucinol, phlorotannins' percursor), a phenolic aldehyde (vanillin), a flavonol (quercetin) and a flavan-3-ol (catechin) were studied. The dry weight of Standards Mixture 1 (SM1) and of the fractions obtained after the adsorption experiment was determined (Table XVIII).

Table XVIII – Gravimetric determination of total dissolved solids in the initial mix of phenolic compounds and in the fractions obtained after adsorption.

Eluent	Fraction	Mass (mg)	%
-	**Initial**	8.67	100
-	**SM1F1**	7.55	87.1
Toluene	**SM1F2**	0.147	1.7
Acetone:Water 7:3	**SM1F3**	0.218	2.5

Methanol:Acetone:Water 2:2:6	**SM1F4**	1.096	12.6
	total	9.011	103.9

Clearly, most of the phenolic compounds mass was recovered from the solvent (SM1F1), with little adsorption. Also, it could be observed that 30% (V/V) of water in acetone (SM1F3) was not enough polar to desorb the small fraction that was adsorbed – indicating that the bonding between cellulose and the adsorbed compounds was relatively strong. The compounds were recovered only when Methanol:Acetone:Water 2:2:6 were used to wash the Avicel (SM1F4). The specificities of this adsorption-desorption dynamics might allow separations of not only polysaccharides *vs.* phenolic compouds, but also of different phenolic compounds classes, by designing the solvent composition for washing the adsorbent-adsorbate matrix. For that reason, we attempted to understand what compounds were being more adsorbed, by analysing the fraction using UV-Vis spectrometry.

Initially, UV-Vis spectra were recorded for the mixture of the five phenolic compounds. However, variations of individual compounds could not be detected due to extensive overlapping of the absorption peaks (Figure 31). Thus, the two most confounding compounds for UV spectrophotometry (gallic acid and vanillin) were removed from the mixture, and the experiment was repeated. This time, each standard solution was scanned by UV-Vis spectrophotometry, so that their massic extinction coefficient could be experimentally determined (Figure 32).

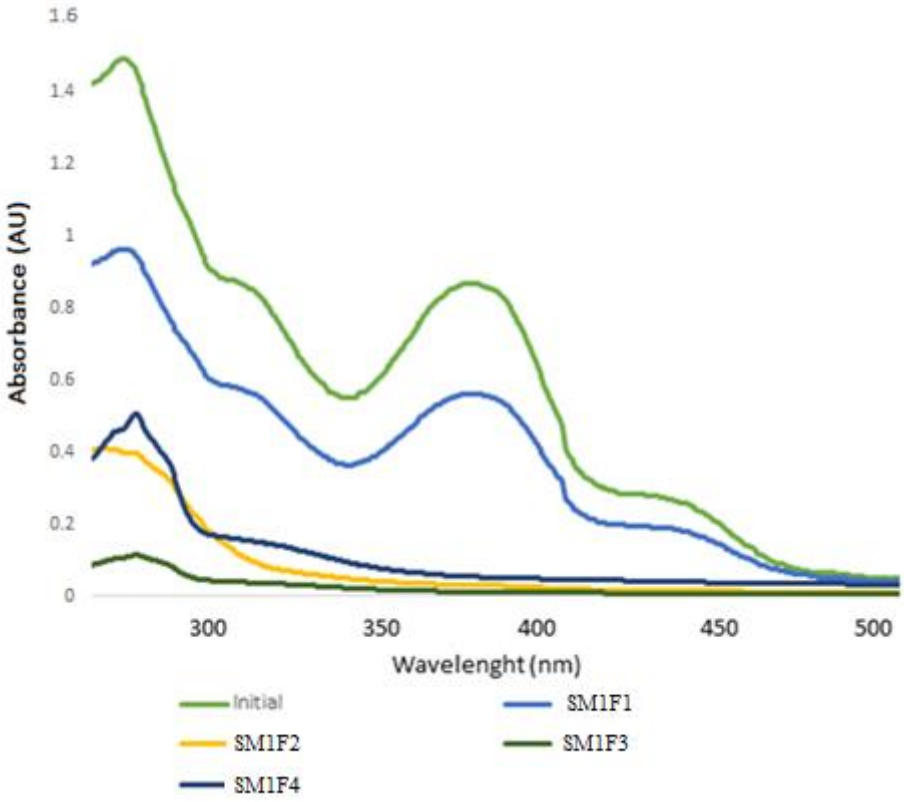


Figure 31 – UV-Vis spectra of the five fractions along the adsorption of a mixture of gallic acid, phloroglucinol, vanillin, quercetin and catechin. Due to overlap, assesment of each compound's presence was difficult and a simplified version of the experiment repeated.

A multiple regression fitting algorithm was then applied in R Statistical Software, to simultaneously compute the absorbances in the 234-430nm range of wavelenghts registered in each fraction and the extinction coefficients of the three compounds (εP, εC and εQ) for the same range of wavelenghts. The concentration of phloroglucinol ([P]), catechin ([C]) and quercetin ([Q]) contributting to the spectra final configuration was theoretically determined according to the equation:

$$Abs\_total(\lambda i) = \varepsilon P(\lambda i) * [P] + \varepsilon C(\lambda i) * [C] + \varepsilon Q(\lambda i) * [Q]$$

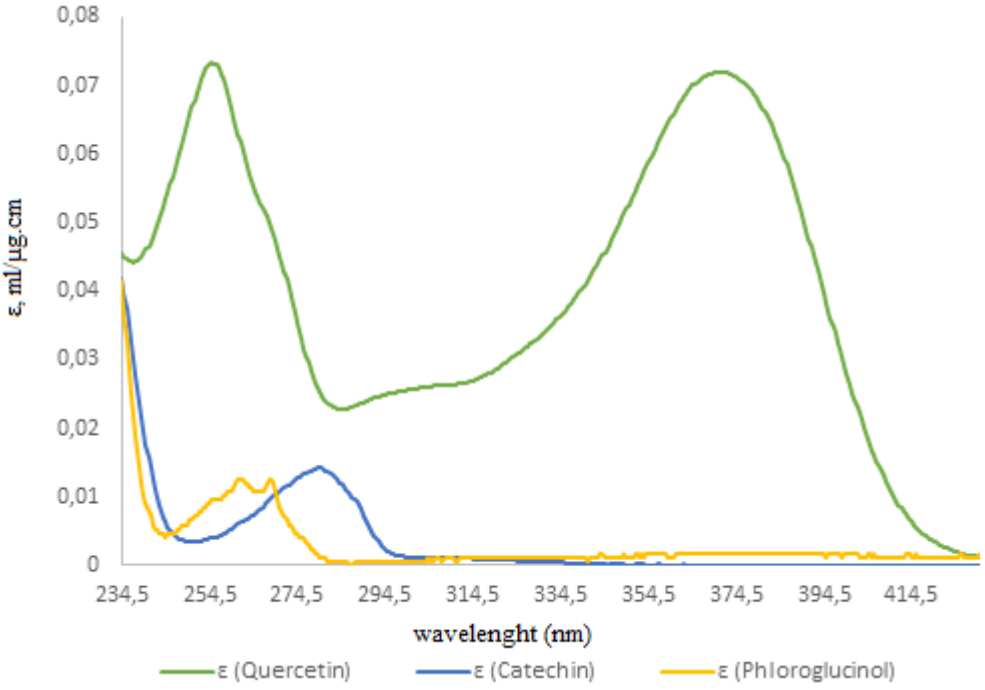


Figure 32 – Massic extinction coefficients of the three phenolic compounds used in the second round of adsorption on Avicel.

Yet, no model that was compatible with the condition $[P] + [C] + [Q] = [total]$ with [total] being the total amount of dissolved solids determined by gravimetry could be obtained. Thus, the better fit was chosen (r^2 = 0.864) for qualitative description. However, the results seemed inadequate, since they were very different from those obtained by gravimetry (Table XIX). Although gravimetric determination might be influenced by several aspects, such as the the residual moisture of the sample, even upon the consideration that our gravimetric determinations could be overestimated the difference between these results and those of UV-Vis spectrophotometry suggests the latter was not sufficiently sensitive. Actually, visual inspection for quercetin (which stains yellow) clearly demmonstrated its elution from the avicel matrix when methanol:water 60:40 was passed through (SM2F3), but a very low concentration of this compound is detected by UV-Vis – or, at least, is recognized in the fitting of models to explain the spectra. For that reason, further studies regarding alternative methods to quantify the diffferent molecules in our solutions had to be though of and tested.

Table XIX – Mass percentage of each phenolic compound and of total dissolved solids as determined by UV-Vis spectra modeling or by gravimetry.

	Fraction	From UV				From gravimetry
		Quercetin	Phloroglucinol	Catechin	%	%
Initial	-	100				
Filtrate	SM2F1	96.82	55.50	91.70	89.1	69.5
Toluene	SM2F2	0.05	1.04	0.25	0.3	3.2
Methanol:H_2O 6:4	SM2F3	0.20	0.30	2	1.1	17.5
MetOH:Acet:H_2O 2:2:6	SM2F4	0.05	0.23	0.50	0.3	9.8

Nonetheless, some information was obtained by these two experiments: first, that alike polysaccharides, phenolic compouds can be retained in Avicel (gravimetric, spectrophotometric and visual evidence); second, that many of the phenolics are nonetheless recovered in the filtrate, even at Avicel concentrations thought to be sufficient to adsorb the polysaccharides (potentially leading to the first enriched fraction); third, that acetone:water 7:3 was not only too hydrophobic to desorb polysaccharides, but it was also too hydrophobic to desorb phenolics; fourth, that the higher recovery of phenolic compounds from solvent washes was obtained at a 60 volumic concentration of methanol (see table XIX); fifth, that of the phenolics studied, phoroglucinol seemed to be the one that is safe to affirm that has been adsorbed.

III.e. Conclusions

In this work, several experiments were carried out to study the use of cellulose as the adsorbent for the separation of seaweed polysaccharides and phenolic components, which are frequently co-extracted. Despite being a procedure preliminarily reported in the literature the basal features of this system were largely unknown and for that reason, this work had to be initiated by testing these phenomena in a simple approach using commercial standards.

Cellulose demonstrated a good potential to be used as an adsorbent for the separation of polysaccharides and phenolic components of algal origin. The results of this study strongly suggest that phenolic components adsorb to cellulose differentially and at very inferior rates to those of polysacharides; also, fucoidan was shown to adsorb in cellulose, which was a critical feature for this system to work. Moreover, we found evidence that recovery of the adsorbate might be possible to achieve by eluting the compounds with different, specifically designed solvent schemes. Importantly, cellulose is a biopolymer widely available, providing a sustainable basis for the implementation of this methodology at larger scales.

Future work towards the development of this method involves the detailed characterization of the adsorption kinetics in a larger set of biomolecules, in which phlorotannins must be included, since their polymeric chains of phenolic moieties might present some very intricate properties concerning interaction with cellulose. Besides, the effect of external variables such as pH, temperature, mechanical stirring, ionic strenght and others should be explored to better understand and to optimize this methodology. Also, a proof-of-concept by applying the method to real extracts from seaweed and characterizing/quantifying the partition of compounds between fractions by HPLC should provide the evidence to motivative the further development of the technique into an industrially viable option.

CONCLUDING REMARKS

The transference of biotechnologies from the research labs to the industrial facilities is what converts the investment in applied sciences into benefits for mankind – both economic and societal. In this thesis, the subject of Seaweed Phenolic Compounds Bioprospecting and Valorization has been tackled, since it has been identified as a very important piece of the Bioeconomy puzzle, for which a big picture was lacking and technical challenges were being overlooked. In this context, a critical analysis of the state-of-the-art, accompanied by a compilation of parameters and results published regarding seaweed phenolic extracts obtainment, processing and analysis has been conducted, resulting in a) systematization of consensual methodologies, of misconceptions and of current trends in seaweed biotechnology research and b) identification of the priority-challenges to be overcomed for a seaweed bioindustry to be developed on the basis of seaweed phenolic compounds.

Essentially, extraction methodologies were found to be the root of most of the problems associated to the slower-than-expected valorization of these components. Currently, aqueous mixtures of alcohol and acetone are consensually being used in the extraction of seaweed phenolic compounds, which results in extracts of high complexity, and which contain polysaccharides and other metabolites in quantities that impair the isolation and characterization of novel phenolic compounds from seaweed – e.g., the HPLC separation and quantification of phlorotannins. Also, this solvent composition is not compatible with industrial volumes of biomass processing – due to environmental and financial reasons. Further, methods to fractionate these extracts and obtain phenolic compounds-enriched fractions are insufficient in preparing the extracts for analytical platforms. It is thus of imperial importance to develop novel extraction and primary fractionation methodologies, that potentiate both the generation of solid scientific data by researchers and the industrial exploration of seaweed biomass.

This document is therefore a practical guide on the subject of seaweed phenolic compounds biotechnology, unlike any other document publicly available. More importantly, procedural information is accompanied by a critical discussion of its contribute for the big picture, providing a resource to better identify adecquate methodologies and emerging problematics worth the efforts of future research.

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