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Exploring Sustainable Extraction Techniques and Analytical Approaches for Giant Kelp Processing

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Exploring Sustainable Extraction Techniques and Analytical Approaches for Giant Kelp Processing

By

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Abstract

The rising global population, coupled with environmental stresses induced by climate change, is exerting extreme pressure on production systems globally, driving the quest for sustainable sources of macro-and micronutrients intended for industrial applications. Macroalgae, or seaweeds, are macroscopic marine algae that hold promise as a sustainable biomass resource with a wide range of potential industrial applications. Their composition, wide availability, and growth-associated environmental benefits (i.e., carbon sequestration, support to fishery habitats, and reduced dependence on freshwater, arable land, and fertilizer) make them of particular interest.

These marine organisms contain a range of desirable compounds with the potential for use in human nutrition, animal feed, production of bio-stimulants or fertilizers for plant growth, biotherapeutic, cosmetic, and food applications. Included in these compounds are carbohydrates (laminarin, fucoidans, alginate), lipids, proteins, and phenolics, which exhibit functional attributes such as emulsification and gel formation properties, as well as bioactive properties such as antioxidant, antihypertensive, and antidiabetic effects. Full utilization of macroalgae's potential hinges on the development of sustainable bio-guided downstream processing strategies which make use of structure and functionality as the benchmark to develop processes capable of both maximizing the extractability of its diverse compounds and safeguarding the functional and biological attributes inherent in the algae extracts. The major goal of this research project was to uncover the effects of pivotal processing conditions (i.e., biomass-to-water ratio, temperature, pH, time) and various extraction methods (i.e., aqueous, enzymatic, and microwave-based extractions) on the extractability, structural composition, and functional/biological properties of the major compounds of the giant kelp species *Macrocystis pyrifera*, typically found in the Pacific Ocean.

The overarching aim was to develop effective and sustainable extraction methods founded on the interplay between structure and functionality to produce algae compounds with the desired properties.

Chapter 1 delves into the current state of the brown macroalgae industry and prevailing extraction methods. It encompasses discussions on environmentally sustainable practices employed in cultivation, harvesting, and industrial integration. This chapter also delves into the intricate structural makeup of brown macroalgae and the multifaceted functional and biological attributes inherent in their compounds. The final section of the chapter offers an in-depth overview of the cutting-edge methodologies employed in the extraction of compounds from macroalgae.

In Chapter 2, the aqueous extraction process (AEP) and enzyme-assisted aqueous extraction process (EAEP) are explored as sustainable, environmentally friendly strategies for extracting the diverse compounds within the macroalgal matrix. A series of experiments beginning with the AEP investigating pH, biomass-to-water ratio (BWR), time, and temperature were used to guide the EAEP. Based upon yield of various components of interest (laminarin, fucoidan, alginate, protein, phenolics), extraction conditions were selected for more in depth analysis looking into their structure (amino acid composition, phenolic profile, monosaccharide and oligosaccharide analysis, alginate characterization by FTIR) and bioactivity (antioxidant, antidiabetic, antihypertensive). These analyses revealed the mechanisms by which enzymes enhance the degradation of the macroalgal matrix, thereby increasing the extractability of intracellular components (i.e., laminarin, intracellular proteins and peptides), and resulting in the production of extracts with higher antihypertensive activity.

Chapter 3 builds upon the results of Chapter 2 by investigating the impact of integrating microwave processing with the AEP and EAEP. Through a series of stepwise experiments,

favorable microwave-assisted extraction (MAE) and microwave-enzyme-assisted extraction (MEAE) conditions were determined based upon yields. Dipole-interactions caused by the microwave resulted in rapid breakdown of the macroalgal matrix resulting in high yields with extractions as short as 15 minutes. As in chapter 2, these favorable conditions underwent more in-depth analysis.

Conventional macroalgae processing strategies are deemed unsustainable because they use hazardous solvents. The series of studies detailed in this report outlines the potential of sustainable processing strategies for giant kelp, an important step for maintaining the environmentally friendly status of these marine organisms. Of special note is the ability of the MAE to produce comparable extracts (i.e., similar yield and activity) in 15 minutes, in contrast to the AEP, which requires 6 hours. This highlights the potential for industrial scale use, as it enables multiple extractions to be performed daily. Additionally, these studies shine light on the composition of *Macrocystis pyrifera*, providing more detail than previously seen in literature. While this giant kelp may be low in phenolics, its other functional and bioactive components render it highly intriguing. Of note is the high antidiabetic activity of all extracts. These insights, along with future work on scaling up the extraction strategies discussed herein, underscore the potential of sustainable and bio-guided downstream processing strategies to introduce this sustainable feedstock to industries worldwide.

Acknowledgements

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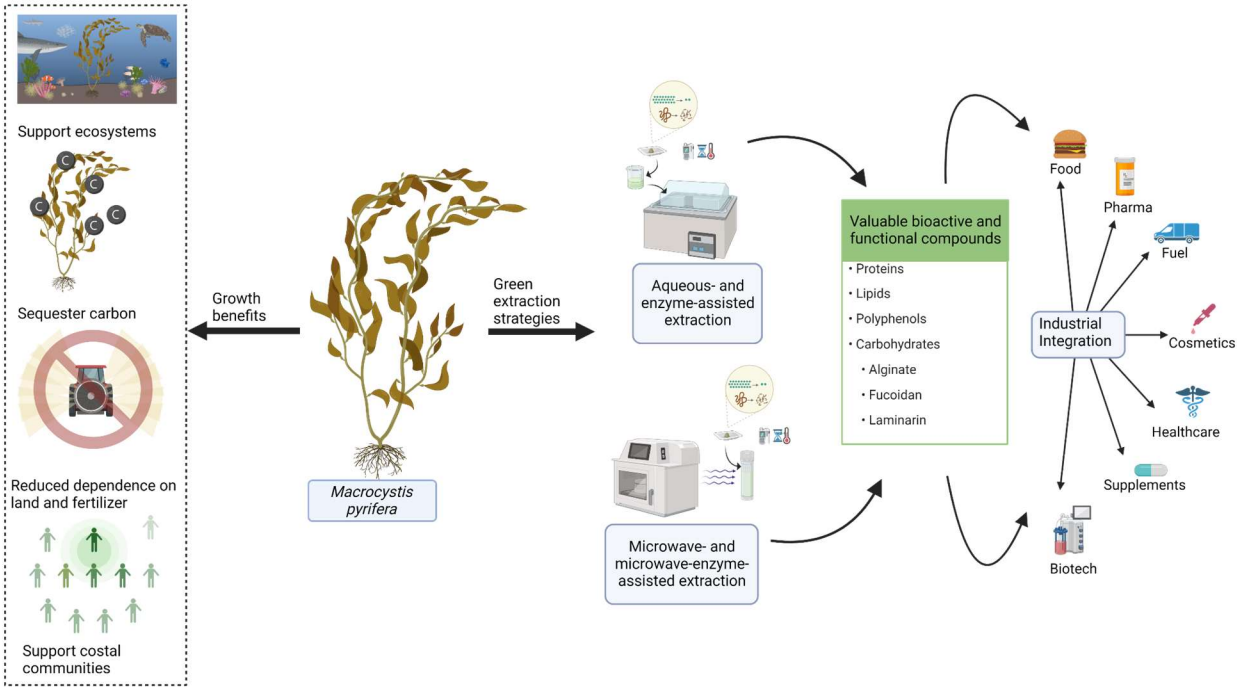
Table of Contents

Glossary

AA: Amino Acids
AAPH: 2,2'-Azobis(2-methylpropionamidine) dihydrochloride
ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE: Angiotensin-converting enzyme
AEP: Aqueous extraction process
AP: Alkaline protease
BWR: Biomass-to-water ratio
CBL: Acidic carbohydrase
DI: deionized
EAEP: Enzyme-assisted aqueous extraction process
FD: Freeze-dried
FTIR: Fourier-transform infrared spectroscopy
GAE: Gallic acid equivalent
GC-FID: Gas chromatography flame ionization detector
HCl: Hydrochloric acid
HPAEC-PAD: high-performance anion exchange chromatography with pulsed amperometric detection
LC-MS: liquid chromatography mass spectrometry
LC-MS/MS: Liquid chromatography-tandem mass spectrometry
ORAC: Oxygen radical absorbance capacity
MAE: Microwave-assisted extraction
MEAE: Microwave enzyme-assisted extraction
NaOH: Sodium hydroxide
NP: Neutral protease
PI: Isoelectric point
RO: reverse osmosis
SFE: Supercritical fluid extraction
SWE: Sub-critical water extraction
TPC: Total phenolic content
TPE: Total protein extractability
UAE: Ultrasound assisted extraction

Chapter 1: *Macrocystis pyrifera* as a biomass in a circular economy: recent advances in cultivation, processing, and industrial integration

Graphical Abstract



Created with BioRender.com

Abstract

The escalating pressure on existing production systems due to an increasing world population, limited availability of arable land, and increasing atmospheric carbon dioxide concentration has prompted the search for sustainable sources of macro-and micronutrients for use in industrial applications. Macroalgae or seaweeds are macroscopic marine algae that have been traditionally consumed in Asian countries for centuries. Because of their composition, wide availability, and growth-associated environmental benefits (i.e., carbon sequestration, support to fishery habitats, and reduced dependence on freshwater, arable land, and fertilizer needs), macroalgae has been highlighted as a potentially sustainable biomass feedstock for the production of a wide range of polysaccharides (i.e., laminarin, fucoidans, alginate), growth hormones,

proteins, and phenolic compounds (phlorotannins) which could potentially be used in human nutrition, animal feed, production of bio-stimulants or fertilizers for plant growth, biotherapeutic, cosmetic, and food applications. However, full utilization of macroalgae's potential relies on the development of bio-guided processing strategies able to not only maximize the extractability of the diverse compounds, but also preserve the functional and biological properties of the extracts. This review highlights the use of brown macroalgae, focusing on the giant kelp *Macrocystis pyrifera* when possible, as a feedstock to produce added-value compounds. The scope encompasses sea farming, harvesting systems, and downstream processing conditions, with emphasis on innovative attempts to develop structure/function-based extraction methods to produce components with the desired properties.

1.1 Introduction

Growing recognition of the challenges associated with feeding an increasing world population, while facing escalating environmental challenges (e.g. limited availability of arable land, increasing atmospheric carbon dioxide concentration, changes in rainfall and temperature patterns), has prompted coordinated efforts between farmers, researchers, policymakers, and the private sector to transform current agricultural systems to be more climate oriented, a needed step to promote food security (1,2). Considering that agriculture accounts for 19-29% of total greenhouse gas emissions (2), innovative strategies to identify and utilize alternative sources of macro-and micronutrients that could be used as feedstocks for food, feed, and other industrial applications are of key relevance for the development of more sustainable food systems.

Seaweed or marine macroalgae have recently been proposed as a potentially sustainable feedstock for use in the development of such a circular economy, as shown in Figure 1.1. Although

macroalgae have been consumed in Asian diets for centuries, it was not until recently that increasing interest in macroalgae-derived products (i.e., carbohydrates, proteins, lipids, polyphenols, plant growth hormones) has spurred intrigue into these organisms and their mass cultivation (3–5). Because macroalgae can grow in aquatic environments at a faster pace than terrestrial crops (5), do not compete with existing crops or infrastructure for land space and fertilization, and are estimated to absorb an estimated 20 times more carbon dioxide per acre than forests on land, their cultivation has been seen as a powerful strategy to store greenhouse gasses and mitigate the effects of climate change, resulting in environmental and societal benefits that could support coastal communities (6,7).

Figure 1.1: Role of *Macrocystis pyrifera* in a circular economy. Created with Biorender.com.

In that view, seaweed production has the potential to make progress towards many of the UN sustainable development goals. According to the seaweed manifesto (8), only 2% of our global food supply comes from oceans, which cover 71% of earth. Ocean cultivation of nutrient-dense seaweeds, ongoing research on medical applications and dietary supplements, and the potential creation of new jobs can help make strides toward zero hunger goals, efficient work and economic growth, and reduced inequalities. However, there are cost-effectiveness and feasibility challenges associated with algal cultivation, harvesting, and processing that need to be overcome to reach the full potential of these marine crops.

Macroalgae can be produced by both wild and farmed cultivation methods. While wild production has remained relatively stable, macroalgae farming has increased greatly in recent years. Reports from the Food and Agricultural Organization for the UN found that global macroalgae production increased by more than 6000% from 1950 to 2019, with nearly 36 million tons of macroalgae produced in 2019, and is projected to continue growing (9). According to Global Market Insights, the compound annual growth rate (CAGR) for the seaweed market from 2021-2027 is projected to be 9.6% (6), with the main drivers for the expected seaweed market growth stemming from increased interest in their functional and biological properties (6,7). While current use of seaweed compounds include drug delivery systems for pharmaceuticals, bioplastic applications, thickening agents, dietary supplements, animal feed and supplements, ingredients in cosmetic products, bio-stimulants, and soil amendments, ongoing research will likely identify new uses and applications for macroalgae's diverse compounds (10).

Macroalgae are mainly classified as red, green, and brown. Brown macroalgae, or Phaeophyta, accounted for over 17 tons of algae production in 2019 (9). They are the second most abundant type with over 2000 species (11), have rapid growth, and contain compounds with

attractive properties. On a dry basis, they are composed of 34-76% carbohydrates, 1-27% proteins, 0.5-3.5% lipids, and 9-41% ash (12). The polysaccharide portion consists of mannitol (5-30% dry basis), cellulose (2-7% dry basis) as well as several polysaccharides of interest, including alginates (10-40% dry basis), fucoidan (5-10% dry basis), and laminarin (22-49% dry basis) (4,12). These compounds possess a wide range of desired functional properties, like gelling and emulsification, and biological properties including anti-inflammatory, immunostimulatory, antioxidant, anticoagulant, antiviral, antiproliferative, antiapoptosis, and antitumor (2).

Although current research has mainly focused on the extraction and use of major macroalgae compounds such as alginate and health promoting carbohydrates like fucoidans, niche research areas with high added value, such as those related to the extraction and use of macroalgae proteins and lipids for human and animal nutrition and other cosmetic applications, will likely increase. However, the development of structure-function based methods needed to maximize the extractability and desired functional and biological properties of macroalgae compounds is in its early stages and will require a comprehensive assessment of current findings and challenges to identify future pathways leading to the development of a cascade biorefinery approach to extract as many of these compounds as possible without impacting their functionality.

In that regard, the development of a biorefinery concept to maximize extraction and applications of algae compounds will likely require a more holistic understanding of the impact of key processing conditions (i.e., disruption of starting material, solids-to-liquid ratio, temperature, reaction time, slurry pH, wet vs. dry extraction) and methods (i.e., aqueous, enzyme assisted, microwave assisted, and subcritical water extractions) on the extractability and structure-function relationship of the extracted compounds. Understanding these relationships, which will also require the use of high-throughput analytical methods, would allow for the development of

structure-function based methods able to not only maximize the extractability of these compounds but to produce them with the desired functionality (Figure 1.2).

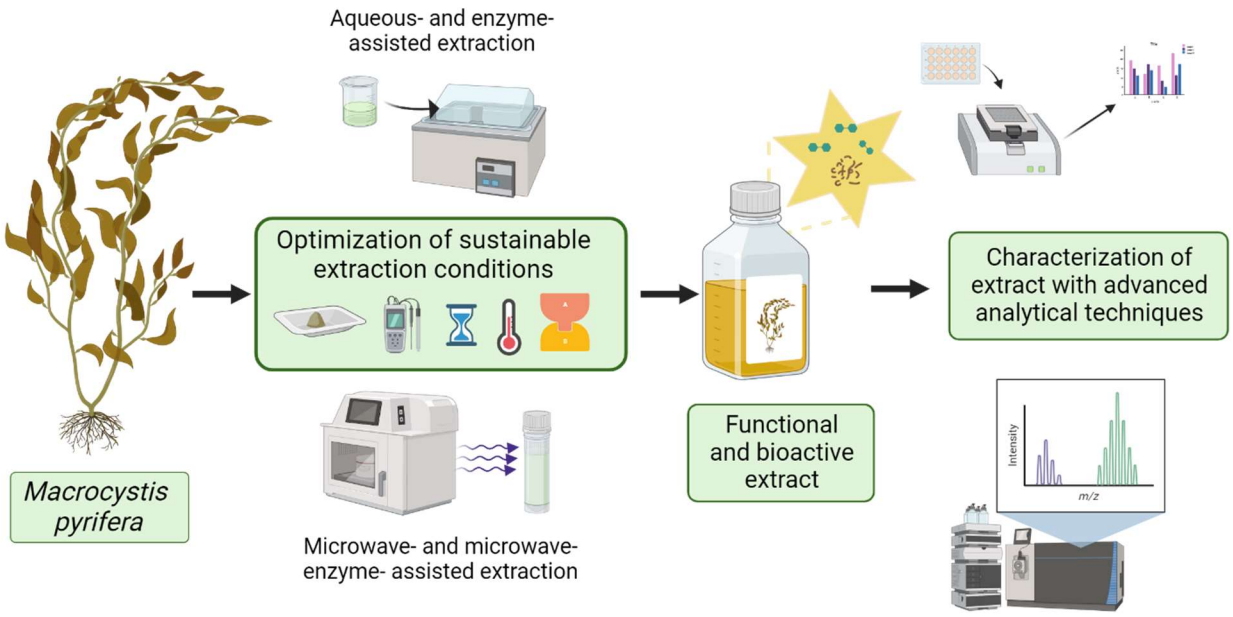


Figure 1.2: Integrating sustainable processing strategies and analytic techniques to maximize the extractability of macroalgae compounds with desired functional and biological properties. Created with BioRender.com.

Albeit different seaweeds species are briefly discussed, this review focuses on the potential use of brown seaweed, specifically the giant kelp *Macrocystis pyrifera*, as the substrate to produce added value compounds for industrial applications and the challenges associated with the development of cost effective and scalable strategies to extract and fractionate target compounds with desired functional and biological properties. The goal of this review is to demonstrate how brown macroalgae fits into a circular economy. This is accomplished by diving into the environmental benefits, industrial integration opportunities, and sustainability aspects associated with macroalgae cultivation and harvesting, and by highlighting the impact of downstream processing parameters and methods on the extractability and structure-function relationship of macroalgae compounds, as well as the potential uses of extracted compounds.

1.2 Environmental sustainability of macroalgae: from cultivation and harvesting to integration with aquaculture and other industries

Macroalgae production and biomass drying are the most energy intensive steps in the seaweed biorefinery (13), spurring research to make these steps more efficient and sustainable. Macroalgae production is a multi-billion dollar industry with over 290 species cultivated worldwide and an annual growth rate of around 10%, with projections to reach 200,000 tons by 2027 (14). This mass-cultivation has the potential to displace a large percentage of terrestrial biomass cultivation to produce sustainable biomaterials.

Cultivation systems include intertidal (the area where the ocean and land meet tides) fixed lines with both vertical and horizontal ropes, floating bottom farms, and growth in cages (15,16). These systems have the potential to scale up for large-scale ocean cultivation. However, existing systems for cultivation and harvesting are labor intensive and require further optimization (15). Cultivation begins in the lab with the release of spores from collected fertile seaweeds (17). The biggest bottle neck in cultivation is the production of juvenile sporophytes on ropes for introduction into marine environments (15). Macroalgae cultivation brings some key advantages compared with terrestrial biomass as they do not require land or freshwater resources and perform photosynthesis 4 times more efficiently (18). However, to fully utilize the potential sustainability of these organisms a thorough understanding of their cultivation characteristics and suitable locations is essential before pursuing further expansion. While not all countries have access to ocean waters necessary for cultivation, diversifying the growing locations of macroalgae will make sourcing the biomass more localized and efficient. Considering that, currently, the majority of the cultivation market is located in Asia, which could lead to early saturation of these waters (19).

Many coastal waters around the world could represent an untapped potential for seaweed cultivation and are a great option for increased production of these marine crops.

Marine macroalgae cultivation is beneficial for marine ecosystems. These marine crops aid in controlling pollution through bioremediation (i.e., removal of contaminants, toxins, and pollutants from the environment by living organism) and monitoring eutrophication (when a body of water becomes overly enriched in nutrients) due to their nutrient intake of nitrogen and phosphorus (20). Such benefit is exemplified by integrated multi trophic aquaculture (IMTA), which combines fish farming with the cultivation of other organisms able to clean the water like kelps and filter feeders that take up the inorganic and organic waste, respectively (21). This system allows for sustainable fish farming as well as macroalgae cultivation and is one example of how the cultivation of macroalgae can be beneficial for our ecosystems and integrate sustainability into a variety of industries.

When selecting species for cultivation, species with high growth rates are often preferred. *Macrocystis pyrifera* is one of the fastest growing biomasses on earth, with a growth rate of up to 2 feet per day (22). It is also important to understand the optimum growing conditions of the various seaweeds as different farm sites will have different conditions that will favor some species over others (21). Light intensity, current flow rate, and nutrient availability are important cultivation parameters that affect macroalgae growth (21). Optimum harvest processes (crop percentage, timing, methods) differ from species to species (21) and are an important factor when considering optimum growth and cultivation. While frequent harvest can help solve issues with growth limitations, it results in increased processing costs and logistics (16). The harvesting process can be done manually by using nets to uproot seaweed or cutting the biomass off

cultivation lines. Alternatively mechanical harvesting methods involve specialized harvesters equipped with rotating blades and cutters, requiring boats or ships to operate them (16).

Ongoing research projects are continually finding new ways to commercialize these crops and utilize macroalgae. While they have been part of the human diet in Asian countries for centuries, more algae-based food products are steadily coming on the market, spanning a ranging of offerings such as pastas, savory snacks, and even thickening agents in ice cream. Furthermore, algae are also being used as supplements, owing to their various bioactive properties that can offer potential health benefits (23). Sulfated polysaccharides like fucoidan have found application in the pharmaceutical industry. Alginate, recognized for its bio-adhesive properties, has been instrumental in wound care, while various other algal polymers have been harnessed as scaffolds in tissue engineering (23). Moreover, the bioactive compounds present in macroalgae have been used in cosmetics to aid in reducing blemishes and brightening and firming the skin (24).

Within the feed and agricultural sector, the incorporation of macroalgae additives has been linked to higher meat and egg quality while the utilization of soil amendments and bio-stimulants has demonstrated the capability to enhance early seed germination and augment overall plant biomass (23). Residues leftover after industrial applications have potential for uses in bioenergy production (23) and fertilizers (26), closing the loop on the circular economy and making macroalgae a zero waste feedstock. However, it's worth noting that macroalgae can accumulate heavy metals from their surrounding environment (24). Consequently, maintaining vigilant monitoring of these metal levels becomes imperative for all consumable products containing macroalgae. Overall, macroalgae has great potential as a feedstock in a circular economy to help keep our oceans healthy and increase sustainability in a wide variety of industries from agriculture

to pharmaceuticals. However, careful research is needed to develop sustainable and scalable strategies to cultivate, harvest, and process macroalgae at a global scale.

1.3 Understanding the structural complexity and composition of brown macroalgae species and their functional and biological properties

The brown macroalgae matrix contains a diverse range of compounds (e.g., proteins, cellulose, hemicellulose, alginates, fucoidan, and polyphenols), as shown in Figure 1.3, with unique functional and biological properties. The development of sustainable processing strategies for extracting compounds from *M. pyrifera* and other species will require a deeper understanding of the impact of innovative processing strategies on the structural composition and biological properties of these bioactive compounds, which will require careful processing optimization of the aforementioned methods for enhancing process feasibility.

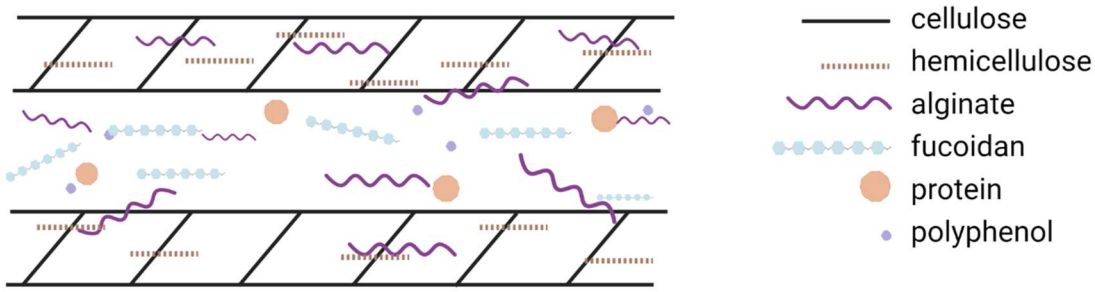


Figure 1.3: Schematic representation of brown macroalgae cell wall. Image based on (12) created with BioRender.com.

Compound characteristics, extraction conditions, and analytical methods are discussed for major brown macroalgae compounds based on existing literature. *Macrocystis pyrifera* data is reported when available.

1.3.1 Proteins

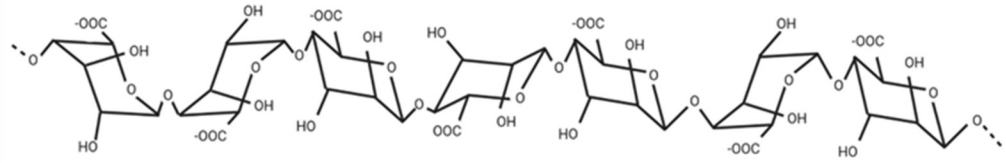
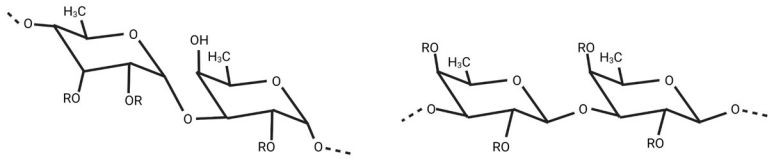
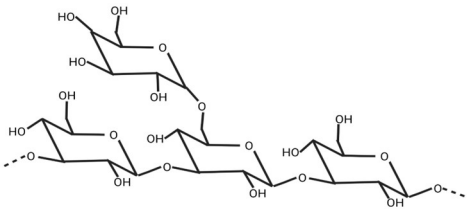
Literature and research on protein extraction from brown macroalgae is limited due to the focus on the extraction of these species' plentiful carbohydrates. Brown algae protein content

ranges from 5 to 15% on dry basis, and it contains all the essential amino acids, making it a complete source of protein for human consumption (27,28). The majority of macroalgae proteins are produced intracellularly and protein extractability is favored by the selection of extraction conditions that favor the solubility and diffusion of the protein from the algae matrix to the extraction medium (29). In that view, tailoring protein extractability requires identifying: i) upstream disruption strategies of the cell wall to facilitate the release of the intracellular proteins to the extraction medium; ii) the isoelectric point of the specific protein to further select pH values that will maximize protein solubility and extractability; and iii) the impact of solids-to-liquid ratio on the overall extractability. Aqueous *(*AEP) and enzyme-assisted aqueous extraction processes (EAEP), that can simultaneously extract a variety of compounds without the need for flammable toxic solvents, have been used to extract proteins from plant-based materials (30,31) and are beginning to be exploited in macroalgae downstream processing. Enzymatic extraction of *M. pyrifera* proteins with the use of a 10% (v/w) cellulase preparation in extraction solution at pH 4.5, 50 °C, and 16 hours achieved extraction efficiency of 74.6%. The extracted proteins exhibited noteworthy bio activity, including antioxidant properties (31).

1.3.2 Carbohydrates

As shown in Table 1.1, *Macrocystis pyrifera* contains several carbohydrates of interest (alginate, fucoidan, and laminarin) due to their functional and biological properties. In addition to these carbohydrates of interest, brown macroalgae also contain significant amounts of mannitol, a sugar alcohol acting as a carbon storage, comprising up to 30% of the macroalgae biomass on a dry basis (32).

Table 1.1: Structure and properties of *M. pyrifera* carbohydrates

Alginate	
Structure	 α-1-4–guluronic acid (G) and β-1-4-mannuronic acid (M) residues forming GG, MM, and M/G blocks
Molecular Weight	76 – 396 kDa
Properties	Gelling, stabilizing, and thickening agents, antioxidant, antibacterial, antidiabetic, antiobesity, and antitumor properties
References	(33–36)
Fucoidan	
Structure	 1-3- and 1-4-linked-α-fucose. R = SO_3^- or H
Molecular Weight	10- 10,000 kDa
Properties	Antioxidant, antitumor, immunostimulatory, anti-inflammatory, anticoagulant, anticancer antibacterial, and antiprotozoal activities
References	(36,37)
Laminarin	
Structure	 β-1-3-glucose backbone with branching β-1-6-glucose units
Molecular Weight	5 – 7 kDa
Properties	Antitumor, antioxidant, anti-inflammatory, immunostimulatory, anticancer
References	(38,39)

Images based on Jonsson et al. (40) and created with BioRender.com.

- Alginates comprise up to 40% of the carbohydrates of brown macroalgae. These linear polysaccharides are composed of β-mannuronic acid (M) and α-guluronic acid (G) (27). Their properties result from the specific ratio of M and G units present within them (41). Alginates are responsible for much of the flexibility in macroalgae, which is important for withstanding marine currents, and are currently used in the food and biomedical industries (27,41). Much of their application is based on the ability of the gels they form to stabilize emulsions (42). Alginate extraction is commonly performed under acidic conditions for several hours (1-24 h) (43,44). However, these conditions can result in degradation of bioactivity of the alginate and of other compounds, like fucoidans (41). Several studies have investigated alginate extraction from *M. pyrifera* with different processing conditions producing alginate with different properties. Extraction in 1 N HCl (pH 2) at room temperature for 1 h followed by a sodium carbonate treatment resulted in around 25% yields with a M/G ratio of 1.29 (45). Alginate extraction with HCl (pH 1) at 42 °C, for 159 minutes from *M. pyrifera* followed by a 0.2 M sodium carbonate treatment at 45 °C for 120 min on the precipitate resulted in 38.9% yields with a M/G ratio of 1.07 (33).
- Fucoidan are sulfated polysaccharides found in brown macroalgae cell walls (2). They constitute up to 25% of the carbohydrates of the macroalgae (27) and are composed of 1-2 or 1-3-linked α-fucose residues along with other monomers (46). The sulphated components in fucoidan cause it to be a highly bioactive compound and therefore of great interest (27). Fucoidan functionality is affected by the monomer composition, molecular weight, number of sulfated components, and extraction technique. They have been shown to have antiviral, anti-inflammatory, immunomodulatory, antithrombotic, and anticoagulant properties, among others (42). The extraction of fucoidans from *M. pyrifera*

is constrained by limitations that hinder scalability. These challenges arise from prolonged extraction times and the frequent utilization of hazardous solvent. One extraction process involves the use of HCL (pH 1) at 42 °C for 159 minutes, followed by ethanol precipitation. This method results in fucoidan yields of 7.59% based on the dry wight of *M. pyrifera* (33).

- Laminarin account for a substantial portion, up to 35%, of the carbohydrate content within the algal biomass (2,38). Serving as a storage carbohydrate, laminarin is predominantly located within the vacuoles of macroalgae cell (42). Its structure consists of linear β-1-3-glucose units interlinked by β-1-6-linkages, which notably control their solubility characteristics (2). Laminarin have exhibited notable bioactivity, encompassing antibacterial, antioxidative, and anticoagulant properties (42). However, the available literature of extractions of laminarin from *M. pyrifera* remains limited.

1.3.3 Phenolics

Polyphenols are found primarily in the form of phlorotannins in brown macroalgae and they naturally help protect macroalgae from microbes and environmental stresses. They are found within the alginate matrix in the cell wall. A hexane pretreatment followed by water extraction at 55 °C for 4 h resulted in a phenolic extraction with 200.5 mg gallic acid equivalent/ 100 g dry *Macrocystis pyrifera* containing two identified phlorotannins (phloroeckol and a tetrameric phloroglucinol) (47). While the hexane pretreatment is deemed unsustainable, these phlorotannins extracted from *Macrocystis pyrifera*, as well as those from other brown macroalgae, have demonstrated bioactivity including bacterial growth inhibition properties and antioxidant activity (47,48).

1.3.4 Lipids

Given the relatively low lipid content, which falls within the range of 0.45–1.4% (dry basis) in *M. pyrifera* (31), the emphasis on extraction and isolation of lipid compounds is not commonly observed. However, macroalgae lipids are of interest due to their fatty acids, which are esteemed for their superior quality compared to those found in terrestrial plants (49). They have an optimum omega-6 to omega-3 ratio, contain many polyunsaturated fatty acids, and are bio-active (27). However, due to their small compositional contribution, research into lipids extraction for *M. pyrifera* remains limited.

1.3.5 Impact of growing conditions on macroalgae composition

The exact ratios of these compounds and the overall macroalgae composition vary based on growth conditions as they are impacted by seasonality, weather patterns, growth depth, and tidal flows (50, 51). Survival in these highly variable environments means that macroalgae are constantly changing to adapt to these stressors. The ever-shifting biomass composition presents some challenges when developing extraction methods for the compounds they contain. Genetic engineering has the potential to play a role in increasing prevalence of desired compounds with growth in diverse environmental conditions (52).

In environments with lower water motion, such as protected coves, macroalgae adapt by developing larger fronds, which enhance light and nutrient absorption. On the other hand, in environments with higher water motion, like intertidal zones, nutrient absorption rates are naturally higher due to the greater availability of nutrients, which is attributed to the mixing caused by ocean currents, leading to the development of smaller fronds (51).

Such impact of wave conditions has been shown to alter the alginate composition of several brown macroalgae species. For example, *Laminaria longicruris* specimens originating from less

exposed, calmer water have shown lower levels of guluronic acids, rendering them less rigid. Conversely, specimens from regions characterized by stronger currents have been shown to contain higher levels of guluronic acid, thereby enhancing their rigidity to withstand these currents (53).

Temperature changes during winter and summer, which are especially pronounced in shallower waters, have been shown to lead to growth and compositional changes. The maximum photosynthetic rates of macroalgae are temperature dependent. Species that thrive in colder waters reach maximum photosynthetic rates at cooler temperatures whereas those that grow in typically warmer waters reach maximum rates at higher temperatures (54). Therefore, warming temperature trends could have impacts on macroalgae growth as they are pulled from their optimum photosynthetic ranges.

During winter and spring, when nutrients are more abundant, growth rates of macroalgae accelerate, allowing them to accumulate higher nitrogen reserves. These reserves, frequently stored in the form of proteins (54), serve as resource for the upcoming summer months (55). Summer samples of *Macrocystis pyrifera* have been shown to be higher in amino acids and minerals, whereas total carbohydrate content remained relatively the same between seasons (56). This study, however, did not investigate the composition of carbohydrates. It is possible that while the total amount of carbohydrates is comparable, the breakdown of specific carbohydrates (i.e., fucoidan, laminarin, and alginate) may have changed.

1.4 State-of-art extraction and utilization of macroalgae compounds and their corresponding functional and biological properties

Conventional extraction methods for obtaining biologically and functionally active compounds (Figure 1.4) from macroalgae are time-intensive and involve the use of hazardous

organic and flammable solvents like methanol, ethanol, and formaldehyde to remove lipids and other pigments prior to the extraction of alginates and other compounds (57–59). The utilization of such solvents poses environmental, health, and safety hazards requiring additional downstream processing for their removal prior to consumption, which leads to increased operational costs associated with the additional unit operations (57–59).

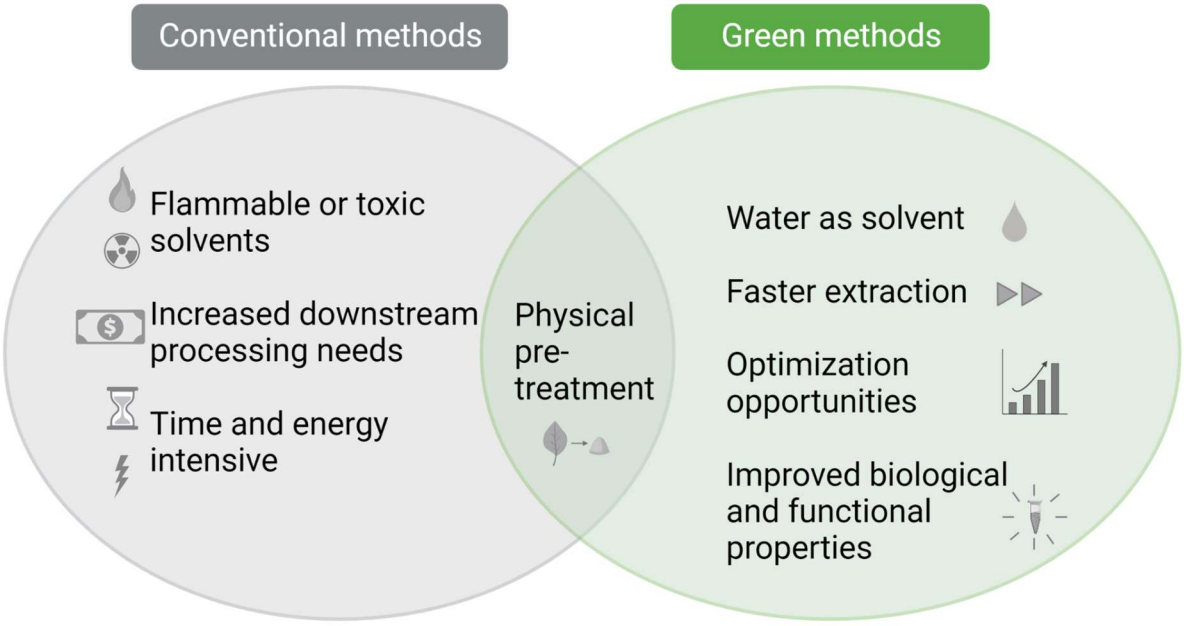


Figure 1.4: Comparison of conventional and green extraction methods. Created with Biorender.com.

Therefore, advancing research in the realm of downstream processing of macroalgae should focus on developing environmentally friendly, alternative methods that circumvent the reliance on hazardous solvents for extracting biologically and functionally active compounds.

However, the implementation of greener extraction techniques must be coupled with a detailed understanding of the impact of various sustainable extraction methods and their extraction fundamental parameters on the extractability of targeted compounds (lipids, proteins, phenolics,

carbohydrates) and their functionality. Processing conditions not only affect component extractability but result in structural modifications in the extracted compounds that directly impact the functional and biological properties of the extracts. Therefore, a holistic understanding of the impact of different extraction methods (i.e., aqueous, enzyme-assisted, ultrasound assisted, and steam injection) and key processing conditions in solid-liquid extractions (i.e., disruption pre-treatments, biomass-to-water ratio (BWR), pH, time, temperature) is needed to develop an extraction process that maximizes the extract properties along with the yields. Because of the limited literature on green extraction methods for *M. pyrifera* (Table 1.2), extraction parameters and current methods will also be discussed for other brown macroalgae species.

Table 1.2: Overview of green extractions methods and conditions employed for *M. pyrifera*.

Extraction Method	Target Compound(s)	Conditions	Yield	Activity	Reference
EAEP	Protein	EAEP in sodium acetate buffer (0.1M, pH 4.5) with 10% enzyme (Cellic CTec3) (v/w), at 50°C for 16 h with 1:50 BWR	74.60% yield	antioxidant, anti-hypertensive	(31)
EAEP treatment, AEP extraction	Carbohydrates	EAEP treatment (alginate lyase, fucoidanase, and 1,3-β-glucanase) at 37 °C, pH 7, 24 h, 1:10 BWR followed by 0.5 N NaOH extraction at 100 °C, 180 min, 1:20 BWR	89.67% yield (composed of 2.72% alginate, 9.81% glucose, 86.96% mannitol, and 0.52% fucose)		(60)
EAEP treatment, AEP extraction	Phlorotannins	EAEP treatment (alginate lyase, fucoidanase, and 1,3-β-glucanase) at 25 °C, pH 7, 36 h, 1:20 BWR followed by 0.5 N NaOH extraction at 100 °C, 180 min, 1:20 BWR	2.14% total phenolic content	antioxidant	(60)
UAE	Proteins	150 W, 40 kHz for 15 min, with 1:10 BWR	57% yield	antioxidant, anti-hypertensive	(61)

Abbreviations: Aqueous extraction process (AEP), Enzyme-assisted aqueous extraction process (EAEP), ultrasound-assisted extraction (UAE)

The use of innovative and greener processing strategies and their integration in macroalgae processing is still in its infancy. The existing literature highlights the potential of these new strategies to enhance extraction yields of macroalgae compounds with reduced extraction time and solvent use (31,57,59). In that view, maximizing the utilization of all macroalgae compounds is crucial for the development of a circular economy and of biorefinery processes that take a holistic view of the multifaceted potential of macroalgae.

An example of a sustainable extraction approach is the aqueous extraction process (AEP), which is a simple, eco-friendly solid-liquid extraction strategy that can fractionate several matrices into protein-, oil-, and fiber-rich fractions, using only water as the solvent (62). Like conventional solvent extractions, AEP is frequently preceded by mechanical pretreatments such as milling, grinding, and blending to increase the surface area-to-volume ratio and improve the diffusion of compounds into the water. In solid-liquid extractions, the process is optimized by altering parameters including the slurry pH, extraction time, temperature, and BWR. Macroalgae extraction yields are highly dependent on both extraction parameters and the species involved. Aqueous extractions for brown macroalgae include fucoidan and protein extractions from *E. maxima* (63 mg fucose/ g dry *E. maxima* and 8.9 mg protein/ g dry *E. maxima) L. pallida* (51 mg fucose/ g dry *L. pallida* and 7.7 mg protein/g dry *L. pallida*), and *S. rugosum* (265 mg fucose/ g dry *S. rugosum* and 17 mg protein/g dry *S. rugosum*). These extractions were carried out at 70 °C for 24 hours with BWR of 1:100 (63).

The AEP can be further enhanced by the addition of enzymes to assist the extraction. Enzyme-assisted aqueous extraction processes (EAEP) work by targeting components of the macroalgal matrix, like cellulose, hemicellulose, and proteins, with enzymes to catalyze their breakdown, thus aiding in the rapid release of desired compounds, while using water as the primary

solvent. AEP and EAEP have been successfully used to simultaneously extract lipids, proteins, carbohydrates, and phenolics from several plant-based matrices including macroalgae (31,62,64). Enzymes used in these processes include carbohydrases, which enables the breakdown of the cell wall cellulose and hemicellulose, thus allowing for greater release of the intracellular compounds, and proteases, which break apart proteins and the lipid body membrane (oleosin) and have been shown to increase extraction yields (64). Both carbohydrases and proteases have been used in macroalgae extraction in the past (65,66). As shown in Table 1.2, proteases have been used to increase the extractability of bioactive proteins from *M. pyrifera* resulting in yields of 74.60% (31). Extractions using an alginate lyase, fucoidanase, and 1,3-β-glucanase to target the breakdown of macroalgae specific compounds prior to extraction for carbohydrates, resulted in extraction yields of 89.67% (composed of 2.72% alginate, 9.81% glucose, 86.96% mannitol, and 0.52% fucose). Moreover, the extracts contained a phlorotannin content of 2.14% (60). However, the enzymes used in the aforementioned study are not commercially available. The lack of macroalgae specific enzymes on the market has limited current research of EAEP for macroalgae. To date, there is no consensus on the best available enzymes to use, and knowledge of the overall impact of enzymes on the extractability and bioactivity of the extracts remains limited.

Considering that the integrity of the cell-wall is a limiting step in the macroalgae processing, microwave-assisted aqueous extraction (MAE) and microwave-enzyme assisted aqueous extraction (MEAE) processes have strong potential to further enhance extractability in AEP and EAEP by further degrading the cell matrix and reducing extraction time through induced dipole rotation. The exposure of the polar aqueous solvent to microwave irradiation creates vibrations among water molecules as they are directed by the frequency of the microwave field. Such water movement generates friction, which creates heat and in turn breaks down the cell wall

matrix and increases mass transfer (42). A challenge with MAE processing is the high capital investment necessary for large scale operation (42). However, microwave processing has been used for several brown macroalgae applications, owing to its ability to effectively disrupt the cell wall matrix. MAE with methanol has been shown to enhance the extractability of cell wall-bound polyphenol molecules from *Carpophyllum flexuosum* by up to 70%, with phenolic extraction yields increasing from 8.6% up to 14.6% (67). MAE of fucoidan from *Ascophyllum nodosum*, with 0.1 M HCl following an ethanol pretreatment, demonstrated the microwave's ability to shorten extraction times from 9 h to 15 min and to reduce solvent volume by 3x compared to a conventional extraction (57). While these studies demonstrate the potential of microwave processing, they employ the use of hazardous solvents making them unsustainable. Despite the potential of microwave processing, there is a lack of studies for MAE and MEAE of *M. pyrifera*.

Ultrasound assisted extraction (UAE) is another novel technique that has shown potential when it comes to extraction of bioactive compounds from the macroalgae matrix (68). UAE is based on the principle of high frequency sound waves traveling through a liquid medium causing cavitation bubbles. Vapor traveling through the medium gets trapped in the bubbles which expand until the bubble collapses. This process generates mechanical energy which disrupts the algal matrix through microturbulence (69). While ultrasound processing has high process energy requirements and high starting capital for large-scale production, UAE is able to operate at low temperatures therefore preserving temperature sensitive compounds (42). UAE of *Macrocystis pyrifera* resulted in higher protein extraction when compared to conventional methods (40% vs. 60% yield). Additionally, UAE extracts exhibited a 27.6% ACE inhibitory activity and a 19.36% antioxidant activity for 1 mg/mL solution (61).

Sub-critical water extractions (SWE) have been highlighted for their ability to modify important extraction parameters (e.g., pressure and temperature) to selectively alter key water properties such as density, polarity, viscosity, and surface tension. This process operates in the sub-critical region (temperature of 100 to 374 °C and pressures between 0.1 and 20 MPa), where water is kept as a liquid but its properties are selectively altered to favor the overall process extractability (42). Changes in water polarity enable the extraction process to mimic that of other efficient organic solvents, thus avoiding the need for using hazardous compounds to extract nonpolar compounds. A search of literature showed a lack of application of this technology for *M. pyrifera*. SWE operated at 180 °C and 40 bar for 23.75 minutes has been used in phenolic (76.02% total phenolic content yield) and antioxidant (1.91 mmol Trolox Equivalent/g dry *Ecklonia maxima*) extraction from the brown macroalgae *Ecklonia maxima*. One drawback of SWE is the high equipment costs associated with the high pressure and temperature conditions required for operation. This equipment results in high initial capital, high maintenance costs, and strict safety measures (42).

Another environmentally-friendly extraction technology is supercritical fluid extraction (SFE), which involves operating the extraction above the critical temperature and pressure values for the fluid, often CO_2 (with supercritical range above 31.1 °C and 73.8 bar) (71,72). This approach enables the manipulation of the physio-chemical properties of the fluid, facilitating precising extraction of targeted compounds (73). CO_2 has limitations by being a non-polar solvent. As a result, co-solvents, like ethanol and water are used to target specific compounds (74). Depending on the co-solvent used, even though it is only a small fraction of the extraction solvent, overall process sustainability can be compromised. SFE extraction has been used primarily for macroalgae lipid extraction, including the extraction of glycolipids from *Sargassum muticum*. In

this process, supercritical carbon dioxide was used with 2% ethanol, as a co-solvent, increasing yields from 60 to 82.2%, compared with conventional methods (75). Despite its potential, a search of literature showed a lack of application of SFE for *M. pyrifera*. As with SWE, SFE has high equipment costs and necessitates strict safety measures (42).

Overall, there is limited knowledge about the impact of downstream processing conditions (i.e., pH, extraction time, temperature, water to biomass ratio, type, and amount of enzyme) and different extraction methods on extraction yields and functional and biological properties of brown macroalgae compounds. This knowledge gap is particularly noticeable for the extraction compounds from *Macrocystis pyrifera*, especially when targeting the extraction of multiple macroalgae compounds, a crucial step to develop sustainable and effective processing strategies conductive to the biorefinery concept. Such limitation is, in part, attributed to the lack of comprehensive throughput analytical methods that can elucidate the impact of these unit operations on the structural composition of these compounds (3,9,11,41,42), which can in turn enhance our understanding of their functional and biological properties.

1.5 Conclusions and future trends

Leveraging the use of *Macrocystis pyrifera* as a sustainable feedstock to produce food, feed, cosmetic, and nutraceutical ingredients has the potential to positively impact the entire kelp chain (cultivation, harvesting, processing, and production of algae-derived products). However, the commercialization of kelp-derived compounds relies on the development of innovative, environmentally friendly, and cost-effective processing strategies (i.e., techniques that fractionate the biomass without using flammable and toxic organic solvents) to convert the ample range of algae biomass compounds into added-value products with unique functional and biological

properties (i.e., antiviral, anti-inflammatory, antioxidant-rich extracts). Despite recent research advances demonstrating the potential role of green processing strategies such as enzyme, microwave, and ultrasound extraction processes, there is limited knowledge of the impact of major extraction parameters on yields, structure, and functionality of brown macroalgae, specifically *M. pyrifera*, which can be partly attributed to the lack of advanced, macroalgae specific, analytic techniques to better characterize and quantitate these compounds.

From a downstream processing perspective, there is a strong need to identify more selective and effective strategies to breakdown the kelp structure and therefore enhance the release of its intracellular compounds, which would benefit from the selective production of commercial enzymes with selective specificity to breakdown the kelp structure. As niche uses for macroalgae compounds emerge, for example as a source of alternative proteins, genetic engineering can play an important role in increasing the prevalence of these compounds and could be exploited to alter their thermal tolerance to increasing ocean water temperatures, making these marine crops even more desirable from an environmental and sustainability perspective.

At present, there is a critical need for the development of a sustainable and scalable biorefinery or cascading extraction-based processes for brown macroalgae, and more specifically for *M. pyrifera*, which, because of its high growth rate and wide availability in the Pacific Ocean, holds great potential as a feedstock for production of added value compounds. However, the development of such a process hinge on the development and optimization of advanced, macroalgae-specific analytical methods to guide the selection of extraction strategies to produce components with desired functional and biological properties. As research into sustainable processing for *Macrocystis pyrifera* and other macroalgae continues, life cycle assessment of the entire production chain (cultivation, harvesting, downstream processing, industrial integration, and

eventual end use) will be needed to provide a holistic understanding of the carbon footprint associated with macroalgae utilization, ensuring the enduring sustainability of these green giants as *Macrocystis pyrifera* becomes a part of everyday life.

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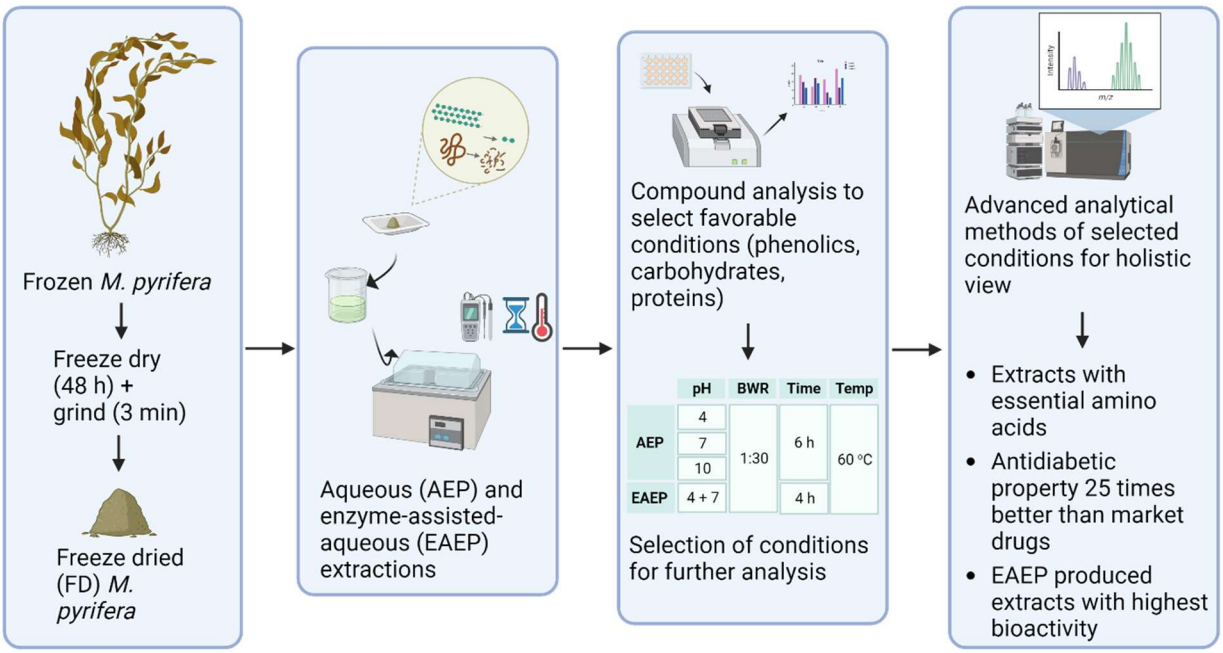
Chapter 2: Extraction of functional and bioactive compounds from Giant Kelp Macrocystis pyrifera using eco-friendly aqueous, and enzyme-assisted aqueous processing

Graphical Abstract



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Abstract

Macrocystis pyrifera, otherwise known as giant kelp, is a species of brown macroalgae found throughout the Pacific Ocean. Aqueous (AEP) and enzymatic extraction processes (EAEP) are eco-friendly strategies that allow for the breakdown of the algae matrix enabling the extraction of a variety of functional and bioactive compounds. To elucidate the effects of AEP and EAEP parameters on the extractability of alginates, laminarin, fucoidan, protein, phenolics, and biological properties of the extracts, the impacts of pH (4, 7, &10), biomass-to-water ratio [BWR, g freeze-dried kelp/mL water] (1:50 &1:30), reaction time (2, 4, 8 & 8 hours), and enzyme use (proteases and/or carbohydrases) were evaluated. The use of a carbohydrase pretreatment followed by proteolysis produced extracts with high yields for most components. AEP (6 h, 1:30 BWR, 60

°C, at pH 4, 7, and 10), and EAEP conditions (5% carbohydrase (w/w) for 2 h followed by 2.5% neutral protease (w/w) for 2 h), were selected as optimum conditions for more complete characterization and profiling. AEP at pH 4 resulted in the highest fucose levels (13.07 mg fucose Eq/ g FD kelp) in the extracts, and alginate with the lowest M/G ratios. The use of enzymes in the EAEP resulted in extracts with the highest bioactivities (antioxidant activity of 213.10 ± 22.58 μmol TE/ g FD extract, 54% ACE inhibition for a 10 mg FD extract/mL solution, and 92% α-glucosidase inhibition for a 0.125 mg FD extract/mL solution). This study provides an in-depth analysis of AEP and EAEP processing strategies for *Macrocystis pyrifera* to produce a diverse array of compounds with potential applications across various industries.

Keywords

Macroalgae; aqueous and enzyme-assisted extraction; fucoidan; laminarin; phenolics; biological properties

Highlights

- AEP at pH 4 resulted in the greatest fucoidan extraction yields.
- Acidic pH in the AEP resulted in alginate with lower M/G ratios associated with more rigid gels.
- Proteolysis led to extracts with the most pronounced bioactivities.

2.1 Introduction

The transition towards sustainability is vital for industries due to increasing environmental and societal pressures. To address the challenges posed by using non-renewable resources and to ensure long-term sustainability, industries from various sectors, including food, pharmaceuticals, fuel, and fertilizers, need to transition to more sustainable and renewable feedstocks. Seaweed or marine macroalgae show promise as a sustainable feedstock due to their abundant reserve of

functional and bioactive compounds such as carbohydrates, proteins, lipids, and phenolics (1,2). The environmental benefits of cultivating macroalgae are noteworthy since they can be grown in aquatic environments without the need for land, additional water, or fertilizer. Notably, macroalgae grow faster than terrestrial crops and can absorb approximately 20 times more carbon dioxide than land-based forests. These characteristics highlight the potential of macroalgae in mitigating climate change and further contribute to their attractiveness as a sustainable feedstock (2–4).

The giant kelp *Macrocystis Pyrifera* is a brown macroalgae typically found in the Pacific Ocean that contains a variety of compounds such as laminarin, fucoidan, alginate, and phenolics (5–7). These compounds possess desirable functional properties like gelling and emulsification, as well as biological properties including anti-inflammatory, immunostimulatory, antioxidant, anticoagulant, antiviral, antiproliferative, antiapoptosis, and antitumor effects (8). However, the successful development of a biorefinery concept for maximizing the extraction and application of these compounds requires a comprehensive understanding of different extraction methods and key processing conditions on their extractability and the structure-function relationship. Overcoming these challenges is crucial for unlocking the full potential of giant kelp as a sustainable and renewable feedstock.

Conventional extraction methods for macroalgae are time-intensive, taking anywhere from 9 to 24 hours (8,9), and involve the use of hazardous organic and flammable solvents, including methanol and formaldehyde. These solvents pose environmental, health, and safety hazards (9–11) and require additional downstream processing to remove them, resulting in increased operational costs (12). The successful integration of the broad range of macroalgae compounds hinges upon the development of eco-friendly extraction methods. These new extraction strategies

not only eliminate the use of hazardous solvents but have also been shown to enhance extraction yields of macroalgae compounds and reduce extraction time (9,11,13).

The aqueous extraction process (AEP) is a solid-liquid extraction strategy that utilizes water as the main solvent. This approach is considered eco-friendly as it eliminates the use of flammable and hazardous solvents and has the ability to fractionate matrices into a soluble fraction containing carbohydrates, proteins, phenolics, and an insoluble fraction rich in fiber (14). As with conventional solvent extractions, the AEP is generally preceded by mechanical pretreatments to reduce particle-size and increase the surface-area to volume ratio, enhancing the overall processing extractability (15). Moreover, the optimization of solid-liquid extractions involves altering important extraction parameters such as pH, time, temperature, and biomass to water ratio (BWR) (15,16). These parameters play a crucial role in determining the efficiency and selectivity of the extraction process, enabling the extraction of target compounds in desired quantities and quality.

The enzyme-assisted aqueous extraction process (EAEP) is an extension of the AEP that, in addition to using water as the primary solvent, incorporates the use of enzymes to catalyze the breakdown of the macroalgal matrix and target components, facilitating the rapid release of desired compounds (14,16). Common enzymes utilized in EAEP include carbohydrases and proteases. Carbohydrases assist in the breakdown of cellulose and hemicellulose present in the cell wall, thereby promoting the release of the intracellular compounds. Proteases, on the other hand, promote the breakdown of proteins including those proteins in the lipid body membrane (17). AEP and EAEP have been successfully used to simultaneously extract lipids, proteins, carbohydrates, and phenolics from several plant-based matrices including macroalgae (12–14).

Although carbohydrases and proteases have been used in macroalgae extractions in previous studies (18,19), their applications have primarily focused on enhancing the extractability

of bioactive proteins from *M. pyrifera* and carbohydrates, such as fucoidan, from other brown macroalgae (12–14). Overall, there is limited knowledge of the impact of enzyme usage (type and amount of enzyme) and other extraction parameters such as pH, extraction time, temperature, and BWR, on extraction yields, structural composition, and functional and biological properties (1,20–23) of *Macrocystis pyrifera* compounds. Further research is needed to better understand the interplay between enzyme use and key extraction parameters to optimize the extraction process efficiency and properties of the extracted compounds. This challenge is further evidenced by the limited availability of enzymes suitable to degrade macroalgae cell walls and comprehensive throughput analytical methods necessary to better understand the impact of downstream processing unit operations on the structure and functionality of target macroalgae compounds.

Therefore, the major goal of this study was to gain a better understanding of the impact of pH, reaction time, and BWR, using sustainable aqueous and enzyme assisted aqueous extraction methods, on the extractability, structure, and functional/biological properties of major compounds of the giant kelp *Macrocystis pyrifera*. To achieve this goal, this work evaluated the role of extraction pH (4, 7 & 10), time (2, 4, 6 & 8 h), and BWR (1:30 & 1:50) on the extractability and composition (fucoidan, laminarin, protein, total phenolic, and alginate contents) of *M. pyrifera* extracts produced by the aqueous extraction process (AEP). Based on best BWR and extraction time from the AEP, the use of carbohydrases and proteases was evaluated, alone and in combination, with respect to the extractability and composition (total phenolic, fucose, sulfate, laminarin, and alginate contents, and total protein extractability) of the extracts produced by the enzyme-assisted aqueous extraction process (EAEP). Subsequently, to better understand the impact of extraction parameters on the composition and properties of the extracts, selected AEP and EAEP extracts were subjected to carbohydrate profiling and quantification by liquid

chromatography mass spectrometry (LC-MS) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), proteomics, amino acid composition, phenolic profiling and quantification by untargeted metabolomics and reverse phase high performance liquid chromatography, and alginate characterization by Fourier-transform infrared spectroscopy (FTIR). The biological properties (antioxidant, antihypertensive, antidiabetic) of selected AEP and EAEP extracts were also evaluated. The use of advanced analytical techniques, along with an in-depth understanding of the biological properties of the extracts and extraction yields, is of critical importance to enhance our understanding of the role of extraction methods and conditions on the composition, extractability, and biological properties of *M. pyrifera* extracts, helping to identify innovative strategies for developing downstream processing conditions and methods to isolate giant kelp compounds with desired composition and bioactivity.

2.2 Materials and methods

2.2.1 Starting material preparation and characterization

Macrocystis pyrifera from the Catalina Sea was generously provided by Primary Ocean. Macroalgae samples were harvested in summer 2021 and kept frozen at -18°C until being processed. Summer samples of *Macrocystis pyrifera* have been shown to be higher in amino acids and minerals (24). Upon thawing, *M. pyrifera* samples were rinsed (3X) with deionized (DI) water, cut into approximately 1-inch pieces, and freeze dried (FreeZone 6 Liter Benchtop Freeze Dry Systems, 77520 Series, Labconco, Missouri, USA) for 72 h. Freeze-dried *M. pyrifera* was ground (COOL KNIGHT Herb Grinder, Spice Herb Coffee Grinder with Pollen Catcher, 7.5 inches) into a powder (FD *M. pyrifera*) and stored at -18 °C until use as the starting material for extraction.

The proximate composition of the starting material (FD *M. pyrifera*) was determined using standard methods. The moisture content was determined after drying the sample in a conventional drying oven at 105 ℃ for 4 h (25). Carbohydrate content was determined via the phenol-sulfuric method (26) with fucose used for the standard curve (27). The lipid content was determined using acid hydrolysis (28) and the ash content was determined after incinerating the samples at 600 ℃ for 2 h (29). Additionally, fatty acid composition was determined by direct transesterification of the *M. pyrifera* powder using an HCL-Methanol solution (8:92 v:v). Fatty acid methyl esters were extracted with hexane and analyzed by gas chromatography coupled with a flame ionization detector (GC-FID), according to Dias et al. (30). Total and free oxylipins were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS)(31). The protein content was determined via the Dumas combustion method (32) using a nitrogen to protein conversion level of five (33). The amino acid profiling of the starting material was determined by the UC Davis Proteomics Core Facility. Lastly, particle size was determined via sieving (#16, 30, 60, 120, 230, and 500 sieves, W.S. Tyler, Mentor, OH, USA).

2.2.2 Extraction Processes

AEP and EAEP conditions are described in Figure 2.1 a and b, respectively. To aid in the selection of optimum extraction parameters of the AEP (Figure 2.1a), the effects of pH (4, 7, and 10), time (2, 4, 6, and 8 h), and biomass (FD *M. pyrifera*) to water ratio (BWR) (1:30 and 1:50) were evaluated. Briefly, FD *M. pyrifera* was weighed and mixed with 20 g of pre-heated DI water in 50 mL (Corning Pyrex) beakers to reach the desired BWR and the slurry pH was adjusted and maintained to the desired pH dropwise with additions of 0.5 M HCl and 0.5 M NaOH. Extractions were performed in a 60 °C water bath (Precision GT20, Thermo Scientific, Waltham, MA, USA) for the desired time (2, 4, 6, and 8 h) with constant stirring at 1400 rpm on a magnetic stir plate

(Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific, Waltham, MA, USA). Each beaker was covered with aluminum foil during the extraction to avoid evaporation. After the extraction, the slurry was transferred to 50 mL falcon tubes, and the solids were separated by centrifugation (Allegra X-14R centrifuge, Beckman Coulter, Indiana, USA) at 4,000 x g for 30 min at 4 °C. The liquid extract was then transferred into a new falcon tube and $CaCl_2$ was added to the extract to create a 2% (w/w) solution, which was vortexed for 1 min to homogenize and then left at 4 °C overnight to enable the precipitation of alginate as calcium alginate. Calcium alginate was separated by centrifugation (Allegra X-14R centrifuge, Beckman Coulter, Indiana, USA) at 4,000 x g for 30 min at 4 °C. The soluble portion (extract) was then transferred into a new falcon tube for further analysis. All extracts were frozen and stored at -18 °C or freeze-dried (FreeZone 6 Liter Benchtop Freeze Dry Systems, 77520 Series, Labconco, Missouri, USA) depending on the analysis performed. Calcium alginate was mixed with 10 mL of sodium carbonate (3% w/v) for 2 h at 60 °C and the slurry was centrifuged to obtain a sodium alginate extract (supernatant). The sodium alginate extract was then freeze-dried and resuspended in reverse osmosis (RO) water. Then, the alginate was precipitated out with 70% ethanol overnight and freeze-dried for 48 hours. Triplicate extractions were performed at each pH, time point, and BWR.

With the goal of validating the extraction temperature of 60 °C and analyzing its potential impact on sensitive components, like phenolics (34), extraction time and BWR were selected from the above experiments and extractions were also performed at 30 °C. Because of the lower extractability observed at 30 °C, 60 °C was kept as the extraction temperature for the subsequent experiments.

Based on the AEP experiments, BWR of 1:30 and extraction temperature of 60 °C, which lead to enhanced extractability of the various components, were selected to guide the EAEP

optimization. The individual impact of using FoodPro's CBL, an acidic carbohydrase provided by the Genecor Division of DuPont™ Danisco® (Rochester, NY, USA), Neutral Protease L (NP), and Alkaline Protease (AP), provided by Bio-Cat (Troy, VA, USA), to assist the overall extractability of *M. pyrifera* was assessed at 1, 2.5, and 5% (w/w), and the slurry pH was adjusted to values according to each enzyme requirement (pH 4 for CBL, pH 7 for NP, and pH 10 for AP). EAEP experiments were performed at 60 °C, 1:30 BWR, under constant stirring of 1400 rpm, for 6 h. Based on the results of the initial enzyme screening, a kinetic study of 5% CBL and 2.5% NP was evaluated for at 2, 4, and 6 h. Lastly, the combined impact of a carbohydrase pretreatment (2 h), using 5% CBL, followed by the subsequent use of 2.5% NP at 2 and 4 h was evaluated. Extraction equipment, centrifugation conditions, and alginate separation were the same as those described for the AEP experiments. Each enzymatic extraction condition was performed in triplicate.

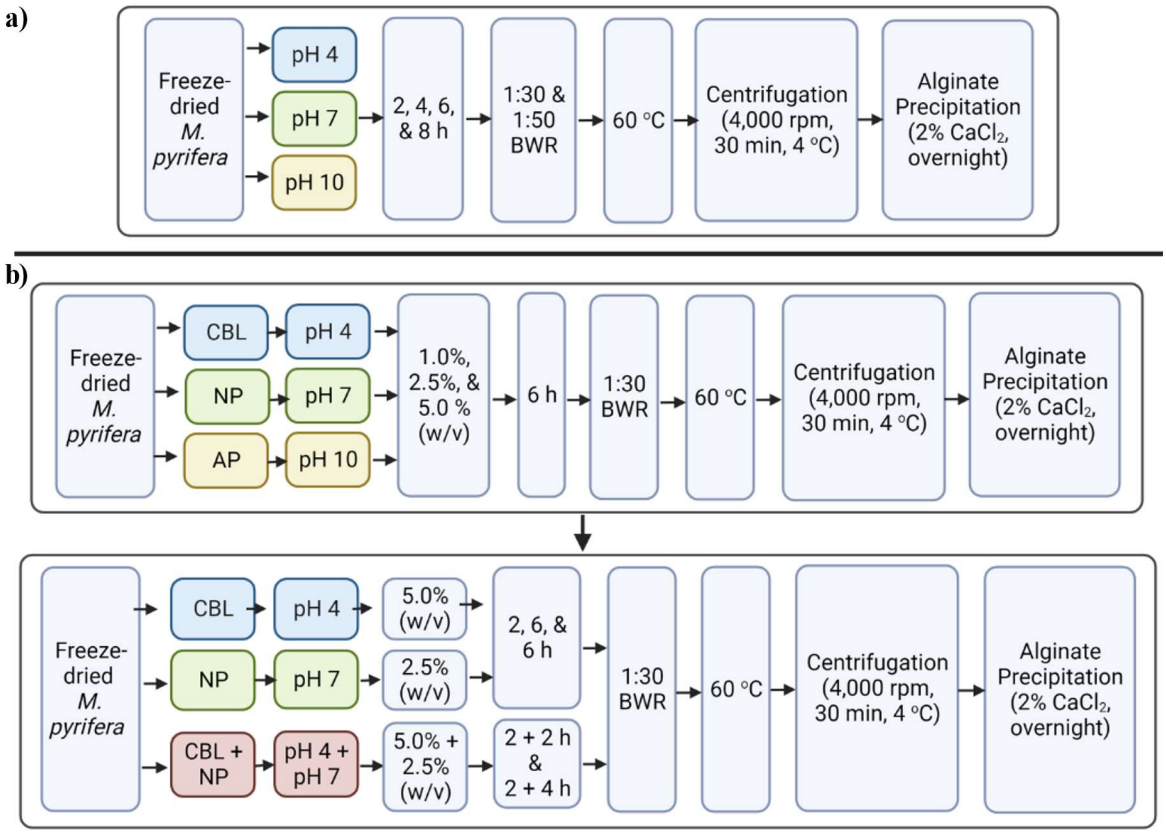

Figure 2.1: Flow chart for the optimization of the AEP (a) and EAEP (b). Created with BioRender.com.

Extracts from all experimental conditions described in Figure 2.1 were analyzed as described in the "Characterization of extracts from all extraction conditions" section 2.2.4. Selected extracts, which were identified based on where yields reached their maximum or where less resource intensive conditions were used (i.e., lower temperature, higher BWR, shorter extraction time) and yields were not statistically different from the maximum, were selected for additional analysis as described in the "Characterization of selected extracts" section 2.2.5.

2.2.3 Characterization of extracts from all extraction conditions

2.2.3.1 Total phenolic content

Total phenolic content (TPC) of the extracts was determined according to the Folin-Ciocalteu method as described by Singleton et al. (35). A 25 µL aliquot of extract (diluted 1:8 v/v or 1:14 v/v in DI water for 1:50 and 1:30 extraction BWRs, respectively) and 125 µL of 10% Folin-Ciocalteu reagent were transferred to a clear 96-well microplate. The mixture was then agitated for 5 min at 300 rpm in the dark at 37 °C. After which, 100 µL of a 7.5% (w/v) sodium carbonate solution was added and the mixture was then agitated for another 30 min at 300 rpm in the dark at 37 °C and left to rest (no agitation) for an additional 90 min. The absorbance was read at 760 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). TPC results were calculated using a standard curve of gallic acid with concentrations of 5–95 µg/mL and were presented as mg gallic acid equivalent (GAE)/ g FD *M. pyrifera*. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

2.2.3.3 Fucoidan content

Fucose content, used to approximate fucoidan content in the extract, was determined based on the method of Dische and Shettles (36), with modifications as described by Rajauria et al. (37). A 40 µL aliquot of the extract (diluted 1:4 v/v or 1:7 v/v in RO water for 1:50 and 1:30 extraction BWRs, respectively), followed by 180 µL of cold 6:1 sulfuric acid: RO water, was transferred to a clear, flat-bottom 96-well microplate. The mixture was then left at room temperature for 3 min and subsequently incubated at 90 °C for 10 min followed by 5 min in an ice bath to stop the reaction. Then, 10 µL of 3% (w/v) L-cystine hydrochloride solution was added. The mixture was left in the dark for 60 min at room temperature and the absorbance was subsequently measured at 396 and 430 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose

CA, USA). Fucose content results were determined by absorbance difference and based on a standard curve created with L-fucose with concentrations of 0–0.2 µg/µL. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

Sulfate content determination of the extracts was based on the method developed by Dodgson (38), with modifications from Torres et al. (39). First, extracts were freeze-dried for 96 h. Freeze-dried (FD) extracts and 0.5 M HCl were added to 2 mL Eppendorf tubes to reach a concentration of 1 mg FD extract/50 µL. The mixture was incubated at 200 °C for 3.5 h with agitation at 300 rpm followed by a brief centrifugation for 2 min at 15,000 x g (accuSpin Micro 17, Fisher Scientific, Hampton, NH, USA). 20 µL aliquot of the supernatants and 140 µL of 0.5 M HCl were transferred to a clear 96-well microplate and the absorbance was read at 405 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). Next, 40 µL of barium-chloride gelatin reagent (75 mg gelatin, 25 mL RO water, 250 mg $BaCl_2$) was added and the mixture was left for 20 min, after which the absorbance was read at 405 nm. The first absorbance reading was subtracted from the second one and the sulfate content was determined by a standard curve created with potassium sulfate with concentrations of 0-2 µg/µL. The sulfate content was expressed as SO_4^{2-} equivalents (Eq). Extraction triplicates were analyzed in duplicate, and the results were presented as an average of six measurements.

2.2.3.4 Laminarin content

Laminarin content was determined by measuring reducing sugars produced after sample hydrolysis with a method based on that of Van Breda et al. (40), with slight modifications. A 400 µL aliquot of extract (diluted 1:2 v/v or 1:3 v/v in RO water for 1:50 and 1:30 extraction BWRs, respectively) and 100 µL of an endo-1,3(4)-β-glucanase enzyme solution (Novozymes, 150 U/mL) was transferred to 2 mL Eppendorf tubes. The mixture was incubated for 2 h at 37 °C. Controls

were created for each sample in the same way but with RO water instead of enzyme solution. After hydrolysis, 500 mL of a reagent solution 0.75% (w/w) dinitrosalicylic acid, 0.75% (w/w) sodium hydroxide, 0.04% (w/w) sodium sulfate, and 10% (w/w) potassium tartrate tetrahydrate was added. Next, the mixture was incubated at 90 °C for 5 min followed by cooling in an ice bath for 3 min. 200 µL of each extract and control sample were added to a clear 96-well plate and absorbances were read at 590 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). Laminarin concentration was determined based on a standard curve prepared with laminarin with concentrations from 0–10 µg/µL and glucose with concentrations from 0–5 µg/µL. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

2.2.3.5 Alginate content

Alginate content determination was based on the method of Mohd Fauziee et al. (41), with the modification of freeze drying the precipitated calcium alginate instead of converting it to sodium alginate prior to freeze drying. Alginate yield was calculated using Equation 2.1, and results were presented as the average of three measurements.

$$Alginate\ yield\ (\%)\left(\frac{w}{w}\right) = \frac{freeze\ dried\ calcium\ alginate\ weight}{initial\ freeze\ dried\ kelp\ powder\ weight}\ x\ 100 \qquad \textbf{(Equation 2.1)}$$

2.2.3.6 Protein extractability

Protein content of the freeze-dried extracts was determined via the Dumas combustion method (32) using a varioMAX cube (Elementar, New York, USA) with a nitrogen to protein conversion level of five (33). Protein extractability was calculated according to Equation 2.2 and the results were presented as an average of three measurements.

$$Protein\ extraction\ yield\ (\%\ w/w) = \frac{protien\ (g)\ in\ FD\ extract}{protien\ (g)\ in\ FD\ kelp\ powder} \times 100 \qquad \textbf{(Equation 2.2)}$$

2.2.4 Characterization of selected extracts

Extraction conditions for more in-depth characterization were selected based on extraction yields of key components (phenolics, fucose, sulfate, laminarin, alginate, and protein) and reduction in resource utilization. When yields were not statistically different from the maximum, the condition that was less resource intensive (i.e., higher BWR, less time, lower temperature) was selected to enhance the environmental sustainability of the process.

2.2.4.1 Metabolomic profiling

Phenolic profiling and quantification were carried out by untargeted and targeted approaches. Freeze-dried extracts were prepped for phenolic analysis by mixing 1:50 (v/w) in 50:50 methanol:RO water + 0.01% HCl and sonicated (Branson 2800, Branson Ultrasonics, Brookfield, CT, USA) for 1 h. Samples were then centrifuged at 13,000 rpm for 5 min and the supernatants were used for subsequent phenolic analysis. The targeted analysis was conducted following the method by the method by Pinton et al (42). Selected AEP and EAEP extracts were analyzed along with a methanolic extraction control (50:50, methanol:RO water) and an acetone extraction control (70:30, acetone:RO water), both using 1:30 BWR (freeze-dried *M. pyrifera*:solvent), and 1 h extraction time in ultrasound at 30 °C. The samples were analyzed by reversed-phase high-performance liquid chromatography using an Agilent 1260 Infinity equipped with an Agilent Zorbax SB-C18 (4.6 × 150 mm, 3.5 µm) column (Agilent Technologies, Santa Clara, CA, USA) at 35 °C, an autosampler with temperature control at 8 °C, and a diode array detector. Two mobile phases were used: mobile phase A composed of MilliQ water with 1.5% o-phosphoric acid (v/v) and mobile phase B composed of 80% acetonitrile and 20% mobile phase A. The following gradient program was used: 10–31% B (0–73 min) and 62% B (73–75 min).

Mobile phase B was held at 62% (75–80 min) and decreased to 10% (82–90 min). The mobile phase flow was maintained at 1 mL/min and the injection volume was 20 µL.

The eluted compounds were monitored and identified by spectral and retention time comparisons to authentic standards at four different wavelengths: 280 nm (gallic acid, protocatechuic acid, (+)-catechin, syringic acid, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, naringenin-7-O-glucoside, (±)-naringenin, and polymeric phenols), 320 nm (chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid), 360 nm (quercetin-3-O-rutinoside, quercetin-3-O-glucoside, quercetin-3-O-glucuronide, isorhamnetin-3-O-glucoside, quercetin, isorhamnetin, and kaempferol), and 520 nm (malvidin-3-O-glucoside and polymeric pigments).

External calibration curves (0.1–200 mg/L) were prepared using authentic standards of gallic acid, (+)-catechin, (-)-epigallocatechin gallate, (-)-epicatechin gallate, chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, quercetin, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, (±)-naringenin, naringenin-7-O-glucoside, isorhamnetin, isorhamnetin-3-O-glucoside, kaempferol, and malvidin-3-O-glucoside. These compounds were quantified as themselves, while syringic acid and protocatechuic acid were quantified as gallic acid equivalents; epicatechin and polymeric phenols as (+)-catechin equivalents; coumaric acid as p-coumaric acid equivalents; quercetin-3-O-galactoside and quercetin-3-O-glucuronide as quercetin-3-O-glucoside equivalents; and anthocyanins and polymeric pigments as malvidin-3-O-glucoside equivalents. Data analysis was performed using Agilent® CDS ChemStation software version D.04 (Agilent Technologies, Santa Clara, CA, USA).

Untargeted analysis of selected phenolic compounds and other small molecules such as dipeptides was performed by the West Coast Metabolomics Center Central Services Core (UC

Davis, Davis, CA, USA) (43). Samples were extracted using 1 mL of 80:20 MeOH:H_2O. Samples were vortexed and centrifuged and 450 µL of the supernatant was dried for analysis. Dried samples were resuspended with 100 µL of a solution 75:25 H_2O:acetonitrile containing internal standards (CUDA, D3-L-Carnitine, Val-Tyr-Val, D4-Daidzein, D9-Reserpine, and D5-Hippuric Acid). Samples were then vortexed for 10 s, sonicated for 5 min at room temperature, and then centrifuged for 2 min at 16,000 x g. 60 µL of supernatant from each sample was transferred into a LC-MS vial containing a glass micro insert. 30 µL supernatant from each sample was then transferred into an Eppendorf tube and vortexed for use as a pool. The samples were then injected onto a Waters ACGUITY Premier BEH C18 1.7 µm, 2.1 x 50 mm column. The gradient used was 0 min 1% (B), 0.5 min 1% (B), 7.50 min 99% (B), 9 min 99% (B), 9.2 min 1% (B), 10 min 1% (B), with a flow rate of 0.6 mL/min. Mobile phase A was 100% LC-MS grade water + 0.1% Formic Acid and mobile phase B was 100% acetonitrile + 0.1% formic acid. Injection volume ranged between 0.1 and 5 µL. A Vanquish UHPLC system (Thermo Fisher Scientific) was used and a Thermo Q-Exactive HF Orbitrap Mass Spectrometry instrument was used in both positive and negative ESI modes to acquire LC-MS/MS data with the following parameters: mass range 80−1200 m/z; full scan MS1 mass resolving power 60,000, data-dependent MSMS with 2 scans per cycle, normalized collision energy at 20, 30, and 40%, data-dependent MSMS mass resolving power 15,000. The instrument was tuned and calibrated per manufacturer's recommendations.

Data was interpreted based on the International Chemical Identifier and presented by peak height. Metabolites were divided into four categories: phenolics, peptides, lipid derived compounds, and other metabolites. They were reported based on the number of unique identified metabolites per extraction condition.

2.2.4.2 Monosaccharide quantification

Monosaccharide quantification in selected extracts was performed by HPAEC-PAD. Due to a lack of commercially available enzymes to aid in the study of fucoidan, fucose, the primary monosaccharide in fucoidan, was used as a surrogate measure of fucoidan after performing acid hydrolysis (44).

All selected extracts were prepared in duplicate for monosaccharide analysis. Polysaccharides were precipitated from extracts by adding 3 volumes of cold ethanol. After centrifugation (14,000 x g, 4 °C, 30 min) and drying, a pellet containing primarily polysaccharides was obtained. Polysaccharides were then hydrolyzed into constituent monosaccharides by trifluoroacetic acid hydrolysis at 100 °C for 2 h with gentle stirring. Samples were briefly cooled, diluted with water, and acid was removed by drying. Hydrolysates were reconstituted in water then filtered by a 0.2 μm polyethersulfone syringe filter (Pall Life Sciences, Port Washington, NY) for HPAEC-PAD analysis. Samples were diluted appropriately to ensure monosaccharide quantification was in the linear range of the method.

To quantify monosaccharides, an HPAEC-PAD system was used to separate and detect fucose, galactose, glucose, xylose, and mannose. An isocratic chromatography method was developed and validated to ensure reproducible results. Commercial standards of the five monosaccharides were mixed and appropriately diluted to create a calibration curve. A linear range from 0.0001-0.01 mg/mL was established. Initial testing of monosaccharides in the AEP samples showed that fucose and galactose were 1 to 2 magnitudes higher in concentration than glucose, xylose, and mannose, consistent with previously reported findings (45). To ensure all monosaccharides were in the linear range for quantification, two separate dilutions were prepared

and analyzed. Additionally, all samples were analyzed in duplicate to assess sample preparation and instrument reproducibility.

HPAEC-PAD analysis was performed on an ion chromatography system with a detector consisting of an electrochemical cell with a disposable, gold, working electrode and a pH-Ag/AgCl reference electrode (Thermo Fisher Scientific, Waltham, MA). Samples were injected onto a Dionex CarboPac PA20 BioLC column (3 x 150 mm, Thermo Fisher Scientific) equipped with a PA20 guard column (3 x 30 mm, Thermo Fisher Scientific) at a flow rate of 0.5 mL/min. Chromatographic separation was performed with an isocratic method consisting of 92% mobile phase A (water) and 8% mobile phase B (20 mM sodium hydroxide in water) for 20 min. A calibration curve containing a mix of all five monosaccharide standards was used to quantify monosaccharide concentration in the extracts. Due to the higher abundance of fucose and galactose compared to glucose, mannose, and xylose, two dilution factors (1:2 and 1:100) were prepared and analyzed to ensure measurements were within the linear range of the calibration curve.

2.2.4.3 Oligosaccharide analysis

To isolate oligosaccharides, 2 volumes of ice-cold ethanol were added to the extracts, then kept at -20 °C for 1 h. Samples were centrifuged (4200 x g, 4 °C, 30 min) and the supernatants were transferred to fresh tubes and dried overnight. The following day, all samples were reconstituted in water, with water volumes normalized to carbohydrate content, as determined by the phenol-sulfur method described previously. Samples were vortexed, then sonicated (10 min, high intensity) to ensure full dissolution. Afterwards, samples were centrifuged (14,000 x g, 4 °C, 30 min) to remove particulates, then cleaned by microplate C18 SPE (Glygen, Columbia, MD), and PGC SPE (Thermo Fisher Scientific, Waltham, MA). Eluted samples from PGC SPE were

dried overnight by speedvac (MiVac Quattro, Genevac Ltd., Ipswitch, Suffolk, UK), reconstituted in water, then diluted 1:80 with additional water for nanoLC-QToF oligosaccharide analysis.

LC-MS/MS analysis was performed with an Agilent 6520 Accurate-Mass Q-TOF instrument equipped with a Chip Cube coupled to an Agilent 1200 Series high performance liquid chromatography interface (Agilent Technologies, Santa Clara, CA) as described by Huan et al (46). Data was annotated by Glyconote (https://github.com/MingqiLiu/GlycoNote) and manually verified using Agilent MassHunter Qualitative Analysis (B.07.00, Agilent Technologies). The data was manually searched for neutral loss of fucose and neutral loss of sulfate which is later discussed in detail. Peaks were manually integrated using Agilent Masshunter Profinder (B.08.00, Agilent Technologies). Microsoft Excel was used for peak area quantitation.

2.2.4.4 Characterization of sodium alginate structure by Fourier transform infrared spectrometry

A FTIR spectrometer (Shimadzu IR Prestige-21, Oregon, USA) equipped with Quest single-balanced attenuated total reflectance accessory was used to investigate and compare the functional groups present in the sodium alginate samples. The freeze-dried sodium alginate was suspended in a 10% w/v solution of 0.1 M PBS buffer, pH 7.4 to avoid instrument damage from exposure to extreme pH values. Samples were scanned from 4000-400 cm^{-1} (41,47) at a resolution of 4 cm^{-1} with 25 scans using absorbance mode. A background scan of the buffer was subtracted for the samples. Scans were analyzed with LabSolutions IR software and absorbance bands corresponding to units of mannuronic and guluronic acids were used to estimate the M/G ratio (47). Each extraction triplicate was analyzed and absorbance for wavelength values was averaged. Microsoft Excel was used to graph the data.

2.2.4.5 Laminarin quantification by β-glucan analysis

β-glucan content was determined using the K-YBGL enzymatic-assay kit from Megazyme International Ltd., Bray, Ireland (48) and was used as an indicator of laminarin presence in the extract as laminarins are composed primarily of β-glucans (37). Briefly, total glucan content was determined by solubilizing β-1-3, 1-6-glucans, β-1-3-glucans, and α-glucans in ice cold 12 M H_2SO_4 and then hydrolyzed in 2 M H_2SO_4. Any remaining glucan fragments were hydrolyzed to glucose using exo-1-3-β-glucanase and β-glucosidase. α-glucans and sucrose were specifically hydrolyzed to glucose and fructose and glucose were measured with amyloglucosidase and invertase using glucose oxidase plus peroxidase and 4-aminoantipyrine in buffer. β-glucan was determined by difference. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

2.2.4.6 Amino acid profiling

Amino acid analysis of freeze-dried extracts was performed by the UC Davis Molecular Structure Facility Proteomics Core (49) with the following adapted protocol (50). For each extract, samples were placed into 3 separate glass hydrolysis tubes (one for analysis of cysteine and methionine, one for analysis of tryptophan, and one for all other amino acids). For cysteine and methionine analysis, samples were oxidized overnight at 2 ºC in performic acid and then dried. For Tryptophan analysis, samples were hydrolyzed in 4.2 N NaOH at 110 ºC, for 24 h, in vacuo, and then neutralized with 4.2 N HCl, diluted with NorLeucine dilution buffer and stored at 2 ºC until analysis. For all other amino acids, samples were hydrolyzed with 6 N HCl, 1% Phenol, at 110 ºC, for 24 h, in vacuo. All samples were then mixed with Sodium Diluent Buffer (Pickering, California, USA) with 40 nmol/mL of NorLeucine added.

Following preparation, 50 µL of sample was injected into a Concise Ion-Exchange column. An amino acid standard solution was used to determine response factors, and thus calibrate the Hitachi 8800 analyzer (Sigma, A-9906) for all the amino acids. Additionally, this standard has been verified against the National Institute of Standards and Technology (NIST) standard reference material 2389a. NorLeucine was included in each injection as an internal standard to allow correction of the results for variations in sample volume and chromatography variables. All samples were injected in triplicate, and data was reviewed by Proteomics Core staff.

2.2.4.7 Antioxidant activity

The 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay for radical cation scavenging activity was performed as described by Al-Duais et al. (51) with some modifications. The radical stock solution was produced by mixing 38.4 mg ABTS and 6.62 mg $K_2O_8S_2$ (potassium persulfate) in 10 mL deionized water. The ABTS•+ solution was incubated overnight for 15 h at room temperature in the dark. Afterwards, the ABTS•+ stock solution was diluted with 95% (v/v) ethanol to obtain an initial absorbance of 0.70 ± 0.20 at 730 nm. 20 µL of each freeze-dried *M. pyrifera* extract (at a concentration of 20 mg/mL in ethanol) was pipetted into a clear 96-well microplate followed by the addition of 200 µL of diluted ABTS•+ solution. A 20 µL sample of ethanol with 200 µL ABTS•+ solution was used as the control. The mixtures were agitated at 300 rpm for 6 min, then the microplate was read at 730 nm using a spectrophotometer (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). A calibration curve using Trolox standard solutions 80–340 µM, was used to quantify the activity in µmol Trolox equivalent (TE)/g FD *M. pyrifera* extract. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

The oxygen radical absorbance capacity (ORAC) assay was performed according to the method described by Zulueta et al. (52). 50 µL of the control (phosphate buffer solution, pH 7.0, 75 mM), 10 mg/mL freeze-dried *M. pyrifera* extract in RO water (diluted 1:100, v/v, in PBS), or standard (Trolox, 20 µM, diluted in PBS) were added to a black 96-well microplate. 50 µL of fluorescein (78 nM, diluted in PBS) was added to each well, and the plate was agitated at 300 rpm for 15 min at 37 °C followed by the addition of 25 µL of 221 mM 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) radical solution. The plate was read using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA), warmed to 37 °C, and set at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Fluorescence measurements were read every 5 min for 1 h. Results were calculated using a standard curve based on area below the fluorescence decay curves (AUC) for 20-100 µM Trolox and were converted to µmol Trolox equivalent (TE)/g FD *M. pyrifera* extract. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

2.2.4.8 Angiotensin-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of the chosen *M. pyrifera* extracts was determined according to a method previously developed (53) and is based on the ability of ACE in hydrolyzing the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO2)-Pro). For this, 50 µL of a 10 mg/mL solution of freeze-dried *M. pyrifera* extract in a 75 mM Tris pH 8.3 buffer was mixed with 50 µL ACE enzyme solution containing 30 mU/mL of ACE dissolved in 150 mM Tris pH 8.3 and pre-incubated for 10 min at 37 °C. The substrate was pre-incubated apart at the same temperature. The reaction was initiated by adding 200 µL of 10 mM Abz-Gly-Phe-(NO_2)-Pro in 0.15 M Tris-base buffer (pH 8.3) containing 1.125 M NaCl. The reaction mixtures were then incubated at 37 °C for 30 min. The

amount of aminobenzoylglycine (Abz-Gly) formed by ACE activity was measured at an excitation wavelength of 355 nm and emission wavelength of 428 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). ACE inhibitory activities of the samples were expressed as percentages of total activity obtained when no inhibitor is present (Equation 2.3).

$$ACE\ Inhibition\ (\%) = \frac{ABS_C - ABS_S}{ABS_C} * 100 \quad \textbf{(Equation 2.3)}$$

Where ABS_C is the absorbance of the control reaction (no sample extract addition: enzyme + substrate + water), and ABS_S corresponds to the absorbance of the sample reaction (enzyme + substrate +extract). Results were compared to a 1 µM solution of Captopril to see how inhibition of extracts compared to current treatment options. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

2.2.4.9 α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was evaluated according to the method described by Ibrahim et al. (54), with modifications. Initially, 50 µL of freeze-dried extracts (0.125 mg/mL in RO water) were incubated with 25 µL of 0.5 U mL^{-1} α-glucosidase from *Saccharomyces cerevisiae* solution in PBS (100 mM, pH 6.8), in a 96-well plate, at 37 °C, for 5 min. After pre-incubation, 25 µL of p-NPG substrate solution (5 mM) in PBS (100 mM, pH 6.8) was added, and the reaction was allowed to proceed for 10 min, at 37 °C. The absorbance was measured at 405 nm in a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). A control reaction and a sample background were used to calculate the inhibitory activity (Equation 2.4).

$$Enzyme\ activity\ (\%) = \frac{ABS_C - ABS_S}{ABS_C} * 100 \quad \textbf{(Equation 2.4)}$$

where ABS_C is the absorbance of the control reaction (no sample extract addition: enzyme + substrate + water), and ABS_S corresponds to the absorbance of the sample reaction (enzyme +

substrate +extract). Results were compared to a 1 μM solution of Acarbose to see how inhibition of extracts compared to current treatment options. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

2.2.5 Statistical Analysis

All extractions and assays were performed in triplicate with results expressed as mean ± standard deviation. One, two, or a three-way Analysis of variance (ANOVA) was performed depending on the number of variables compared. This was followed by Tukey HSD test to determine significant differences among the experiments at $p < 0.05$. All statistical analyses were performed using JMP Statistical Discovery LLC (Cary, NC, USA).

2.3. Results and discussion

2.3.1 Starting material composition (freeze-dried *Macrocystis pyrifera*)

The proximate composition of the freeze-dried *M. pyrifera* is shown in Figure 2.2a. *M. pyrifera* contained 5.06 ± 0.45% moisture, 1.80 ± 0.10% lipids, 23.16 ± 0.11% ash, 6.51 ± 0.05% protein, and 50.08 ± 3.00% carbohydrates (dry basis). Particle size was determined via sieving, and 98.99 ± 0.24% of the powder had a diameter less than 0.25 mm. Figure 2.2b shows the amino acid composition of the FD *M. pyrifera* and highlights that *M. pyrifera* is a complete source of essential amino acids (EAA), in agreement with other compositional analysis of *M. pyrifera* which indicates the presence of all EAA but in slightly different ratios than our starting material (55). These differences may be due to seasonality and growing conditions.

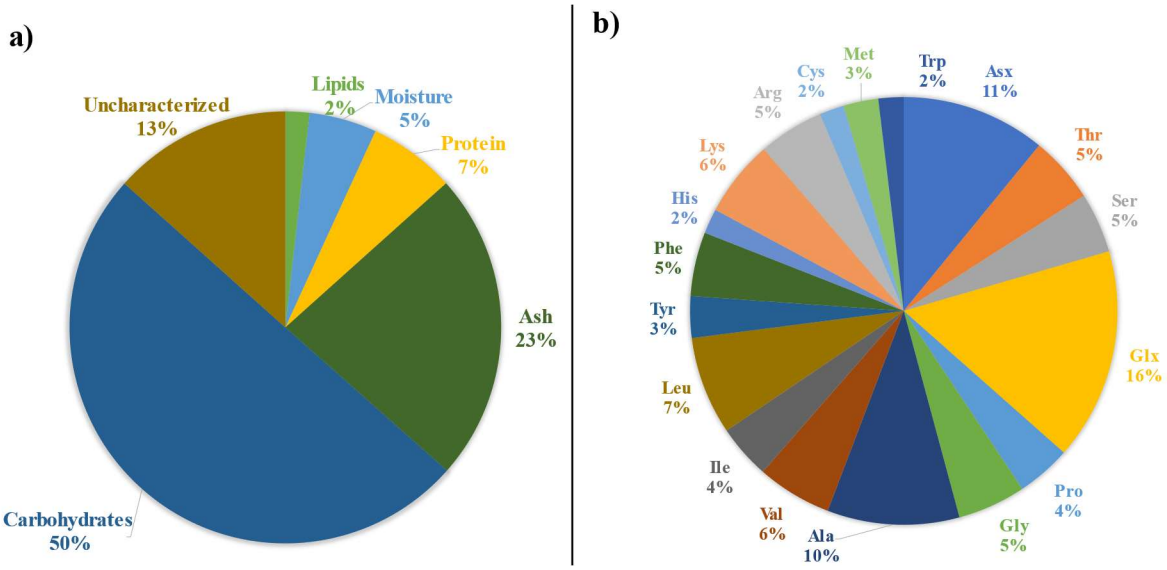

Figure 2.2: a) Proximate composition and b) amino acid composition of freeze-dried *M. pyrifera* (starting material). Abbreviations: Methionine (Met), Tryptophan (Trp), Asparagine (Asx), Threonine (Thr), Serine (Ser), Glutamine (Glx), Proline (Pro), Glycine (Gly), Alanine (Ala), Valine (Val), Isoleucine (Ile), Leucine (Leu), Tyrosine (Tyr), Phenylalanine (Phe), Histidine (His), Lysine (Lys), Arginine (Arg), Cystine (Cys)

The FAO provides guidelines for the recommended amount of each EAA in relation to total protein, and the amounts of EAA present in the freeze-dried *M. pyrifera* meet these requirements. This is extremely important for the potential inclusion of *M. pyrifera* into the alternative protein value chain, and these requirements are later discussed in the results section describing the amino acid composition of the extracts.

The fatty acid profile of the starting material is shown in Table 2.1. *M. pyrifera* fatty acid composition included primarily myristic (C14:0) (11%), palmitic (C16:0) (27%), oleic (C18:1, n-9) (21%), linoleic (C18:2, n-6) (7%), arachidonic (C20:4, n-6) (20%), and eicosapentaenoic acids (C20:5, n-3) (9%), followed by small amounts of other fatty acids. Our results differ from the ones previously found for *M. pyrifera* (55), where a higher concentration of n-3 fatty acids, resulting from a greater percentage of eicosapentaenoic acid, was measured in our starting material (~11% vs. ~ 6%). These differences may be due to variations in environmental growth conditions and

harvesting time. Regardless of the differences observed, both studies demonstrated high levels of unsaturated fatty acids and a n-6/n-3 ratio well below 10/1, indicating that while low in lipids, the quality of fats in *M. pyrifera* is high (55).

Table 2.1: Fatty acid composition by GC-FID of *M. pyrifera* lipids (starting material).

Fatty Acid	Composition of identified fatty acids (% w/w)
Lauric acid, C12:0	0.26 ± 0.02
Myristic acid, C14:0	11.33 ± 0.14
Tetradecenoic acid, C14:1	0.60 ± 0.03
Palmitic acid, C16:0	26.81 ± 0.15
Palmitoleic acid, C16:1 *n-7*	1.32 ± 0.01
Stearic acid, C18:0	0.44 ± 0.00
Oleic acid, C18:1 cis *n-9*	21.40 ± 0.31
Linoleic acid, C18:2 *n-6*	6.65 ± 0.35
α-linolenic acid, C18:3 *n-3*	1.46 ± 0.01
Arachidonic acid, C20:4 *n-6*	20.22 ± 0.11
Eicosapentaenoic acid, C20:5 *n-3*	9.21 ± 0.13
Docosahexaenoic acid, C22:6 *n-3*	0.29 ± 0.01
Total *n-6*	26.87 ± 0.46
Total *n-3*	10.96 ± 0.15

Oxylipins are biologically active oxygenated derivatives of polyunsaturated fatty acids and their derived metabolites have recently gained attention for their potential to be used as sensitive oxidation markers to identify early oxidation (31). To the best of our knowledge, while individual *M. pyrifera* oxylipins have been studied (56), the oxylipin profile of *M. pyrifera* has not been previously reported. The distribution of bound and free oxylipins in *M. pyrifera* lipids is shown in Table 2.2. The results indicate high levels of arachidonic and linolenic acid metabolites. Additionally, the majority of oxylipins were present primarily in the esterified form, with only 11-HETE, 12-HETE, 15-HETE, 14.15 DiHETrE and 15-HEPE present in majority free form. While this could be of interest because free oxylipins are the bioactive forms, compared with esterified or bound oxylipins (31), additional research is needed to determine which forms of oxylipins

would be more absorbed by humans. Importantly, the presence of anti-inflammatory oxylipins derived from n-3 fatty acids is an important finding.

Table 2.2: Concentrations (fmol/mg) of total, free, and % esterified oxylipins by LC-MS/MS in *M. pyrifera.*

	Compound	Free Oxylipins (fmol/mg)	Total Oxylipins (fmol/mg)	% Esterified
ARA-metabolites	14(15)-EpETrE	303.33 ± 26.58	1670.00 ± 197.70	82%
	8(9)-EpETrE	22442.60 ± 670.51	85692.50 ± 1723.97	74%
	5-HETE	680.43 ± 11.78	2070.76 ± 76.48	67%
	11-HETE	1554.06 ± 26.51	2161.61 ± 43.58	28%
	12-HETE	1043.95 ± 17.47	2051.71 ± 53.70	49%
	15-HETE	8058.10 ± 35.62	15200.32 ± 95.67	47%
	5,6-DiHETrE	95.12 ± 3.65	1441.32 ± 40.15	93%
	14.15- DiHETrE	1008.43 ± 51.26	1888.63 ± 17.85	47%
LA-metabolites	9-HODE	658.30 ± 26.58	2885.97 ± 148.51	77%
	13-HODE	22442.60 ± 670.51	57128.33 ± 1723.97	61%
	9-oxo-ODE	101.38 ± 10.48	589.64 ± 48.23	83%
	13-oxo-ODE	760.45 ± 57.92	1786.34 ± 52.53	57%
	12(13)-EpOME	21.62 ± 2.06	171.83 ± 20.15	87%
	9(10)-EpOME	23.91 ± 0.90	204.64 ± 3.81	88%
	12,13-DiHOME	45.00 ± 11.98	164.10 ± 7.03	73%
	9,10-DiHOME	19.11 ± 1.09	53.12 ± 1.09	64%
	9,10,13-TriHOME	267.34 ± 8.33	1688.55 ± 69.58	84%
	9,12,13-TriHOME	648.71 ± 15.66	4831.02 ± 313.81	87%
ALA-metabolites	13-HOTrE	3561.55 ± 87.33	10428.62 ± 168.72	66%
	9-HOTrE	1253.42 ± 14.07	3664.90 ± 52.22	66%
EPA-metabolites	17,18-DiHETE	29.87 ± 3.23	89.60 ± 3.20	67%
	15-HEPE	8703.56 ± 41.38	12633.24 ± 118.78	31%
	5-HEPE	12.30 ± 1.80	49.80 ± 0.90	75%
DHA-metabolites	14(15)-EpETE	33.60 ± 2.54	123.40 ± 4.00	73%
	19(20)-EpDPE	12.00 ± 2.30	84.32 ± 3.40	86%
	13(14)-EpDPE	9.12 ± 0.17	31.68 ± 1.46	71%
	7(8)-EpDPE	7.79 ± 0.69	23.56 ± 7.39	67%
	17-HDoHE	93730 ± 53.10	2144.17 ± 66.46	56%

Abbreviations: linoleic acid (LA), arachidonic acid (ARA), α-linolenic acid (ALA), eicosapentaenoic Acid (EPA), and docosahexaenoic acid (DHA)

2.3.2 Effects of extraction conditions on the overall extractability of the AEP and EAEP

The effects of the key extraction parameters on the extractability of compounds from *M. pyrifera* and other brown macroalgae have been minimally investigated. The following results and discussion shed light on the impact of important extraction conditions (BWR, pH, time, temperature, and amount and type of enzyme) on the extractability (yield), structure/composition, and functional/biological properties of the extracts.

2.3.2.1 Total phenolic content of AEP and EAEP extracts

The effects of extraction BWR (1:30 and 1:50), time (2, 4, 6, and 8 h), and pH (4, 7, and 10) on the TPC of the AEP extracts are shown in Figure 2.3a. A maximum TPC of 15.56 ± 0.45 mg GAE/g FD *M. pyrifera* was achieved for AEP extracts obtained at 1:30 BWR, 6 h, and pH 7. Although reduced BWR increases the gradient concentration between solutes and extraction media, often favoring overall extractability (57), reducing BWR from 1:30 to 1:50, at the same pH value, did not significantly enhance overall phenolic extractability, allowing for the selection of reduced water usage during the extraction.

The existing literature suggests that in terrestrial plant matrices, pH plays a significant role in phenolic extraction mechanisms. In alkaline conditions, increased TPC contents have been attributed to the cleavage of ester and ether linkages between phenolics and other compounds such as carbohydrates and proteins, resulting in the release of bound phenolics and consequently higher TPC values (42,58). Conversely, acidic conditions have been linked to cleaving glycosidic linkages, but not ester linkages, which may lead to lower phenolic yields during the extraction (42,58,59). However, little is known about the effects of pH on the release of phenolics from the macroalgal matrix. Macroalgal phenolics exhibit high sensitivity, readily undergoing degradation and being susceptible to variations in storage and extraction

circumstances (34). The role of extraction pH on the TPC of *M. pyrifera* extracts is shown in Figure 2.3a, with lower TPC values observed at acidic (pH 4) and alkaline (pH 10) conditions, suggesting that these pH values could have been too extreme or harsh for the sensitive phenolics in *M. pyrifera*. Overall, at same BWR, the TPC of the extracts was favored by neutral pH, with a small increment observed at higher extraction times (6 - 8 h), which is attributed to higher solubilization of phenolics and other compounds that could impact phenolic extractability.

Additionally, the use of high temperatures has also been shown to cause the oxidation of phenolic compounds in macroalgae (57). Our results, however, demonstrate that the TPC of AEP extracts obtained at 30 °C (Figure 2.3b), which ranged from 7.98 ± 0.25 mg GAE/g FD *M. pyrifera* at pH 4 to 11. 58 ± 0.21 mg GAE/g FD *M. pyrifera* at pH 7, was lower than the ones at 60 °C, which ranged from 10.18 ± 0.43 mg GAE/g FD *M. pyrifera* at pH 10 to 15.56 ± 0.45 mg GAE/g FD *M. pyrifera* at pH 7. These results indicate that the enhanced breakdown provided by higher temperature was more important for extraction yields than the possible protection provided using lower temperatures.

The use of enzymes to assist in extracting *M. pyrifera* compounds resulted in increasing TPC with the addition of increasing amounts of NP (Figure 2.3 c and d). Although highest TPC values (17.75 ± 2.20 mg GAE / g FD *M. pyrifera*) were achieved when using 5% NP (w/w) (6 h) to assist the extraction, these values were not statistically different from the ones using 2.5% NP (w/w) (15.71 ± 2.33 mg GAE / g FD *M. pyrifera*) (6 h) (Fig. 2.3c) and lead to the selection of 2.5% NP (w/w) for further enzyme testing, with consideration for extraction time and integrated optimization of enzymes.

Extraction kinetics (Figure 2.3d) showed that the use of 2.5% NP (w/w) for two hours were sufficient to achieve TPC yields (16.19 ± 0.59 mg GAE / g FD *M. pyrifera*) that were not

statistically different from the ones achieved by the EAEP at 6 h (16.65 ± 0.43 mg GAE / g FD *M. pyrifera*) and were higher than the ones from the AEP at 2 h (1:30 BWR, pH 7) (11.56 ± 0.53 mg GAE / g FD *M. pyrifera*). This demonstrates the ability of enzymes to shorten extraction times due to their ability to enhance the breakdown of the algal matrix. Additionally, the use of a 5% CBL (w/w) pretreatment for 2 h, followed by the use of 2.5% NP (w/w) for an additional 2 h, resulted in the highest TPC value (16.79 ± 0.38 mg GAE/g FD *M. pyrifera*), suggesting that the use of the carbohydrase pretreatment likely enhanced the degradation of the complex carbohydrate matrix of *M. pyrifera*, thus facilitating the protease access to matrix and dissolution of phenolics into the extraction medium. Increased TPC caused by the addition of enzymes can be attributed to enzymes' ability to enhance cell disruption and cleave intracellular proteins (12,16,17) releasing more phenolics and other compounds, like amino acids, that can be detected by the assay.

The lack of consensus on optimum parameters for the extraction of phenolics from macroalgae is reflected in the available macroalgae literature, which report optimum extraction temperatures from 25 to 80 °C (5,34), BWRs ranging from 1:15 to 1:70 (5,60,61), and optimum extraction times from 3 up to 32 h (5,60,61), with limited attention to the potential effects of pH on phenolic extractability and composition. The TPC results reported herein are higher than others reported previously for water-based extractions (1:10 BWR, 40 °C, 2 h) from *M. pyrifera,* where TPC values of 1.47 mg GAE/ g dry seaweed have been achieved by the use of a hexane pretreatment followed by water extraction (5). This may be due to the preparation of the starting material (air dried vs. freeze-drying, which could result in the degradation of phenolics) as well as the decreased extraction temperature and time. Although there are no reports describing the impact of enzymatic extraction on the TPC of *M. pyrifera* extracts, the effectiveness of 5 carbohydrases

(Viscozyme, AMG 300, Cellucast, Termemyl, Ultraflo) and 3 proteases (Flavourzyme, Alcalase, Neutrase) on the TPC of the extracts of 7 brown seaweeds (*Sargassum boveanum*, *Sargassum angustifolium*, *Padina gymnospora*, *Canistrocarpus cervicornis*, *Colpomenia sinuosa*, *Iyengaria stellata*, and *Feldmannia irregularis)* has been reported. Highest TPC values of 84.0 ± 6.7 mg GAE/ g of extract from *S. angustifolium* were achieved with the use of 0.1% of the protease Flavourzyme at pH 7, 1:100 BWR, 20 h, 50 °C. (62). The aforementioned study demonstrates that phenolic extractability from different seaweed species is influenced by different enzymes, indicating the importance of the matrix effect and enzyme specificity on phenolic extractability.

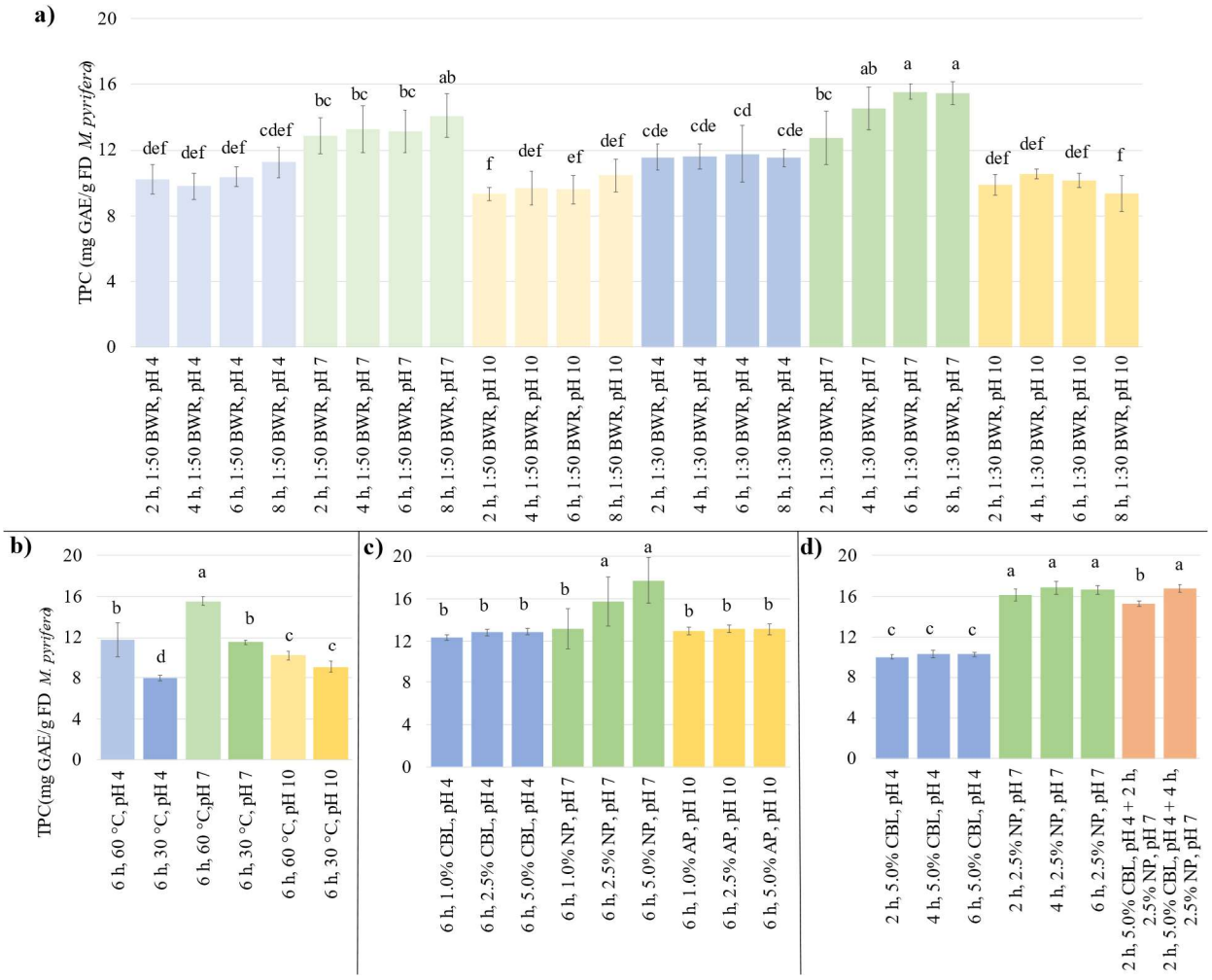

Figure 2.3: Impact of BWR, pH, and incubation time on the TPC of AEP extracts at 60 °C (a), impact of temperature and pH on the TPC of AEP extracts at 6 h, 1:30 BWR (b), impact of type and amount of enzyme on the TPC of EAEP extracts at 6 h, 1:30 BWR, 60 °C (c), and integrated impact of extraction time and combined enzyme usage on TPC of EAEP extracts at 1:30 *BWR,* 60 °C (d). TPC was determined via Folin-Ciocalteu assay.

It is important to note that, while the TPC assay is commonly used to estimate the total phenolic content of a wide range of samples, it is a colorimetric assay that is not highly specific for phenolics and can be influenced by the presence of other molecules in the extract. The reagents used in the TPC assay can react with other oxidizable compounds like aromatic amines, amino acids, and reducing sugars (63,64). Therefore, TPC values obtained with colorimetric assays should be interpreted with caution as other reducing compounds extracted from *M. pyrifera* could

also contribute to the TPC readings. To obtain a more comprehensive understanding of the phenolic profile and composition of *M. pyrifera*, selected extracts were subjected to subsequent HPLC analysis (see section 2.3.3.1).

2.3.2.3 Fucoidan content of AEP and EAEP extracts by using fucose and sulfate content indicators

There is no consensus in the literature regarding the optimum extraction parameters for the extraction of fucoidan, a sulfated polysaccharide found in brown macroalgae and other marine organisms. Because fucose is the major monomer in fucoidan, its quantification provides a good gauge of the fucoidan content in the extracts (44). It is important to note that these findings for fucose content do not directly translate to fucoidan content. While fucose is the most common monomer in fucoidan, around 50% of fucoidan molecules are composed of other monosaccharides including galactose, glucose, xylose, mannose, and rhamnose (45,65). Aside from its monosaccharide composition, fucoidan is distinguished by the inclusion of sulfate groups within its structure. These sulfate groups have been quantified to serve as markers for fucoidan presence and to assess potential bioactivity of the extracts. Higher sulfation levels have been linked to higher bioactivity in extracts. While the measurement of fucose and sulfate groups does not directly correlate to the extraction of fucoidan, it likely relates to the bioactivity of the extracts (20,44).

The impact of extraction pH, BWR, and reaction time on the fucose content of the *M. pyrifera* extracts is shown in Figure 2.4a. Highest fucose content in the AEP extracts (15.23 ± 1.81 mg fucose Eq/ g FD *M. pyrifera*) was achieved at 1:50 BWR, 8 h, and pH 4. However, this value was not statistically different ($p<0.05$) from yields achieved at 4 and 6 h (1:50 BWR, pH 4) or from yields achieved at 6 h, 1:30 BWR at pH 4 (13.49 ± 1.73), pH 7 (12.79 ± 1.64), and pH 10 (12.16 ± 1.45), allowing for the selection of reduced water usage during extraction without

sacrificing yields. As observed for TPC extraction, higher temperatures (60 vs. 30 °C) also lead to higher fucose extractability by an average increase of 4.02 mg fucose Eq/ g FD *M. pyrifera* for each pH (Figure 2.4b), illustrating the ability of increased temperature to enhance mass transfer, cell wall permeability, and solubility of the matrix compounds into the extraction medium (42).

The use of enzymes to assist the extraction increased fucose extractability and shortened its extraction time (Figures 2.4 c and d). Overall, the use of higher concentrations of CBL and NP enhanced fucose extractability (Figure 2.4c). The highest fucose yield of 22.84 ± 0.86 mg fucose E/ g FD *M. pyrifera* was achieved when using 5% CBL (w/w) and was 69% higher than the yield without the use of enzymes at same conditions (13.49 ± 1.73 mg fucose Eq/ g FD *M. pyrifera* at 6 h, 1:30 BWR, pH 4). The use of 5% NP (w/w) resulted in 19.94 ± 2.30 mg fucose/g FD *M. pyrifera*, a 56% increase in extraction compared to AEP (12.80 ± 1.65 mg fucose Eq/ g FD *M. pyrifera* at 6 h, 1:30 BWR, pH 7). The use of AP, however, did not increase fucose yields to the same degree as CBL and NP. A 16% increase was observed for the use of 5% AP (w/w) (14.15 ± 0.94 mg fucose Eq/ g FD *M. pyrifera*) compared to the AEP (12.16 ± 1.45 mg fucose Eq/ g FD *M. pyrifera* at 6 h, 1:30 BWR, pH 10). Additional optimization of extraction time and elucidation of the integrated impact of enzymes use (Figure 2.4d) showed no statistical differences between fucose yields when using 5% CBL alone for 2 h (18.89 ± 0.88 mg fucose Eq/ g FD *M. pyrifera*) or 5% CBL for 2 h, followed by the use of 2.5% NP for 2 h (18.56 ± 0.57 mg fucose Eq/ g FD *M. pyrifera*) or 5% CBL alone for 2 h followed by the use of 2.5% NP for 4 h (20.44 ± 2.13 mg fucose Eq/ g FD *M. pyrifera*). These results demonstrate that the use of carbohydrates, rather than the protease, plays a pivotal role in enhancing extractability, allowing for the selection of conditions with shorter extraction times and reduced types and concentration of enzymes.

Fucoidans are located within the cell wall structure, underscoring the significance of breaking down the cellulose and protein network that constitutes the algal matrix. While this process is critical for extraction of these compounds, the prevalence of proteins in the cell wall structure is less common in brown macroalgae (66). In general, acidic conditions aid in the extraction of carbohydrates by aiding in breaking glycosidic bonds present in carbohydrates (7,59,67) and, as a result, are often used in the extraction of fucoidan from brown macroalgae (68,69). Our results demonstrate that the use of CBL alone was sufficient to increase fucoidan extractability when compared with the combined use of CBL followed by NP. These results can be attributed to the low amounts of proteins in brown macroalgae cells and to the ability of cellulases to breakdown the cellulose in the cell wall, thus enhancing the release of fucoidan from the cell wall.

The impact of BWR, pH, and extraction time on the sulfate content of AEP extracts is shown in Figure 2.5a. While the highest sulfate content for AEP extracts was achieved at 8 h, 1:50 BWR, and pH 7 (21.56 ± 2.02 mg SO_4^{2-} Eq/ g FD *M. pyrifera*), this value is not statistically different from the ones achieved at 2 h, 1:30 BWR and pH 4 (20.97 ± 1.51 mg SO_4^{2-} Eq/ g FD *M. pyrifera*), allowing for the selection of extraction conditions with reduced water usage and shorter extraction time. The use of lower temperatures resulted in lower sulfate yields (Figure 2.5b), with 17.23–20.44 mg SO_4^{2-}/g FD *M. pyrifera* at 60 °C versus 12.15–14.33 mg SO_4^{2-}/g FD *M. pyrifera* at 30 °C. The reduced sulfate extractability at 30 °C agrees with the reduced fucose extractability at the same temperature (Figure 2.4b), highlighting the extractability benefits associated with the use of a higher extraction temperature. The sulfate content of the extracts in the enzyme screening (Figure 2.5c) did not vary significantly within different enzyme concentrations and enzymes evaluated at 6h of extraction time. However, the use of NP during the extraction increased sulfate

yield from 17.23 ± 0.17 mg SO_4^{2-}/g FD *M. pyrifera* (AEP 6 h, 1:30 BWR, pH 7) to 27.84 ± 2.87 mg SO_4^{2-}/g FD *M. pyrifera* (2.5% NP (w/w), 6 h, 1:30 BWR, pH 7). The sulfate content of the extracts obtained using CBL and AP, individually, across the different enzyme concentrations, was close to their AEP counterparts (pH 4 and pH 10, respectively). Figure 2.5d shows that maximum sulfate extractability can be achieved by using 2.5% NP (w/w) at 6 h (27.84 ± 2.87 mg SO_4^{2-}/g FD *M. pyrifera*) and by using 5% CBL (w/w) for 2 h, followed by the use of 2.5% NP (w/w) for 4 h (23.96 ± 3.09 mg SO_4^{2-} Eq/ g FD *M. pyrifera*).

While the use of CBL resulted in greater fucose yields (22.84 mg fucose Eq/ g FD *M. pyrifera*, Fig 2.4d), the use of NP resulted in higher sulfate yields (27.84 mg SO_4^{2-}/g FD *M. pyrifera*, Fig 2.5d). When using a 5% CBL pretreatment followed by 2.5% NP, the resulting extracts are high in fucose (18.56 mg fucose Eq/ g FD *M. pyrifera*, Fig 2.4d) and have a relatively high sulfate content as well (23. 96 mg SO_4^{2-}/g FD, Fig 2.5d) demonstrating the benefits of using both enzymes in tandem.

The comparison of our data with the literature is hindered by the limited availability of a comprehensive evaluation of aqueous processing parameters on the extractability of *M. pyrifera* compounds. Acidic extraction of *M. pyrifera* (HCl, pH 1, 42 °C, 159 min, 1:10 BWR), after an ethanol pretreatment, resulted in a fucoidan fraction containing 12.16 mg fucose/ g dry *M. pyrifera* and 14.12 mg sulfate/ g dry *M. pyrifera* (7). These values are similar to those presented here. Additionally, as with the *M. pyrifera* extracts of this study and the sulfate to fucoidan ratio in the former study is greater than 1.

It is interesting to note that our results on fucose extractability from *M. pyrifera* are within the same range, albeit lower, compared to the findings of a study that evaluated the impact of hot water (70 °C, 24 h, 1:100 BWR) and acidic extraction (0.15M HCl, 65 °C, 2 h, 1:100 BWR) on

fucose extractability from three South African brown macroalgae species (*Ecklonia maxima*, *Laminaria pallida*, and *Splachnidium rugosum*) (70). These findings underscore the variability in extract composition among different species, with fucose and sulfate yields, as well as their relative ratios, varying when comparing one species to another (70). Hot water and acidic extractions from *Ecklonia maxima* resulted in extracts with 63 and 26 mg fucose/g dry seaweed and 44 and 136 mg sulfate/g dry seaweed, respectively. For extractions with *Laminaria pallida*, extracts with 51 and 33 mg fucose/g dry seaweed and 29 and 55 mg sulfate/g dry seaweed were achieved when using hot water and acidic extractions, respectively. However, higher yields were observed for extracts from hot water and acidic extractions from *Splachnidium rugosum*, which contained 265 and 220 mg fucose/g dry seaweed and 157 and 226 mg sulfate/g dry seaweed, respectively.

In contrast to the EAEP results discussed above, a study with *N. zanardinii*, tested different enzymes and found that the use of Alcalase, an alkaline protease, at pH 8, had highest yields (5.58% yield dry seaweed basis, from which 34.76 and 20.05% were attributed to carbohydrates and sulfate, respectively) when compared to other proteases and a cellulase (19). This difference can be attributed to the difference in pH required by each enzyme, enzyme specificity, and inherent differences between the macroalgal species.

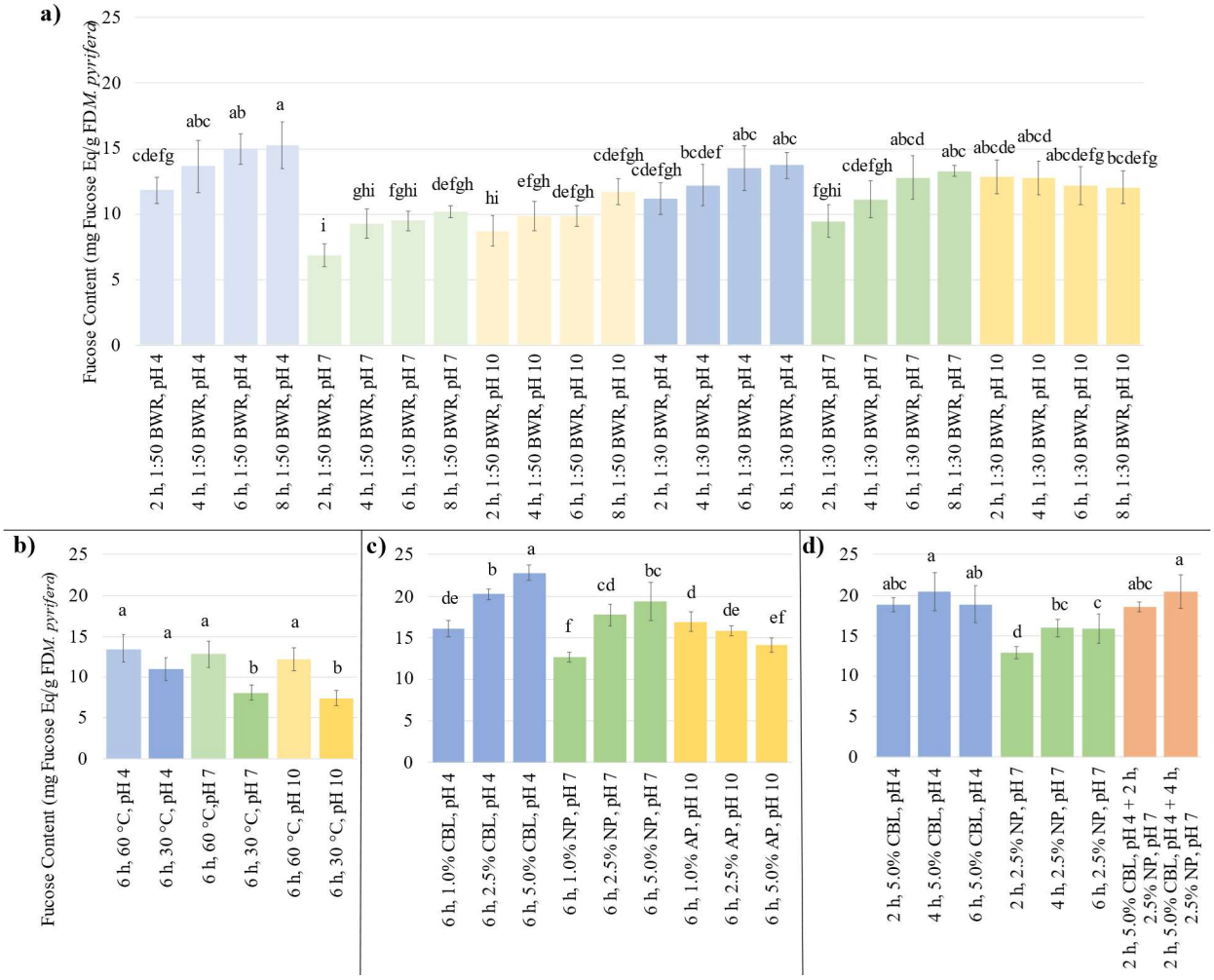

Figure 2.4: Impact of BWR, pH, and incubation time on the fucose content of AEP extracts at 60 °C (a), impact of temperature and pH on the fucose content of AEP extracts at 6 h, 1:30 BWR (b), impact of type and amount of enzyme on the fucose content of EAEP extracts at 6 h, 1:30 BWR, 60 °C (c), and integrated impact of extraction time and combined enzyme usage on the fucose content of EAEP extracts at 1:30 BWR, 60 °C (d). Fucose content was determined via the Dische and Shettles method.

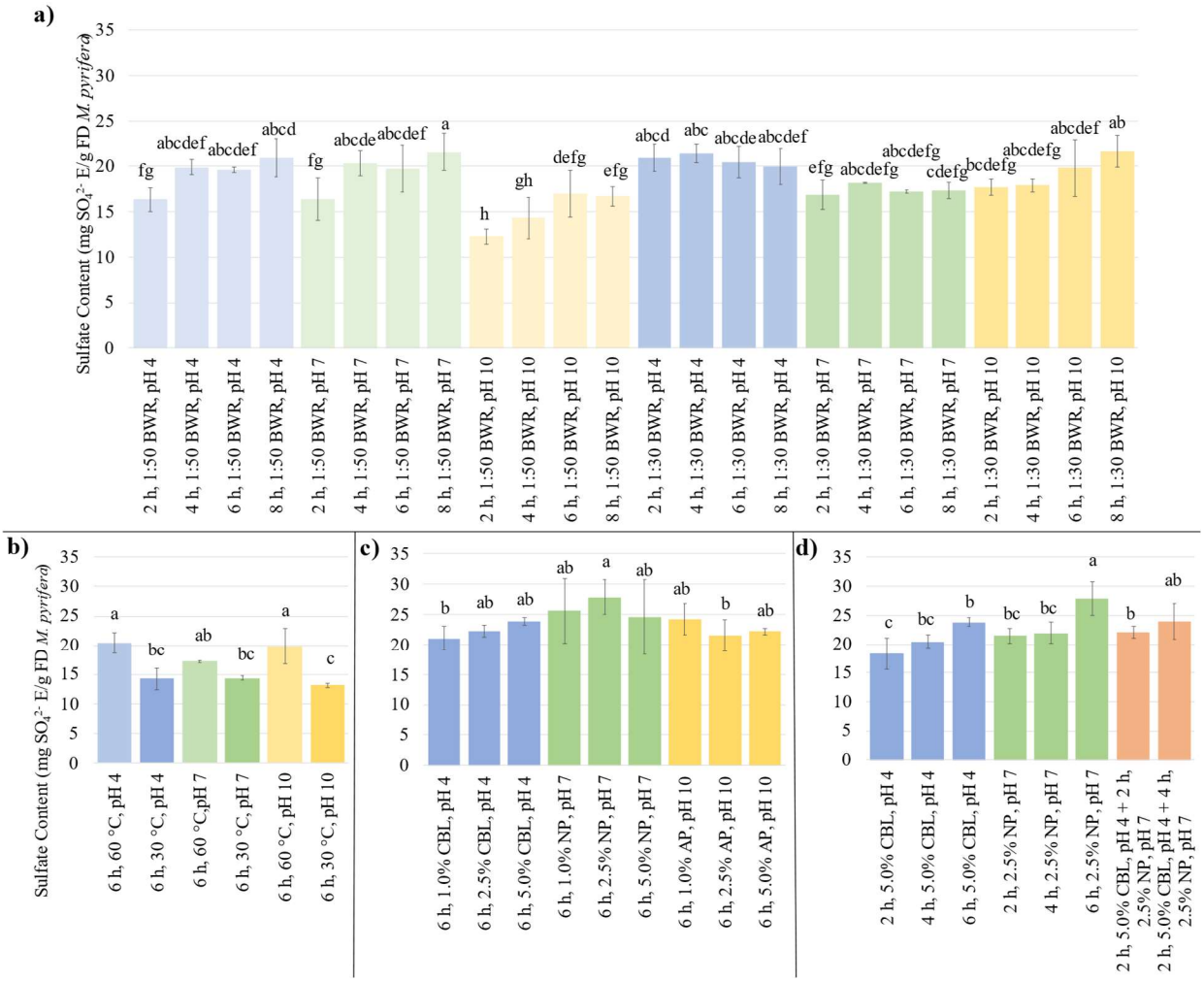

Figure 2.5: Impact of BWR, pH, and incubation time on the sulfate content of AEP extracts at 60 °C (a), impact of temperature and pH on the sulfate content of AEP extracts at 6 h, 1:30 BWR (b), impact of type and amount of enzyme on the sulfate content of EAEP extracts at 6 h, 1:30 BWR, 60 °C (c), and integrated impact of extraction time and combined enzyme usage on the sulfate content of EAEP extracts at 1:30 BWR, 60 °C (d). Sulfate content was determined via the barium-chloride assay.

2.3.2.4 Laminarin content of the AEP and EAEP extracts

The effects of extraction BWR (1:30 and 1:50), time (2, 4, 6, and 8 h), and pH (4, 7, and 10) on the laminarin content of the AEP extracts are shown in Figure 2.6a. Maximum yields of 12.96 ± 1.06 mg laminarin Eq/ g FD *M. pyrifera* were achieved at 2 h, 1:30 BWR, pH 7. Overall, extraction time did not play a large role in laminarin extractability. Yields achieved at 2 and 8 h were not significantly different, regardless of the pH (4, 7, or 10) and BWR (1:30 or 1:50).

Furthermore, reducing BWR from 1:30 to 1:50, at the same pH value, did not significantly enhance overall laminarin extractability, allowing for the selection of reduced water usage and shorter extraction time. As observed for the extraction of other compounds, the use of a lower temperature (30 vs. 60 °C) (Figure 2.6b) resulted in lower yields at pH 4 and 7, but not at pH 10. Laminarin exists in forms of both high and low branched chains of β-glucans, with highly branched chains being soluble in both hot and cold water (71). These results indicate that laminarin solubility, and its subsequent diffusion into the extraction medium, can vary depending on pH, temperature, and structure of the laminarin chains. Although structural characterization of the extracted laminarin was not evaluated, the lack of significant difference between laminarin extractability at pH 10 at 30 and 60 °C is likely due to the extraction of the highly branched chains, which are soluble in hot and cold water.

The benefit of adding enzymes to assist the extraction of laminarin is shown in Figure 2.6c. Laminarin extractability increased from 10.37 to 13.20 mg laminarin Eq/g FD *M. pyrifera* when CBL concentration increased from 1 to 5%, respectively, ending higher than the AEP results (11.00 mg laminarin Eq/g FD *M. pyrifera*) at the same conditions (6 h, 1:30 BWR, pH 4). However, the use of NP to assist the extraction resulted in lower extractability (9.05 to 9.73 mg laminarin Eq/ g FD *M. pyrifera*) compared with the AEP results at the same conditions (12.58 mg laminarin Eq/g FD *M. pyrifera*, 6 h, 1:30 BWR, pH 7), likely due to the powder form of the NP enzyme, which likely resulted in increased slurry viscosity. Additional processing optimization (Figure 2.6d) revealed that the use of 5% CBL (w/w) achieved maximum yields after just 2 h (14.38 ± 0.42 mg laminarin Eq/g FD *M. pyrifera*), reducing extraction time from 6 to 2 h.

Laminarin are water soluble polysaccharides found within vacuoles of brown algae cells and serve as energy reserves (71). Their extractability is highly dependent on the extraction

process's ability to breakdown the algal cell wall. However, unlike with other compounds that make up the matrix of the cell wall, laminarin extraction does not require complete breakdown of the algal matrix, which explains its high extractability at short extraction times. Increased laminarin extractability when using carbohydrases to assist the extraction is likely due to the ability of CBL to breakthrough the network of cellulose and hemicellulose making up the cell wall, allowing for greater release of laminarin.

The AEP results presented herein are in good agreement with the laminarin content of aqueous extracts from *Sargassum mcclurei* where maximum yields (11.98 ± 0.49 mg laminarin Eq/ g dry seaweed) were achieved at 2 h and pH 7 (72). Unlike the results presented above, Van Breda et al. found the use of carbohydrases less effective than non-enzymatic dilute acidic extractions on laminarin extraction (1 g laminarin Eq/ liter vs. 2.5 g laminarin Eq/ liter) from *E. maxima* (40). Possible explanations for this may be the specific enzymatic cocktails used and differences in the algal matrix of different species. To the best of our knowledge, there are no reports describing the impact of enzymatic extraction on the extractability of laminarin from *M. pyrifera*.

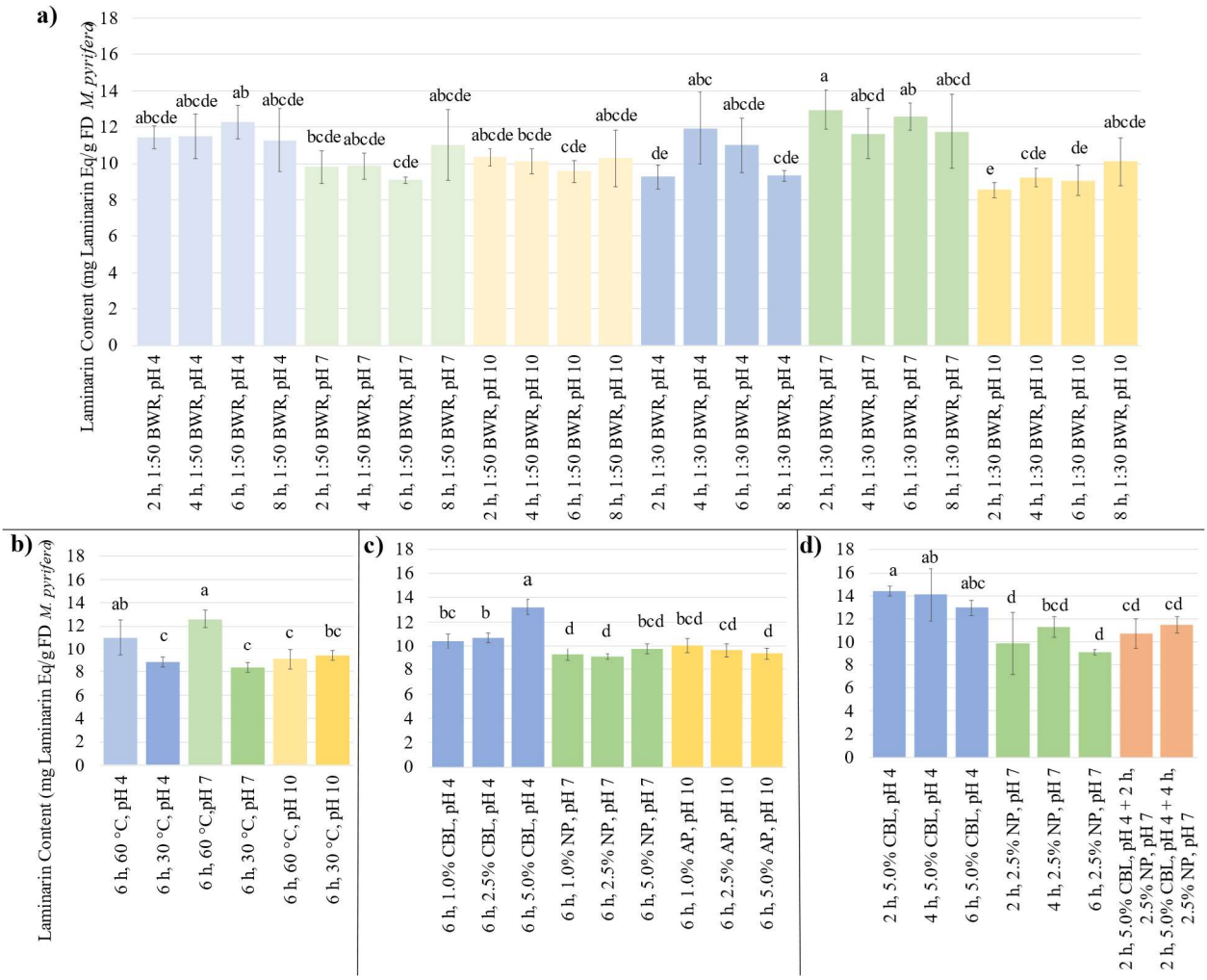

Figure 2.6: Impact of BWR, pH, and incubation time on the laminarin content of AEP extracts at 60 °C (a), impact of temperature and pH on the laminarin content of AEP extracts at 6 h, 1:30 BWR (b), impact of type and amount of enzyme on the laminarin content of EAEP extracts at 6 h, 1:30 BWR, 60 °C (c), and integrated impact of extraction time and combined enzyme usage on laminarin content of EAEP extracts at 1:30 *BWR,* 60 °C (d). Laminarin content was determined via a reducing sugar assay.

2.3.2.5 Alginate content of the AEP and EAEP extracts

Maximum alginate content of 181.50 ± 9.84 mg calcium alginate/g FD *M. pyrifera* was achieved for AEP extracts produced at 1:30 BWR, 8 h, and pH 10, as shown in Figure 2.7a. At a higher BWR (1:30), higher extractability was achieved at the alkaline condition (pH 10), compared with other pH values. However, at a lower BWR (1:50), where the gradient concentration between solutes and extraction media is increased and the overall processing extractability is enhanced (57),

both acidic and alkaline conditions led to higher extractability at longer extraction times (6 and 8 h) when compared with neutral conditions (128–153 mg FD calcium alginate/ g FD *M. pyrifera* versus 76-112 mg FD calcium alginate/ g FD *M. pyrifera*). These results reflect the role of acidic and alkaline pH in disrupting the kelp biomass (71), which favors compound extractability, including alginate. However, at higher BWR, which increases the medium viscosity, alkaline pH seems more effective to enhance alginate extractability (73), evidencing the integrated impact of both extraction parameters. Higher alginate extractability at alkaline pH has been linked to the deprotonation of carboxylic acids molecules in the alginate, which enhances its solubility in the extraction medium by increasing electrostatic repulsion between the negatively charged alginate molecules (74). The impact of temperature and extraction pH on alginate extractability (Figure 2.7b) illustrates the compounding effect of temperature in alkaline conditions. At lower temperatures (30 °C), alkaline conditions did not result in significantly higher yields than neutral conditions, but the combined disruptive effect of higher temperatures and alkaline conditions contributed to higher yields at 60 °C (114 mg calcium alginate/ g FD *M. pyrifera*).

In the enzyme screening (Figure 2.7c), the addition of enzymes did not lead to increased alginate yields compared with AEP. In fact, the addition of NP at all enzyme loadings (1, 2.5, and 5%) led to decreased yields (60–79.5 mg calcium alginate/g FD *M. pyrifera* for NP vs. 94 mg calcium alginate/g FD *M. pyrifera* for AEP at 6 h, 1:30 BWR, pH 7), possibly due to increased slurry viscosity caused by the addition of the powder enzyme. It is important to note, that additional compounds like proteins and polyphenols may have been precipitated along with the alginate following the addition of calcium chloride (41). At pH 10, protein extractability was higher (see protein extraction section), and some proteins in the extracts may have been precipitated in the

alginate fraction, leading to an overestimation of alginate. Further alginate structural analysis for selected conditions will be discussed later.

The AEP results are in agreement with the literature, where the benefits of alkaline extraction of alginate from *Durvillaea potatorum*, over acidic extraction conditions, were reported (68). Our results differ from those of Rostami et al. where, in extractions following an ethanol pretreatment, the use of cellulase increased alginate yields from *C. peregrina* when compared with water extractions (6.60 vs. 3.80% yield) (75). Such differences can be due to differences in seaweed structure, pretreatment effects, and purification of alginates prior to yield calculation. This suggests that alginate extracted enzymatically may be purer than that extracted by the AEP, which is supported by the extraction of alginate from *A. angustifolium*, where alginate extracted with Alcalase and Cellulase, when compared to water and acid extracted alginate, contained the lowest levels of proteins and polyphenols (11).

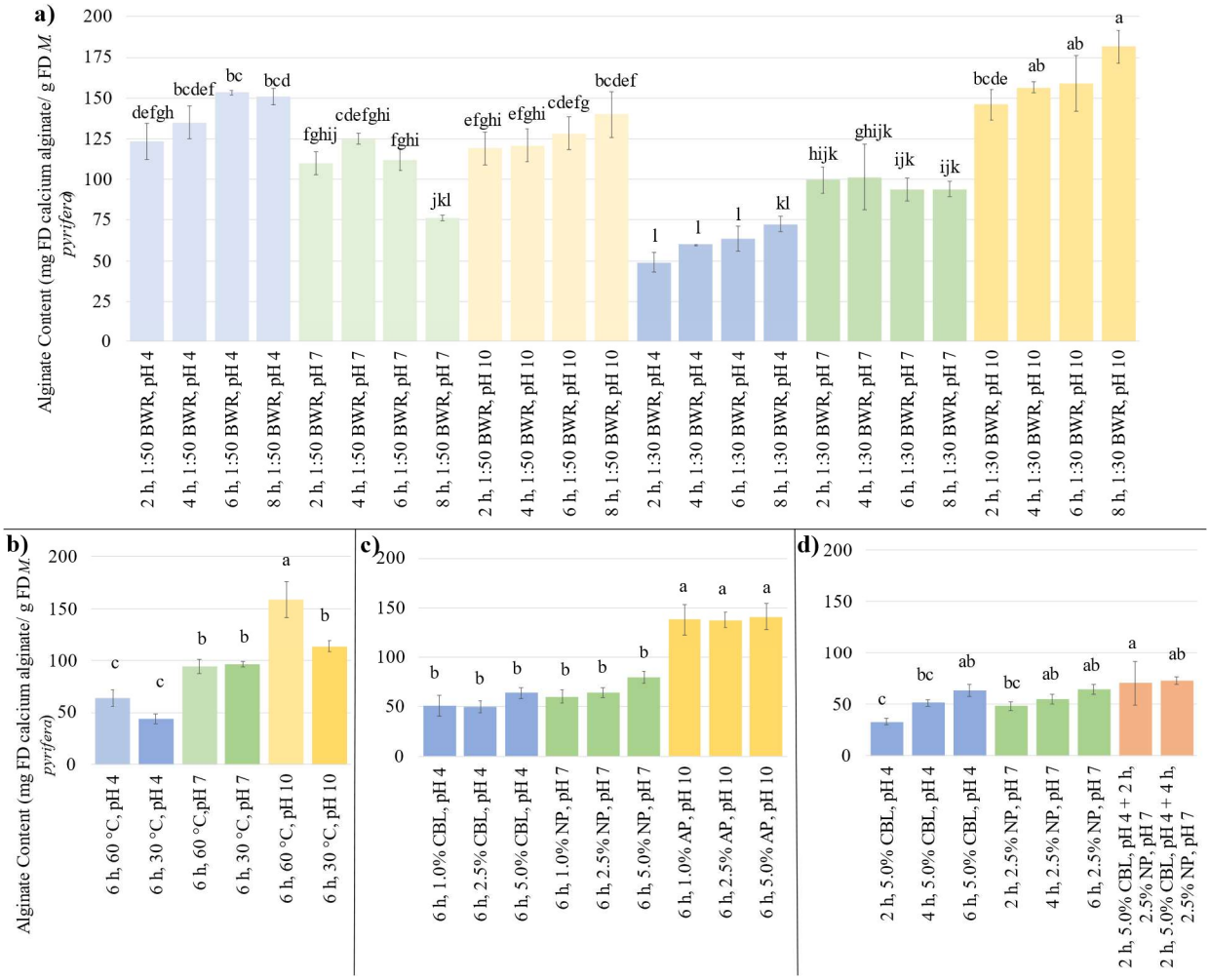

Figure 2.7: Impact of BWR, pH, and incubation time on the alginate content of AEP extracts at 60 °C (a), impact of temperature and pH on the alginate content of AEP extracts at 6 h, 1:30 BWR (b), impact of type and amount of enzyme on the alginate content of EAEP extracts at 6 h, 1:30 BWR, 60 °C (c), and integrated impact of extraction time and combined enzyme usage on alginate content of EAEP extracts at 1:30 *BWR,* 60 °C (d). Alginate content was determined by mass of freeze-dried calcium alginate.

2.3.2.6 Protein extraction yields of the AEP and EAEP extracts

The impact of BWR, extraction time, and pH on protein extractability in the AEP is shown in Figure 2.8a. Although a small increment in protein extractability was observed when extraction pH increased from 4 to 10, the magnitude of the increment observed was not significant in many instances. Maximum protein extractability (36.02% total protein extractability (TPE)) was reached at pH 7, 1:50 BWR, 8 h. These yields are not statistically

different from those achieved at pH 7 and 10 regardless of extraction time or BWR. Overall, increasing BWR from 1:50 to 1:30 did not lead to a statistically significant reduction in TPE, enabling the selection of conditions with reduced water usage.

That trend agrees with the literature (12,15) which demonstrates that protein extractability is enhanced when extractions are carried out away from the protein isoelectric point (PI). Although the PI of *M. pyrifera* proteins is unknown, the PI for other seaweeds is reported to be between pH values of 3 and 4 (76), explaining the slightly lower protein extractability of *M. pyrifera* proteins at pH 4. Additionally, this study focused on the simultaneous extraction of several compounds from *M. pyrifera* to gain a holistic view of the impact of the processing conditions evaluated, which might have impacted accurate quantification of the extracted proteins. It is possible that, when precipitating the alginate from the extract, some protein loss might have occurred, resulting in lower yields in the extract.

Unlike other compounds (phenolics, fucoidan, laminarin, alginate), Figure 2.8b shows that protein extractability was not significantly affected by temperature (30 v. 60 °C). This indicates that the enhanced mass transfer, cell wall permeability, and solubility of compounds provided by the higher temperatures (42), affected the extractability of algal carbohydrates more than that of the proteins.

The literature on enzyme assisted macroalgae extractions most commonly uses a 5% enzyme loading, much higher than the amount used for other plant matrices (19,75,77). To identify the best enzyme concentration and strategy for the EAEP of *M. pyrifera*, the impact of enzyme loading was screened and evaluated for 1, 2.5, and 5% (w/w) (Figure 2.8c). TPE greatly increased from maximum AEP value of 36.02% to 41.77-54.72% and 60.42-64.35% when 1-5% NP (w/w) and 1-5% AP (w/w) were used, respectively. Higher TPE with the use of AP is likely

due to the synergistic effects of the protease and alkaline conditions (pH 10) (12). The use of CBL (18.14-20.18% TPE with 1, 2.5 and 5% CBL (w/w)) did not increase the protein content of the extracts with respect to its AEP counterpart. Due to the ability of NP to enhance the extractability of a higher number of *M. pyrifera* compounds (phenolics, fucose, sulfate) compared to AP, NP was selected as the protease for further optimization.

When evaluating the impact of extraction time and sequential use of enzymes (Figure 2.8d), overall, high TPE (47.75% TPE) was reached by the sequential use of 5% CBL (w/v) for 2h followed the use of 2.5% NP (w/v) for an additional 2 h. However, this result is not significantly different from that when using NP alone at 2 h (43.14% TPE), indicating, as observed with other plant matrices, that a carbohydrase pretreatment is often not effective to substantially increase TPE (12).

Higher protein extractability in the EAEP is attributed to the ability of carbohydrates and proteases to break down the algal matrix and hydrolyze the proteins, thus allowing for greater release of proteins from the matrix, which , for macroalgae, are primarily produced intracellularly (78). Current literature has demonstrated that enzymatic extraction can enhance overall protein extractability from macroalgae (79). Previous studies with EAEP targeting the extraction of proteins (18 h, pH 4.5, 50 °C, 1:50 BWR, 10% CellicCTec3 (v/w)) had higher extraction yields (74%) than our process (13). Differences in TPE may be related to the use of different protein quantification methods (Dumas combustion vs. BSA), differences in the specific enzymatic cocktails and extraction conditions used (i.e., extraction time), and differences in sample preparation (air dried v freezing and freeze-drying).

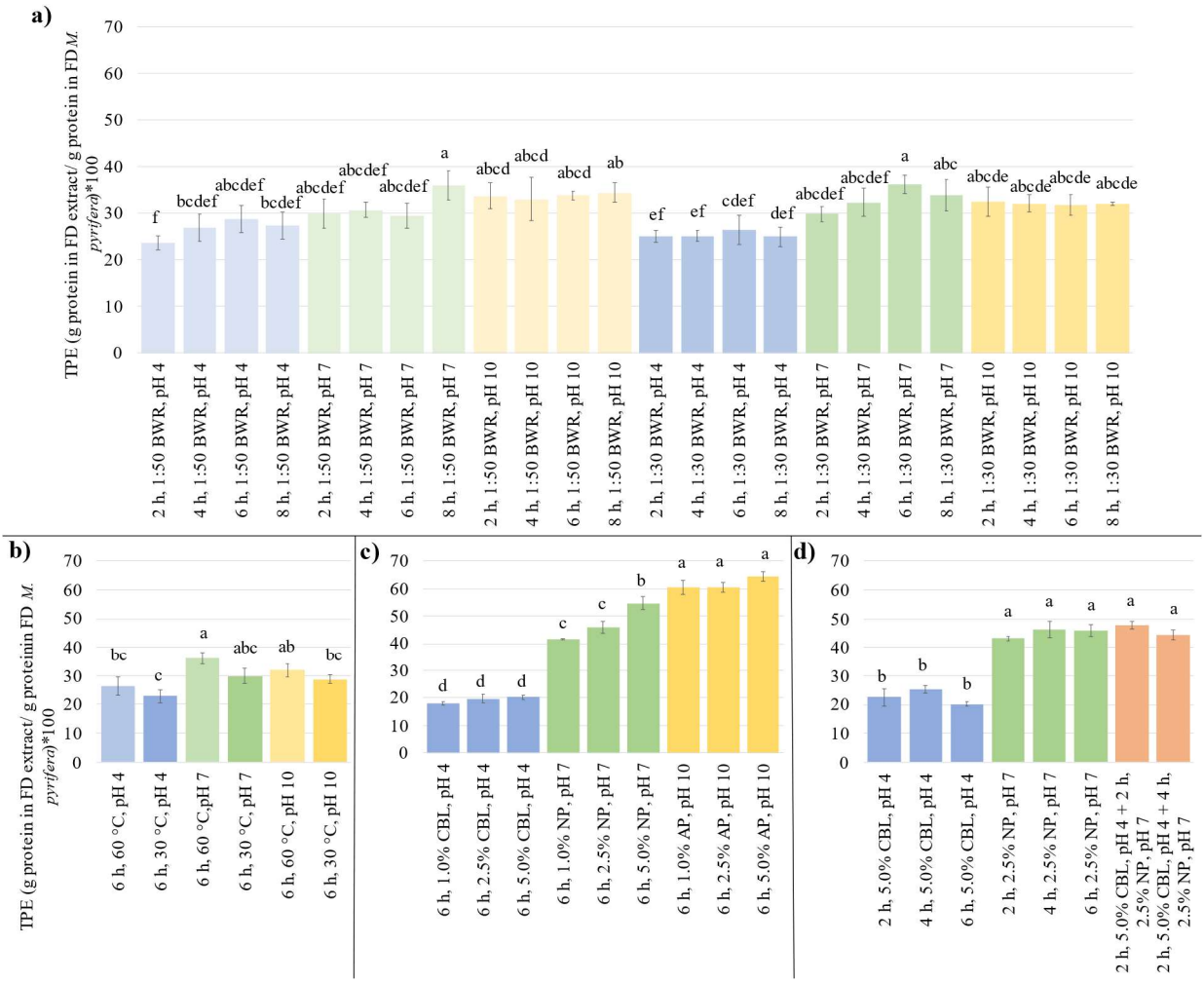

Figure 2.8: Impact of BWR, pH, and incubation time on the TPE of AEP extracts at 60 °C (a), impact of temperature and pH on the TPE of AEP extracts at 6 h, 1:30 BWR (b), impact of type and amount of enzyme on the TPE of EAEP extracts at 6 h, 1:30 BWR, 60 °C (c), and integrated impact of extraction time and combined enzyme usage on TPE of EAEP extracts at 1:30 *BWR,* 60 °C (d). TPC was determined via the Dumas combustion method.

2.3.3 Selecting extracts from specific extraction conditions for comprehensive analytic characterization and determination of biological properties

Because the use of higher dilutions (1:50 BWR) did not significantly enhance the extractability of most biomass compounds, a higher BWR (1:30), which results in reduced water usage, was selected for subsequent experiments. Extraction time of 6 h was selected to enable adequate time for all compounds to reach their maximum extractability. Overall, the use of a higher extraction temperature (60 vs. 30 °C) resulted in higher extractability for most macroalgal

compounds, highlighting the impact of higher temperatures on reducing the slurry viscosity and enhancing diffusion. Based on these results and on the potential impact of pH on the structure and functionality of the extracted compounds, extracts generated at 1:30 BWR, 6 h, and 60°C at pH 4, 7, and 10 were selected for subsequent in-depth analyses with respect to their structural composition (using advanced analytical techniques) and biological properties as the extractability of most compounds was maximized under the aforementioned conditions.

As discussed above and shown in Figures 2.4c and 2.6c, the use of 5% (w/w) CBL resulted in maximum overall extractability, with significantly higher fucose and laminarin yields when compared with 2.5% (w/w) CBL. Although 5% NP (w/w) resulted in higher TPE when compared with the use of 2.5% NP (w/w), the extractability of phenolics, fucose, sulfate, laminarin, and alginate at 2.5% was not statistically different from that at 5.0% enzyme use. When compared against the extraction performance of CBL and NP, AP only increased alginate and protein extractability, resulting in lower TPC values than the comparable AEP condition (BWR 1:30, pH 10, 6 h). For these reasons, 5% CBL (w/w) and 2.5% NP (w/w) were selected for further optimization of extraction time (2, 4, and 6 h) and the integrated impact of a carbohydrase pretreatment (5% CBL (w/w)) followed by protease extraction (2.5% NP (w/w)) was evaluated, with the goal of determining the impact of the initial breakdown of cellulose and hemicellulose by the carbohydrase on subsequent matrix penetration by the protease. The sequential use of enzymes (5% CBL for 2 h followed by 2.5% NP for an additional 2 h) reached maximum, or not statistically different from the maximum, yields for fucose and alginate compared to the use of 5% CBL alone for 4 and 6 h respectively and for sulfate, alginate, and protein compared to 2.5% NP alone for 6, 6, and 2 h respectively. TPC and laminarin yields when using 5% CBL for 2 h followed by 2.5% NP for an additional 2 h were not at their maximum but were between that of the yields for 5%

CBL (2 h) and 2.5% NP alone (2 h). However, unlike when using either CBL or NP which each selectively increased yields of certain components, the enzyme combination was able to reach high yields for all components. Therefore, the use of 5% CBL (w/w) for 2h followed by 2.5% NP (w/w) for an additional 2h was selected as the enzymatic condition for further analysis.

Based upon the justifications above, selected extraction conditions for more in-depth analytical characterization are summarized in Table 2.3: 1:30 BWR, 60 °C, 6 h, pH values 4, 7, and 10 for the AEP and 1:30 BWR, 60 °C, 5% CBL (w/w) for 2 h followed by 2.5% NP (w/w) for 2 h for the EAEP. These were selected for their ability to reach maximum yields for a wide variety of compounds while reducing water use and time.

Table 2.3: Selection of extracts from optimum AEP and EAEP conditions for comprehensive analysis

Process	pH (enzyme)	Temperature	BWR	Time	Abbreviated title
AEP	pH 4	60 °C	1:30	6 h	AEP pH 4
AEP	pH 7	60 °C	1:30	6 h	AEP pH 7
AEP	pH 10	60 °C	1:30	6 h	AEP pH 10
EAEP	pH 4 followed by pH 7 (carbohydrase followed by protease)	60 °C	1:30	4 h	EAEP

2.3.3.1 Metabolomic profiling

Targeted phenolic profiling of the extracts revealed the absence of any peaks for phlorotannins, the type of phenolic compound characteristic of brown macroalgae, or polyphenols in general. Although an increase in gallic acid concentration was observed in the extracts obtained by solvent extraction (50:50, methanol:water), solvent extracts still lacked phlorotannins. This suggests that *M. pyrifera* may not contain many phenolic compounds, including phlorotannins. It is important to note that phlorotannins are very sensitive compounds and easily degraded (34).

Although samples were shipped frozen and were kept (-20 °C) frozen for 6-12 months until analysis, it is possible that during handling, storage, and freeze-thaw cycles, the phlorotannins in the starting material may have been degraded. Other studies employing a hexane wash prior to water extraction have identified a limited number of phlorotannins in low concentrations (2.14 mg phlorotannins/ g dry seaweed) in *M. pyrifera* extracts via High Precision Liquid Chromatography Mass Spectrometry (HPLC-ESI-MS/MS), identifying phloroeckol and a phloroglucinol tetramer (80) and phloroglucinol (5). Unlike our analysis, these studies both used a hexane wash which may have enhanced the extractability of the phlorotannins.

Untargeted phenolic profiling of the extracts supported the targeted analysis findings. No phlorotannins and very few phenols were found. Untargeted analysis was able to confirm the presence of peptides, lipid derivatives, and other metabolites. The results, in Figure 2.9, show the relative amounts of each metabolite (lipids, phenols, peptides, and other metabolites) found at each extraction condition.

The number of unique identified metabolites are shown in Figure 2.9 and are reported as the average of each metabolite class for each extraction condition. Apart from peptides, extraction conditions contained nearly identical amounts of each metabolite category (phenols, lipid derivatives, and other metabolites). When looking at the peptides, the EAEP resulted in the release of a greater number of peptides compared to the other conditions. Figure 2.10 shows the peak heights for the 10 most abundant peptides illustrating that, in addition to having more identified peptides than other conditions, the use of enzymes resulted in many peptides released in greater abundance. It is important to note that the untargeted metabolomics approach used has limitations when it comes to peptide identification. The results reported are of interest but are an

underestimation of the peptides in the extracts as the library used for comparison does not contain all possible peptides and hydrophilic peptides could not be identified with the methods employed.

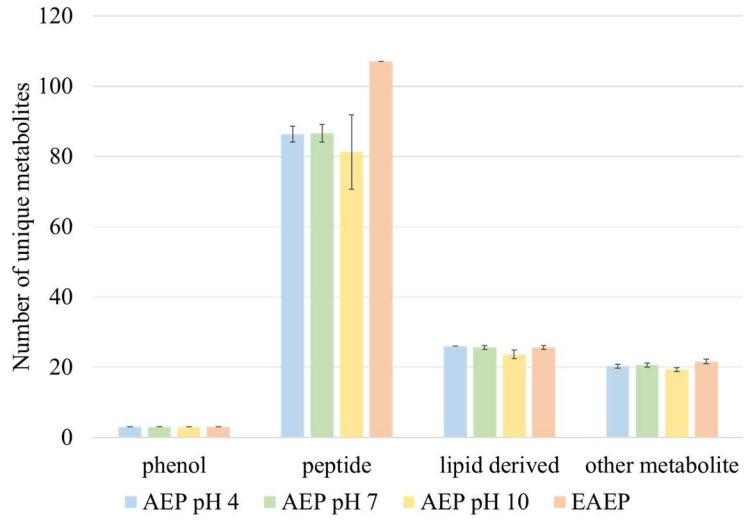

Figure 2.9: Impact of AEP and EAEP on the metabolomic profile of the extracts using a LC-MS untargeted metabolomic approach

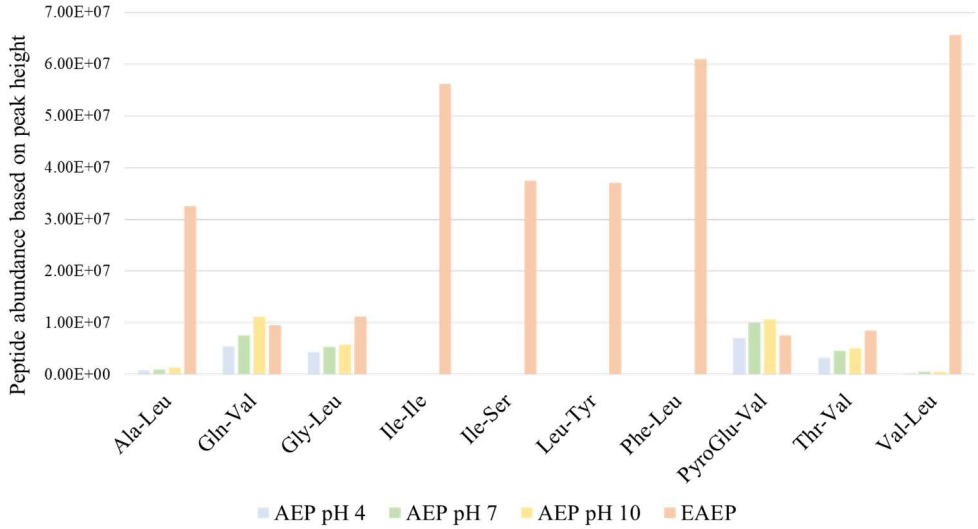

Figure 2.10: Impact of AEP and EAEP on the relative abundance of the 10 most prevalent peptides using a LC-MS untargeted metabolomic approach

2.3.3.2 Monosaccharide quantification

Monosaccharide quantification data for fucoidan's two most abundant monosaccharides, fucose and galactose (44), are shown in Table 2.4. Highest concentrations of fucose and galactose of 13.07 and 5.97 mg /g freeze dried *M. pyrifera*, respectively, were achieved for AEP extracts

obtained at pH 4. Acidic conditions are typically used to hydrolyze polysaccharides and release monosaccharides, which has been attributed to the ability of acidic conditions to cleave glycosidic bonds (7,59,67).

While the fucose yields are below those for aqueous extractions reported by Leyton et al. for *M. pyrifera* (39.7 mg fucose/ g dry seaweed), the galactose yields fall within the range found by Leyton et al. (5.7 mg galactose/ g dry seaweed) (5). These differences can partly be attributed to different harvest seasons (81). However, they also likely stem from the alginate precipitation step, not performed by Layton et. al., which might have precipitated some of the extracted fucose. Other studies involving alginate precipitation from the extracts have reported the presence of fucose in the alginate fraction (7,68). It is possible that the part of the fucose extracted in the current study might have precipitated along with the alginate (not measured), leading to lower fucose content in the extract.

Table 2.4: Impact of extraction condition on fucose and galactose yields obtained by HPAEC-PAD

Process	mg Fucose/ g FD *M. pyrifera*	mg Galactose/ g FD *M. pyrifera*
AEP pH 4	13.07 ± 1.50	5.97 ± 3.70
AEP pH 7	8.01 ± 1.07	5.60 ± 0.61
AEP pH 10	9.04 ± 0.12	5.85 ± 0.27
EAEP	7.77 ± 0.11	5.74 ± 0.32

2.3.3.3 Oligosaccharide analysis

Oligosaccharide analysis results are shown in the heatmap below (Figure 2.11). The number of identified oligosaccharides for the AEP extracts increased with pH (34 identified at pH 4, 39 at pH 7, and 46 at pH 10). For the EAEP, conservative estimates of the remaining compounds after enzyme subtraction (CBL and NP) are reported. Because enzyme contribution could not be quantitatively subtracted from the total amount detected, any identified compound present in the enzyme blank was completely removed from EAEP extract measurements. With such a

conservative approach, true values of the number of identified oligosaccharides for EAEP samples are likely higher than the 29 reported.

Oligosaccharides are reported as a code (key along the y-axis of Fig. 2.11) corresponding to the number of constituent monosaccharides. Of the 57 oligosaccharides that remain after enzyme blank subtraction, 16 were identified as potentially containing fucose. Full identification of the oligosaccharide was not possible due to a lack of, or obscure, fragmentation data. For these compounds, neutral masses are reported along the y-axis in Figure 2.11. It is likely that these unknowns contain fucose units that are linked to other molecules aside from the five monosaccharides (fucose, galactose, glucose, xylose, and mannose) used in the data search and annotation. Due to the novelty of this research, there are no currently similar findings in scientific literature. As such, the results cannot be compared with any published findings.

Among all oligosaccharides, two structures containing fucose but with unknown identities stand out. A fucose containing compound with neutral mass of 473.223 Da and the dimerized form of this unknown (neutral mass 946.446 Da) were detected in much greater abundance compared to all other oligosaccharides. This compound was found in greatest abundance in extracts obtained at pH 4, supporting the high fucose yields observed at acidic pH. Other abundant oligosaccharides include 0_0_3_0_0 (an oligosaccharide consisting of 3 fucose units, likely a fucoidan fragment) and dimerized 0_0_3_0_0, which were detected among all extraction conditions with comparable abundances. Again, these were most prevalent at pH 4, supporting our original findings. EAEP extracts also featured two fucose-containing but unknown compounds in relatively large abundance (neutral masses 512.214 Da and 527.231 Da) indicating that the enzymes used enhanced the breakdown of the algal matrix, releasing compounds not seen in the AEP extracts.

It is expected that fucoidan would contain primarily sulfated oligosaccharides with many fucose subunits (82), however none were detected through the methods described herein. While fragmentation data does show many abundant oligosaccharides with neutral loss(es) of sulfate (data not shown here), the sulfate groups prevent the production of comprehensive fragmentation data that can be used to deduce the structure of the parent molecule. Therefore, it is likely that all features reported in Figure 2.11 as "containing fucose" are unsulfated oligosaccharides.

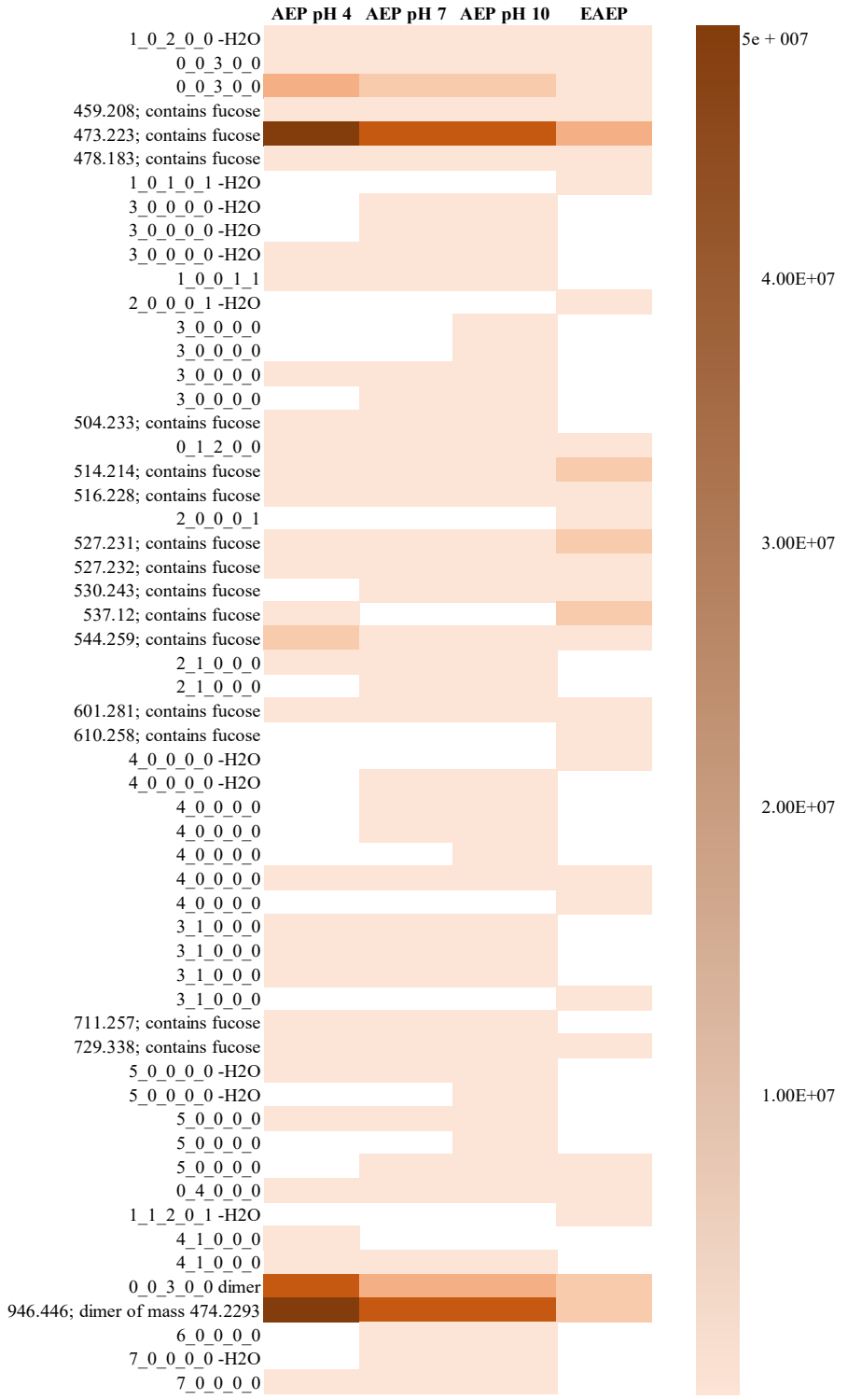

Figure 2.11: Glycoprofile results obtained by nanoLC-QToF following enzyme blank subtraction for all extraction conditions. Identified oligosaccharides are reported by a 5-digit code, corresponding to the number of constituent monosaccharides shown along the y-axis. Unknown oligosaccharides containing fucose are reported by their neutral mass.

2.3.3.4 Characterization of extracts sodium alginate structure by FTIR

The FTIR spectra of sodium alginate from the 4 chosen extraction conditions is shown in Figure 2.12.

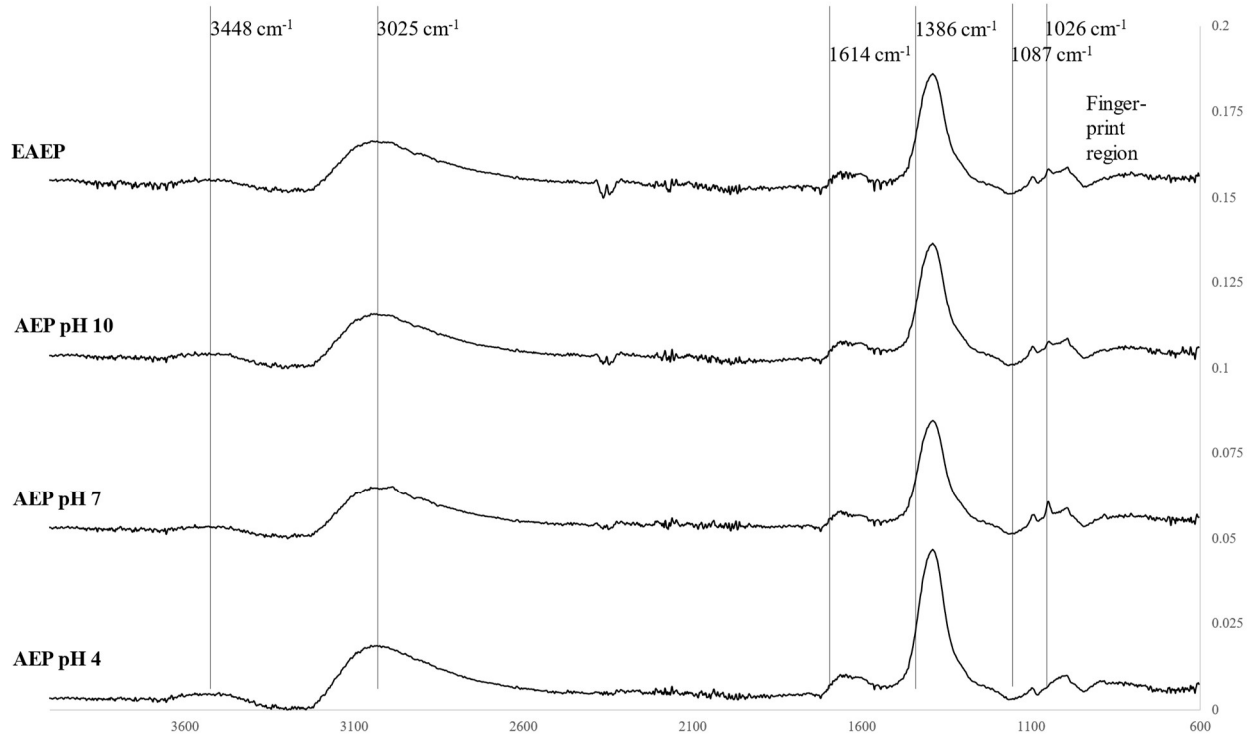

Figure 2.12: Impact of extraction conditions on FTIR spectra of sodium alginate.

Overall, the conditions produced alginate with similar spectra. The peaks from around 3448-3423 cm^{-1} can be assigned to O-H stretching vibrations (41,83,84) while the peak centered at 3025 cm^{-1} can be attributed to C-H stretching vibrations of uronic acids (41). The peaks around 1614 cm^{-1} correspond to asymmetric stretching of carboxylate vibrations (83,84) and the peak at 1386 cm^{-1} can be attributed to the symmetric carboxylate group stretching vibration (COO−) of the mannuronate and guluronate constituents (41,47,75,83). Peaks between 1087-1093 cm^{-1} indicate C-O stretching vibration of mannuronic acids while peaks around 1012-1028 cm^{-1} indicate C-O or C-C stretching of pyranose rings of guluronic acids (41,47). C-O or C-C stretching of

pyranose rings of guluronic acids was more pronounced in AEP extracts obtained at pH 7 and 10, indicating a higher mannuronic to glucuronic acid (M/G) ratio.

The M/G ratios shown in Table 2.5 were estimated using these peak heights. The lower the M/G ratio, the stronger and more rigid is the gel formed by the alginate. The M/G ratio is species dependent, but the estimated M/G ratios described in Table 2.5 fall within the ranges typically found for macroalgae (84). The M/G ratio for the selected extracts were all below 1, indicating stiffer gels (47), with lowest M/G ratio (0.676) observed for AEP extracts obtained at pH 4 and highest for pH 7 (0.930) and pH 10 (0.900).

Table 2.5: Impact of extraction conditions on sodium alginate M/G ratios by FTIR

Condition	Ratio
AEP pH 4	0.676 [b]
AEP pH 7	0.930 [a]
AEP pH 10	0.900 [a]
EAEP	0.795 [ab]

2.3.3.5 Laminarin quantification by β-glucan analysis

β-glucan content of the extracts (mg β-glucan/ g of FD extract) is shown in Table 2.6. This measurement was used as an alternative to the laminarin assay reported in the "Laminarin content of the AEP and EAEP extracts" section because of its higher specificity compared to the previously used method. Laminarin are made of linear β-1,3-glucose units with β-1,6-linkages which control their solubility (8). This assay uses enzymes that can cleave the laminarin molecules at both their 1-6 and 1-3 glycosidic bonds and provides the results as β-glucan content. Most of the data available in the literature report the effectiveness of similar assays to the data presented previously with an enzyme that can only cleave the branching 1-6 bonds.

Laminarin extricability for the AEP was greatest for pH 10 (1.40 β-glucan/g FD extract) and lowest for pH 7 (0.072 β-glucan/g FD extract). We also saw that alkaline conditions aided in

the release of proteins, many of which are intracellular. These results indicate that the high alkalinity was able to release intracellular compounds by breaking through the algal matrix. The results show the ability of the enzymes used in the EAEP to breakdown the algal matrix, resulting in the highest extractability, with yields of 4.56 ± 0.60 mg β-glucan/g FD extract. Laminarin are storage molecules inside the algal cell (71), meaning that greater release of laminarin is inherent to breakdown of the cell wall. Higher extractability under enzymatic conditions can be attributed to the ability of the enzymes to enhance breakthrough of the algal matrix. It is important to note that the commercial enzymes used contain significant amounts of carbohydrates and that contribution was not subtracted from the results reported herein. Therefore, the EAEP yields reported, although higher than the AEP ones, could be lower than the values reported.

It is difficult to compare the β-glucan content results to the current literature, since other studies have used methods that characterize the total glucan content (vs. β-glucan content) in their starting material (37), but not in the extracts. The lack of laminarin quantification of extracts based upon β-glucan content indicates a gap in the existing literature. Furthermore, in addition to the increased yields shown for the EAEP extracts (Table 2.6), enzymatic hydrolysis has been shown to increase the antioxidant activity of extracts when compared to solvent extracted laminarin, making enzymatically extracted laminarin-rich extracts of great interest for various applications in industries such as pharmaceuticals, nutraceuticals, and functional foods.

Table 2.6: Impact of extraction conditions on the β-glucan content of extracts obtained by aqueous and enzyme-assisted aqueous extractions

Condition	β -glucan (mg β-glucan/ g FD extract)
AEP pH 4	0.22 ± 0.02^{c}
AEP pH 7	0.072 ± 0.01^{c}
AEP pH 10	1.40 ± 0.27^{b}
EAEP	4.55 ± 0.60^{a}

2.3.3.6 Amino acid profile

The results of the amino acid (AA) profile in Table 2.7 show that *M. pyrifera* extracts contain all the nine essential amino acids (EAA), except for the AEP extract obtained at pH 4. The first column indicates the protein guidelines from the Food and Agriculture Organization of the UN (FAO) (85). These guidelines provide the percent abundance (w/w) that each EAA contributes to the total protein content. While the starting material contained all EAA, in the required concentrations, the extracts, although containing all EAA, did not meet the concentrations specified by the FAO. However, as mentioned earlier, some of the proteins may have precipitated along with the alginate. An optimized extraction process targeting the extractability of protein from *M. pyrifera* has the potential to meet these requirements. The AA profile of the EAEP closely matches the FAO requirements. This is likely due to enhanced breakdown of the cellular matrix, leading to the release of proteins previously trapped in the algal matrix. As a result, the EAEP extract has a different composition than those extracted without enzymes.

A study focusing on protein extractability also reported relatively high abundance of Asparagine (Asx) and Glutamine (Glx) in the extracts, while Taurine levels (not found in our starting material or extracts) were highly species dependent (86). This information is crucial to the holistic understanding of the impact of extraction conditions and is important when considering broader uses of the extracts.

Table *2.7*: Amino acids composition of AEP and EAEP extracts (g amino acid/100 g total protein)

Amino Acid	FAO guidelines	Starting material	AEP pH 4	AEP pH 7	AEP pH 10	EAEP
Asx		10.92 ± 0.02	9.26 ± 0.96	10.10 ± 0.04	9.69 ± 0.17	11.77 ± 0.10
Thr	2.3	5.04 ± 0.01	4.24 ± 0.21	4.41 ± 0.05	4.48 ± 0.11	5.99 ± 0.06
Ser		4.56 ± 0.01	3.69 ± 0.27	3.76 ± 0.15	3.59 ± 0.09	5.27 ± 0.04
Glx		15.99 ± 0.11	30.98 ± 4.42	28.27 ± 2.70	31.36 ± 1.12	19.78 ± 0.14
Pro		4.1 ± 0.22	2.10 ± 0.24	2.18 ± 0.72	2.29 ± 0.25	3.71 ± 0.38
Gly		5.13 ± 0.03	4.58 ± 1.53	4.12 ± 0.36	4.16 ± 0.08	4.90 ± 0.04
Ala		9.96 ± 0.10	25.22 ± 4.86	17.83 ± 2.28	19.71 ± 0.62	12.17 ± 0.20
Val	3.9	5.68 ± 0.01	3.01 ± 0.02	3.55 ± 0.96	3.72 ± 0.08	5.07 ± 0.02
Ile	3	4.1 ± 0.01	2.19 ± 0.74	3.21 ± 0.06	2.96 ± 0.13	3.32 ± 0.04
Leu	5.9	7.45 ± 0.03	2.33 ± 0.35	4.12 ± 0.50	3.51 ± 0.20	5.91 ± 0.03
Tyr	3.8 (total Tyr	3.13 ± 0.01	1.31 ± 0.29	2.02 ± 0.19	1.75 ± 0.06	4.14 ± 0.04
Phe	+ Phe)	4.86 ± 0.02	2.74 ± 0.52	3.13 ± 0.08	2.82 ± 0.18	1.41 ± 0.03
His	1.5	1.89 ± 0.01	1.78 ± 0.18	2.78 ± 0.38	1.55 ± 0.10	4.19 ± 0.05
Lys	4.5	5.83 ± 0.02	1.74 ± 0.41	2.92 ± 0.58	2.11 ± 0.07	3.57 ± 0.05
Arg		4.89 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Taurine		-	1.92 ± 0.34	2.32 ± 0.12	1.97 ± 0.15	1.73 ± 0.04
Cys acid	0.6	1.86 ± 0.05	0.49 ± 0.08	0.86 ± 0.10	0.83 ± 0.05	1.26 ± 0.05
Met sulfone	1.6	2.63 ± 0.07	0.00 ± 0.00	0.61 ± 0.55	0.41 ± 0.37	2.10 ± 0.64
Trp	0.6	1.91 ± 0.04	1.12 ± 0.05	1.47 ± 0.26	1.35 ± 0.06	2.98 ± 0.17

Abbreviations: Methionine (Met), Tryptophan (Trp), Asparagine (Asx), Threonine (Thr), Serine (Ser), Glutamine (Glx), Proline (Pro), Glycine (Gly), Alanine (Ala), Valine (Val), Isoleucine (Ile), Leucine (Leu), Tyrosine (Tyr), Phenylalanine (Phe), Histidine (His), Lysine (Lys), Arginine (Arg), Cystine (Cys) (-, not identified in the sample). FAO guidelines are based upon the individual amino acid mass contribution to the total protein mass (85).

2.3.3.7 Antioxidant activity

The ABTS and ORAC antioxidant activities of selected extracts are shown in Figure 2.13 a and b respectively and are reported in µmol Trolox Equivalent per gram of freeze-dried extract. All extracts exhibited antioxidant activity. ABTS results, which indicate radical cation scavenging

activity, are relatively uniform across the extracts at 47.48–48.40 μmol Trolox Eq/ per g FD extract. Antioxidant activity has been tied to several macroalgae compounds including phenolics, proteins, and fucoidan (13,69,87). The ORAC results (Figure 2.13b), which indicate oxygen radical absorbance capacity, show that the highest antioxidant activity (213.10 ± 22.58 μmol TE/ g FD extract) is observed in the EAEP extracts. This is likely due to the increased release of compounds like fucoidan and peptides, which are tied to the antioxidant activity of the extracts. The antioxidant capacity was lowest for the AEP pH 10 which can be attributed to lower extraction of bioactive compounds (i.e., less fucoidan) at this pH.

While there is a lack of data comparing the antioxidant activity of macroalgae extracts across pH values, the results for radical cation scavenging activity presented herein (47.48–48.40 μmol Trolox Eq/ per g FD) are higher than those reported for other aqueous *M. pyrifera* extracts (1:10 BWR, 40 °C, 2 h), with 38.4 mg Trolox Eq /100 g dry seaweed (equivalent to 12 μmol Trolox Eq/g dry seaweed) (5). This increase may be a result of 2 h not being long enough to extract as any bioactive compounds. Similarly, the ORAC results presented herein (113–213 μmol Trolox Eq/ per g FD extract) are also higher than those found previously for enzyme-assisted extracts from *M. pyrifera* (18 h, pH 4.5, 50 °C, 1:50 BWR, 10% CellicCTec3 (v/w)), which presented an antioxidant capacity of 35 μmol Trolox Eq/ per g dry seaweed (13). In this case, the extraction process was focused on protein extraction. While antioxidant activity has been tied to many macroalgae compounds, proteins are likely not the major contributors to the higher antioxidant activity of the extracts compared to other compounds (i.e., fucoidan and laminarin).

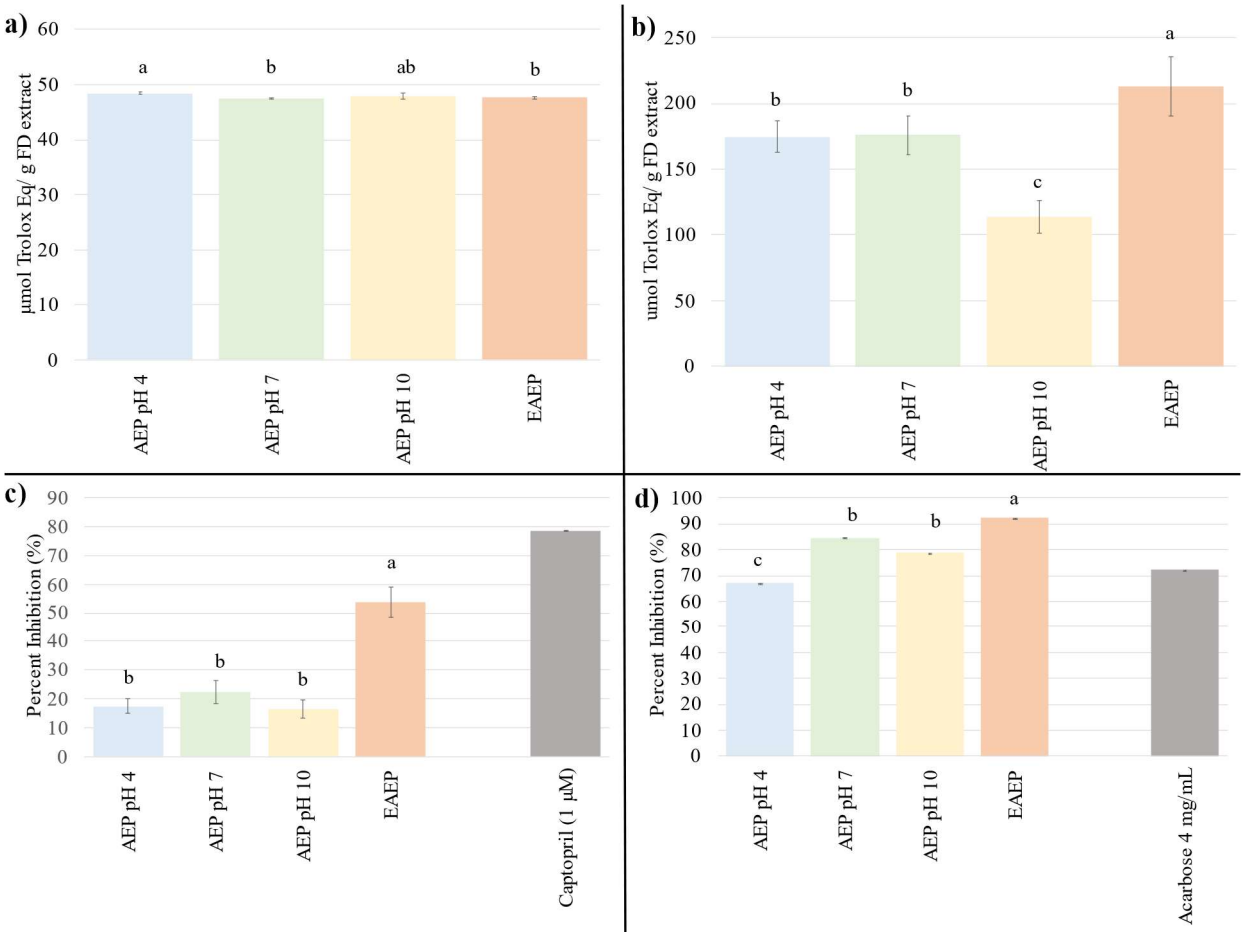

Figure 2.13: Impact of extraction conditions on radical cation scavenging (ABTS) activity (a), oxygen radical absorbance capacity (ORAC) (b), ACE inhibition (c), and α- glucosidase inhibition (d) of selected extracts.

2.3.3.8 Angiotensin-converting enzyme (ACE) inhibitory activity

The ability of the selected extracts to inhibit ACE, which can be exploited as a strategy to relieve hypertension, is shown in Figure 2.13c. Results are shown as percentage inhibition of a 10 mg FD extract/mL of solution. ACE inhibitory activity of the AEP extracts varied from 16.56-22.44%, not being significantly impacted by the extraction pH. However, the use of enzyme in the EAEP increased the ACE inhibitory activity of the extracts to 53.69%. The higher ACE inhibitory activity of the EAEP extracts is likely due to the higher amount of peptides released by the protease in the EAEP extracts (see metabolomics data), which has been previously tied to higher ACE

inhibition activities (88). As with previous studies, ACE inhibitory results of the extracts were lower than those exhibited by the commercial drug, captopril (13,89). These values are lower than that of a *M. pyrifera* extract richer in proteins (38.83% inhibition with a 1 mg extract/mL solution) (13). However, as mentioned earlier, this study focused on the simultaneous extraction of several compounds from *M. pyrifera* to gain a holistic view of the impact of the processing conditions evaluated. Had more protein extraction been optimized herein, it is likely more bioactive peptides related to ACE inhibition would have been released resulting in higher inhibitory effects. This research demonstrates the potential use of these extracts as a functional food to aid in ACE inhibition and the opportunity for further optimization to maximize this inhibitory activity.

2.3.3.9 α-glucosidase inhibitory activity

α-glucosidase inhibition is an important strategy in managing diabetes. The α-glucosidase inhibition activity of the selected extracts is shown in Figure 2.13d and is presented as percent inhibition of a 0.125 mg FD extract/mL of solution. The EAEP extract presented highest inhibition at 92% while the AEP extract at pH 4 resulted in the lowest inhibition at 67%. Antidiabetic effects have been tied to algal polysaccharides, like fucoidans, laminarin, and polyphenolic extracts, and previous studies have found antidiabetic potential in *Macrocystis pyrifera*. Additionally, bioactive macroalgae peptides displaying antidiabetic activity have been identified (88). Likely some of these peptides were released in the EAEP extracts, the extraction condition with the highest peptide release (as shown in the metabolomics data above), helping explain the higher inhibitory activity of the EAEP extracts. These results relay important information when compared to the market-available drug, acarbose, which was run as a control. A 4 mg/mL solution of acarbose resulted in 72% inhibition, demonstrating the strong potential of these extracts as a therapy for diabetes. It is

challenging to compare these findings with literature, because, while other studies have screened for antidiabetic potential (88,90,91), information on the impact of aqueous extraction conditions on antidiabetic activity is lacking. That being said, a study by Yuan et al. with a 70% methanol extraction (4 h, 1:10 BWR, room temp) using 10 mg extract/mL of solution found 0% inhibition for *Lessonia nigrecens,* 55% inhibition for *Laminaria japonica* extracts, 75% inhibition for *A. nodosum*, and 100% inhibition for *Lessonia trabeculate* demonstrating how α-glucosidase inhibitory activity can differ greatly among species (23,92). Additionally, in comparison, the AEP and EAEP extracts from this study were able to produce extracts with higher α-glucosidase inhibitory activity without the need for methanol.

2.4. Conclusions

This study focused on evaluating green extraction strategies on the extractability and structure/functionality of compounds from the brown macroalgae *Macrocystis pyrifera*. While conventional extractions utilize flammable and hazardous solvents, aqueous and enzyme assisted aqueous extraction processes using water as the solvent and enzymes to further enhance yields and biological activities of the extracts from *M. pyrifera* were developed. Through a series of processing optimizations, selected extracts produced by aqueous extraction (60 °C, 1:30 BWR, and 6 - much shorter than that of conventional methods that can take over 24 h (8,9) - across acidic, neutral, and alkaline pH values) and by enzyme-assisted extraction (60°C, 1:30 BWR, 5% CBL for 2 h followed by the use of 2.5% (w/w) NP for 2 h) methods were analyzed for their biological properties, amino acid, carbohydrate, and phenolic composition. The use of LC-MS, HPAEC-PAD, and FTIR were useful tools to characterize the complex carbohydrates of *Macrocystis pyrifera*. Monosaccharide and oligosaccharide profiling demonstrated that aqueous extraction at

acidic pH resulted in highest fucoidan extractability. Extraction pH had a significant impact on the M/G of the extracted alginate (neutral and acidic conditions resulting in highest (0.930) and lowest ratios (0.676), respectively) and could be exploited to tailor the properties of the extracted alginate to suit specific applications in various industries. Except for the use of AEP at pH 4, all extraction conditions produced extracts with significant amounts of all essential amino acids, showing great potential for use in the alternative protein industry. Despite the high TPC of the extracts, targeted and untargeted metabolomics demonstrated that *M. pyrifera* is not a substantial source of phenolic compounds. Nevertheless, the extracts produced were highly bioactive, highlighting the potential bioactivity of other compounds in *M. pyrifera*. The use of a carbohydrase pretreatment, followed by a neutral protease, allowed for greater extractability of compounds, including laminarin and peptides. EAEP extracts exhibited the highest bioactivities, including antioxidant activity of 213.10 ± 22.58 μmol TE/ g FD extract, ACE inhibition of 54% inhibition (10 mg FD extract/mL solution), and α-glucosidase inhibition of 92% (0.125 mg FD extract/mL solution). This research plays a critical role in elucidating the impact of downstream processing unit operations (e.g., pH, extraction time, temperature, water to biomass ratio, type and amount of enzyme) and extraction methods (AEP, EAEP) on yields, structural composition, functional and biological properties of *M. pyrifera* compounds, thus contributing to unlocking the full potential of *M. pyrifera* as a valuable resource for food, pharmaceutical, and biotechnical applications.

2.5 Supplementary materials

Table S2.1: Average peak heights of phenolic compounds in freeze-dried selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (min)	Average peak heights for extraction conditions				
						FD *M. pyrifera*	AEP pH 4	AEP pH 7	AEP pH 10	EAEP pH 4+7
4-Hydroxyphenyllactic acid	[M-H]-	2	181.0508	neg	1.456	5368365	3031402	2813668	1688056	2948375
5,7-Dihydroxy-4-methylcoumarin-3-acetic acid	[M-H]-	2	249.0403	neg	0.309	299559	365672	581197	36273	346512.3
Homovanillic acid sulfate	[M-H]-	2	261.0076	neg	0.936	4199382	3782430	3547978	3136600	4074002

Table S2.2: Average peak heights of peptides in freeze-dried selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (min)	Average peak heights for extraction conditions				
						FD *M. pyrifera*	AEP pH 4	AEP pH 7	AEP pH 10	EAEP
Ala-Ala	[M+H]+	2	161.0922	Pos	0.279	1090013.729	439299.305	1552831	1857808.505	8332720.809
Ala-Ile	[M+H]+	2	203.1391	Pos	1.254	9031.398902	772253.1479	539298.3737	683309.4439	2044186.566
Ala-Leu	[M+H]+	2	203.1391	Pos	1.415	1655254.89		1002541.681	1349261.788	32579558.17
Ala-Leu-Ala-Leu	[M+H]+	2	387.2601	Pos	2.421		17846.66667	7731.534063	29395.03097	1443526.057
Ala-Met	[M-H]-	2	219.0808	Neg	0.565	19229.66667	230630.7109	8164.5		1755649.667
Ala-Phe	[M+H]+	2	237.1233	Pos	1.671	443755.2476	1165332.715	277123.0932	379777.1182	15003332.72
Ala-Pro	[M+H]+	2	187.1077	Pos	0.435	1628161.873		1701941.045	1800307.521	2420871.496
Ala-Trp	[M+H]+	2	276.1343	Pos	1.906	455.7017101	32224.77875		1930.532841	1500336.218
Ala-Tyr	[M+H]+	2	253.1187	Pos	0.658	137733.7284	1275888.163	48847.80089	78335.39995	6174588.223
Ala-Val	[M+H]+	2	189.1234	Pos	0.508	2679978.495	9760.067185	1679800.428	2011622.622	9576524.665
Arg-Leu	[M+H]+	2	288.2034	Pos	0.851	906922.6713	3957.987489	30687.27649	31943.29273	160034.4072
Asn-Ile	[M+H]+	2	246.1452	Pos	1.174	472266.6946	457652.9787	193326.4367	623.9052274	384513.473
Asn-Leu	[M+H]+	2	246.1449	Pos	1.329	15493.46139	47663.53536	598605.2629	659641.9964	2845650.952
Asn-Phe	[M+H]+	2	280.1292	Pos	1.583	101861.6585		71728.27962	79268.50917	2078892.206
Asn-Tyr	[M+H]+	2	296.124	Pos	0.653		1636592.774			1923749.926

Asn-Val	[M+H]+	2	232.1295	Pos	0.487	2975836.872	702192.5757	2119983.792	3070045.755	2257327.849
Asp-Leu	[M+H]+	2	247.1287	Pos	1.402	1223606.077	2446.5	934927.7516	1100273.931	2774307.586
Asp-Tyr	[M-H]-	2	295.0933	Neg	0.698	4971	36556054			1920579
gamma-glutamyl-alanine	[M-H-H2O]-	2	199.0723	Neg	0.481	36249866.67	1212585.667	29210986	26072851	36516570.67
Gln-Ala	[M-H]-	2	216.0992	Neg	0.277	1881638.667	160490.3333	954217.3333	868168	1422850.333
Gln-Phe	[M-H]-	2	292.1304	Neg	1.597	384212.3333	5498254.01	119785.3333	138587	604437.6667
Gln-Val	[M+H]+	2	246.1452	Pos	0.505	15333730.32	1557893.907	7549499.973	11191927.64	9556932.964
Glu-Ala	[M+H]+	2	219.0977	Pos	0.284	955380.6485	962857.01	1823154.168	1775344.891	2685507.322
Glu-Ile	[M+H]+	2	261.1447	Pos	1.324	1356386.394	1196229.367	1160743.664	1101661.466	1232812.108
Glu-Leu	[M+H]+	2	261.1444	Pos	1.464	1800180.05	29317.33333	1423327.952	1473254.484	3655397.159
Glu-Tyr	[M-H]-	2	309.1091	Neg	0.774	17678.66667	638207.6667	30674.33333	20852.5	561598
Glutathione, oxidized	[M-H]-	2	611.145	Neg	0.437	27967	4281236.486	373772.6667	10174.66667	391345.6667
Gly-Leu	[M+H]+	2	189.1234	Pos	1.423	8542339.605	387588.8981	5414529.82	5812247.77	11255062.6
Gly-Phe	[M+H]+	2	223.1077	Pos	1.621	734383.8052	1691132.671	470039.5893	559763.5093	4684895.172
Gly-Val	[M+H]+	2	175.1082	Pos	0.529	3399445.075	174133.6744	2502251.168	2375275.021	3499328.685
Ile-Ala	[M+H]+	2	203.1392	Pos	0.704	154762.5279	17116.32066	268234.5997	294764.1256	15492735.73
Ile-Asn	[M+H]+	2	246.1448	Pos	0.445	76771.71314	24667.10237	40101.75191	67005.10401	25351859.45
Ile-Glu	[M+H]+	2	261.1444	Pos	0.581	97304.94741	122616.6846	63662.00652	40225.53934	5574635.858
Ile-Ile	[M+H]+	2	245.1858	Pos	2.238	289320.7506		196257.1164	204860.1968	56195193.67
Ile-Leu	[M+H]+	2	245.1859	Pos	2.768		14328.39466			581061.3622
Ile-Met	[M+H]+	2	263.1423	Pos	1.737	57089.87791	47219.65848	16055.46702		10105666.48
Ile-Ser	[M+H]+	2	219.134	Pos	0.475	2941760.377	101070.8236	110631.6992	73855.93215	37453463.18
Ile-Thr	[M+H]+	2	233.1496	Pos	0.494	287494.3449		159409.5567	192129.3954	18503410.46
Ile-Trp	[M+H]+	2	318.1813	Pos	2.401		22922.5387		3020.137066	1902038.92
Ile-Tyr	[M+H]+	2	295.1651	Pos	1.66	60426.18635	227700.828	34854.36738	46644.79949	12095894.26
Ile-Val	[M+H]+	2	231.1703	Pos	1.559	604369.2351	22658.42892	337341.1925	396436.1191	19924497.98
Leu-Arg	[M+H]+	2	288.203	Pos	0.462	9867.973203	1575690.165			14904474.58

Leu-Asp	[M+H]+	2	247.1288	Pos	0.529	2275661.262	75529.80722	2142782.543	1788715.421	15225242.96
Leu-Gln	[M+H]+	2	260.1604	Pos	0.497	225170.6089	38506.85248	105342.9938	171664.1612	9959392.417
Leu-Gly	[M+H]+	2	189.1234	Pos	0.739	1163.738476	12436.17213	73927.82555	73582.91158	12997291.93
Leu-Gly-Leu	[M+H]+	2	302.2074	Pos	2.457	30248.58727		19623.37358	36072.12468	1164872.836
Leu-Gly-Lys	[M+H]+	2	317.2181	Pos	0.482	15311.44546	6036.941015			9852504.092
Leu-Leu-Lys	[M+2H]2+	2	187.1442	Pos	1.562	1944.56556		5657.378714	8076.348785	1352087.037
Leu-Lys	[M+H]+	2	260.1968	Pos	0.399		52966.25384			8465058.996
Leu-Phe	[M+H]+	2	279.1703	Pos	2.342	115439.3938	355355.3816	76498.00964	93389.70674	23872371.06
Leu-Pro	[M+H]+	2	229.1546	Pos	1.739	834161.8845		528580.8784	524659.8954	2052004.086
Leu-Thr-Lys	[M+H]+	2	361.2444	Pos	0.447					5567329.411
Leu-Trp	[M+H]+	2	318.1812	Pos	2.503		16499.22306		3178.677779	3789315.97
Leu-Tyr	[M+H]+	2	295.1652	Pos	1.741	40867.71904	226135.8116	35663.80747	37030.48225	37098580.78
Leu-Val	[M+H]+	2	231.1703	Pos	1.694	83065.47112	10364.18068	303524.2752	336475.9706	15598759.75
Leucyl-leucine methyl ester	[M+H]+	2	259.2015	Pos	2.842	4626.943346	8172.151058	3377.489046	5604.458424	631982.5969
Met-Ala	[M+H]+	2	221.0954	Pos	0.484	33014.27253	24764.31167			8609054.446
Met-Ile	[M+H]+	2	263.1424	Pos	2.017	78033.97031	42804	41273.73586	40156.58497	10910148.87
Met-Met	[M-H]-	2	279.0841	Neg	1.544	42553.33333	2730.226672	44442.33333	157877.5	685636.6667
Met-Phe	[M+H]+	2	297.1266	Pos	2.241	7476.221172		4363.218235		2880266.468
Met-Tyr	[M+H]+	2	313.1214	Pos	1.565	4740.566796	4600.666667	3294.350135		3601908.707
Met-Val	[M-H]-	2	247.1121	Neg	1.454	15581.33333	116480	3297.333333		1564602
N-Acetyl-valine	[M-H]-	2	158.0822	Neg	1.612	155529.6667	25028.96905	100188	144068	183640
Phe-Ala	[M+H]+	2	237.1233	Pos	1.439	60405.98529		47016.28662	56981.6069	16172802.3
Phe-Asn	[M-H]-	2	278.1145	Neg	0.675		8137.957756			2218861.667
Phe-Asp	[M+H]+	2	281.1131	Pos	0.963			10317.60579	10086.79459	3947601.753
Phe-Gln	[M+H]+	2	294.1448	Pos	0.942		18826.54101			2861630.697
Phe-Glu	[M+H]+	2	295.1288	Pos	1.299	12444.76305	18346.66667	26609.86089	17849.73292	2679498.788
Phe-Gly	[M-H]-	2	221.0931	Neg	1.401	30626.66667		20176.33333	26987	3483217.333

Phe-Gly-Phe	[M+H]+	2	370.176	Pos	2.739		10279.3667			646748.7943
Phe-Ile	[M+H]+	2	279.1702	Pos	2.277	24254.62148	84887.21898	28205.84873	23704.13805	1144254.268
Phe-Leu	[M+H]+	2	279.1703	Pos	2.457	170654.8801		169397.1496	169963.6542	61022363.74
Phe-Met	[M+H]+	2	297.1267	Pos	2.092		14449.9968			4713663.199
Phe-Phe	[M+H]+	2	313.1545	Pos	2.567	26687.45921	4048.333333	38233.73081	46206.7465	11488450.2
Phe-Ser	[M-H]-	2	251.1036	Neg	0.81	5160	2429.5	3362.333333	3855	4469093.667
Phe-Thr	[M-H]-	2	265.1193	Neg	0.983	3600.666667		3271.333333	8268	7719557.333
Phe-Trp	[M+H]+	2	352.1654	Pos	2.645		1769.199271			559825.3378
Phe-Tyr	[M+H]+	2	329.1494	Pos	1.957	5028.462314	17783.61516	4492.791948	4980.783251	6002404.378
Phe-Val	[M+H]+	2	265.1546	Pos	1.912	50751.87306	315987.8278	39896.96274	44252.26138	3002354.346
Pro-Ile	[M+H]+	2	229.1552	Pos	1.442	972539.437	133774.8797	367936.1004	466122.4664	575491.3347
PyroGlu-Met	[M+H]+	2	261.0903	Pos	1.6	229204.4725	7017949.328	139801.2917		411409.8282
PyroGlu-Val	[M+H]+	2	229.1187	Pos	1.589	10970750.85	1424094.822	9935624.624	10685978.26	7521082.081
Ser-Leu	[M+H]+	2	219.134	Pos	1.305	56985.69444	112469.9783	1747433.809	1972982.841	8870567.612
Ser-Phe	[M+H]+	2	253.1181	Pos	1.562	222140.5765	15815.66667	153426.8543	190887.8629	6554862.777
Ser-Tyr	[M-H-CH2O]-	2	237.0881	Neg	0.646	43515.66667	1204988.877	10835.66667	12200	1480521
Ser-Val	[M+H]+	2	205.1185	Pos	0.485	2123291.233	1481515.482	1544405.297	1812571.505	2427700.04
Thr-Leu	[M+H]+	2	233.1496	Pos	1.442	3227822.297	81478.33333	1888408.049	2169062.933	7216600.496
Thr-Phe	[M-H]-	2	265.1193	Neg	1.663	120535.3333	4661.462871	63775.33333	86363	1824187.667
Thr-Tyr	[M+H]+	2	283.1288	Pos	0.765	6337.745192	3261905.644			1874706.729
Thr-Val	[M+H]+	2	219.134	Pos	0.532	6728725.785		4640131.766	5169029.54	8470346.414
Trp-Asn	[M+H]+	2	319.1401	Pos	1.218		1385.940441			990785.6916
Trp-Leu	[M+H]+	2	318.1812	Pos	2.649	1415.955243	18335.22354	2242.189851	3277.865368	1101338.251

Table S2.3: Average peak heights of lipid derived metabolites in freeze-dried *M. pyrifera* powder selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (mins)	Average peak heights for extraction conditions				
						FD *M. pyrifera*	AEP pH 4	AEP pH 7	AEP pH 10	EAEP
LPA 16:0	[M+Na]+	2	507.2696	pos	5.28	1125.24592	229812.8052	298997.696	7661.578	211768.264
LPA 18:1	[M+H-H2O]+	2	493.2926	pos	5.4		281603.4919	461641.153	15943.7368	251829.182
LPC 14:0	[M+Na]+	2	490.2908	pos	5.067	2485.05155	130944.9975	202105.8	34166.4794	118737.856
LPC 15:0	[M+H]+	2	482.3247	pos	5.367	75412.5777	1172614.546	154917.318	99824.6969	461069.061
LPC 18:1	[M+Na]+	2	544.3375	pos	5.809		137976.2403	228804.42	2336.55285	104605.066
LPC 20:0	[M+H]+	2	552.4024	pos	6.863		48823.73553	104860.969		35430.8252
LPE 14:0	[M+H]+	2	426.2614	pos	5.041	85417.7009	777933.7569	1054515.15	70524.7919	716443.675
LPE 16:0	[M+H]+_[M+Na]+	2	454.2928_476.2749	pos	5.6385	36373.7832	1357229.132	1726137.11	40098.7005	1046965.41
Palmitoyleicosapentaenoyl phosphatidylcholine	[M+H]+	2	780.5536	pos	7.025		92314.08063	153542.37	24167.5581	2846.44515
PC 18:1_14:0	[M+H]+	2	732.5532	pos	7.102	81959.1392	69829.85229	266285.653	64414.0277	165241.162
PC 20:4_20:4	[M+H]+	2	830.5688	pos	9.532		113429.0043	111647.568	164233.551	225730.65
(3.beta.,5.alpha.)-4,4-Dimethylcholesta-8,14,24-trien-3-ol	[M+H-H2O]+	2	393.3516	pos	7.03		90624.07789	156947.166		116351.611
1-(Benzoyloxy)propan-2-yl benzoate	[M+H-C7H6O2]+	2	163.0757	pos	3.784		8075.007886	53191.0116	12322.1711	11967.7802
1,11-Undecanedicarboxylic acid	[M-H]-	2	243.1603	neg	4.105	283807.667	218608.3333	175633.333	223253	311634.333
2-hydroxyisocaproic acid	[M-H]-	2	131.0715	neg	2.065	440935	172004.3333	173690	118141.667	394297.333
6-Oxooctadecanoic acid	[M+NH4]+	2	316.2843	pos	4.712	18230.8566	16504.19021	62627.5591	174350.082	1062128.4
9-(2,3-Dihydroxypropoxy)-9-oxononanoic acid	[M-H]-	2	261.1345	neg	2.604	397821.333	97988.33333	122003.333	21112.3333	126202.667
Acetyl-carnitine	[M+H]+	2	204.1235	pos	0.382	9370271.8	3314214.162	5148720.11	64470.9101	5420927.12
Azelaic acid	[M+H-H2O]+	2	171.102	pos	2.707	307939.728	41480.33143	84436.9844	92496.7583	79366.363
Dinorprostaglandin E1	[M-H-H2O]-	2	307.1917	neg	4.549	2415743.67	832619.6667	991203	643144.333	637294.667

Hymeglusin	[M-H-H2O]-	2	305.176	neg	4.423	265166.667	260977.667	591400	164041	493823
LPI-(1-oxo-9,12-octadecadienyl)	[M-H]-	2	595.2891	neg	4.913		438256	494665.667	27621	340510.667
MG 16:0	[M+H]+	2	331.2842	pos	6.041	24731.1499	526619.6272	913210.288	55590.4125	517953.523
Sebacic acid	[M-H]-	2	201.1135	neg	3.079	798881	441548.3333	539777.333	621401	1189171
Suberic acid	[M-H]-	2	173.0818	neg	2.287	1771342.33	1302653	1470126.33	1660742.33	3999526.67
Succinic acid	[M-H]-	2	117.0193	neg	0.443	9632511.67	8949751.333	8211898.33	9090779	8215354.33

Table S2.4: Average peak heights of other metabolites in freeze-dried *M. pyrifera* powder selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (min)	Average peak heights for extraction conditions				
						FD *M. pyrifera*	AEP pH 4	AEP pH 7	AEP pH 10	EAEP
1-(2-Hydroxyethyl)-2,2,6,6-tetramethyl-4-piperidinol	[M+H]+	2	202.1801	pos	0.454	8341818	6656574	5666768	8833519	5108881
1,5-Pentanediamine	[M+H-NH3]+	2	86.0967	pos	0.503	12798953.98	12008179	10606730	11764031	14435775.86
13,14-Dihydro-15-ketoprostaglandin A2	[M+Cl]-	2	369.1836	neg	4.888	934842.3333	1312264.667	1980021	909838	1193416
2-Hydroxyglutaric acid	[M-H-H2O]-	2	129.0193	neg	0.32	940145.667	984286.6667	991168.667	839739	852140.6667
4-Oxododecanedioic acid	[M-H]-	2	243.124	neg	2.909	75880.33333	90293	157695.667	112428.667	144560.3333
5'-S-Methyl-5'-thioadenosine	[M+H]+	2	298.0973	pos	1.63	4070437.936	4678383	1585904	5418652	3516279.971
Carnitine	[M+H]+	2	162.1126	pos	0.265	28714709.54	24542567	21789591	21725657	19322158
Dicyclohexylamine	[M+H]+	2	182.1907	pos	2.686		79137	118068	102360	
Flavine mononucleotide	[M+H]+	2	457.112	pos	1.802	606968.1605	484397	307066	593251	337703.7902
Glyceric acid	[M-H]-	2	105.0193	neg	0.268	138100.3333	409143	977732.667	503571.667	227611.3333
Glycerol 1-myristate	[M+H-H2O]+	2	285.2423	pos	5.414	313022.1966	217167	68201	299360	279861.198
Guanosine	[M-H]-	2	282.0842	neg	0.465	123655	137773	147281.667	125495	642871.3333
N-Acetyltyramine	[M+H]+	2	180.1024	pos	1.811	222315.5235	243720	240303	232260	181876.2018
Pantothenic acid	[M-H]-	2	218.1033	neg	1.283	204731	210024.6667	211975.333	192443	421000
Pentadecylamine	[M+H]+	2	228.2686	pos	6.209	522646.2246	335947	103869	487407	177442.3247
Phenylalanine, methyl ester	[M+H]+	2	180.1023	pos	1.918	185758.866	227230	168548	151615	251265.4148

Pheophorbide a	[M+H]+	2	593.2757	pos	6.927	1347991.376	907499	376557	1107999	814613.3618
Phloroglucinocarboxaldehyde	[M-H]-	2	153.0193	neg	1.979	157507.3333	266608	286556.6667	350425.6667	170179
Propionylcarnitine	[M+H]+	2	218.1391	pos	0.665	1302844.558	1317258	573688	1218652	1077862.636
Rubinaphthin A	[M-H]-	2	365.0877	neg	2.464					
Undecanedioic acid	[M-H]-	2	215.129	neg	3.441	507742.6667	621065.6667	621249.3333	580705.3333	495687
Violaceol I	[M-H-C7H6O2]-	2	139.0402	neg	1.606	189080.6667	153167	17944.66667	77657.33333	203089.3333

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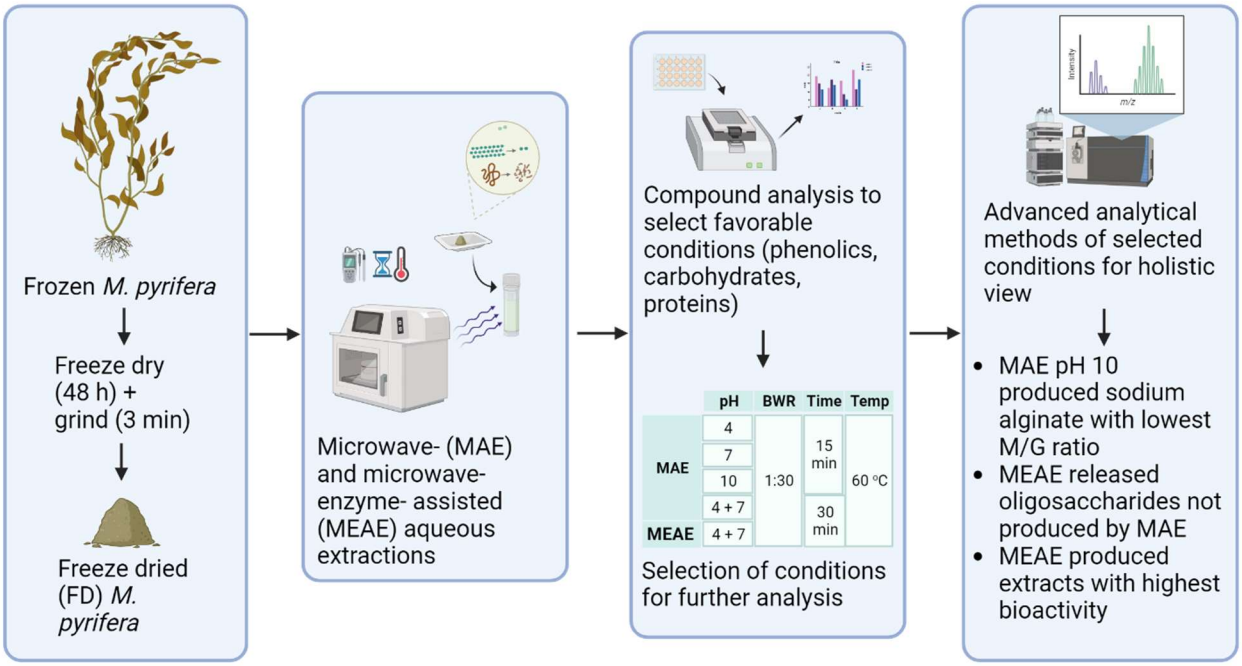
Chapter 3: Integrated impact of microwave processing and enzyme-assisted aqueous extraction process for extracting functional and bioactive compounds from *Macrocystis pyrifera*

Graphical Abstract



Abstract

To explore the potential of microwave-assisted (MAE) and microwave-enzyme-assisted (MEAE) aqueous extraction processes on the extractability of alginates, laminarin, fucoidan, protein, and phenolics, from *Macrocystis pyrifera*, the impact of pH (4, 7, & 10), biomass-to-water ratio (BWR) (1:50 & 1:30), time (15, 30, 45, and 60 min), temperature (60, 70, and 80 °C), and enzyme use (carbohydrase and protease) were evaluated. Overall, acidic conditions led to a greater release of carbohydrates, as reflected by the higher fucose content of the extracts generated at pH 4. Similarly, the use of higher temperatures led to enhanced extractability, likely due to decreased slurry viscosity. Dipole-dipole interactions from microwaves enhanced compound extractability allowing for less water use and faster extractions, which was reflected in the high yields obtained

at 1:30 BWR and 15 min. Furthermore, the integration of enzymes and microwave processing enhanced the breakdown of carbohydrates and proteins during MEAE, releasing more intracellular compounds such as laminarin and peptides. Based on yields and efficient resource utilization, MAE (15 to 30 min, 1:30 BWR, 70 °C, pH values of 4, 7, 10, and 4 shifted to 7) and MEAE conditions (5% carbohydrase (w/w) pretreatment for 15 min followed by the use of 2.5% neutral protease (w/w) for 15 min) were selected for further characterization with respect to their composition (monosaccharide and oligosaccharide profiling, alginate M/G ratio by FTIR, amino acid composition, and metabolomic profiling) and biological activities (antioxidant, antihypertensive, antidiabetic). MEAE yielded extracts displaying the most remarkable bioactivities, including an antioxidant capacity of 210.67 µmol TE/ g freeze-dried extract, 46% ACE inhibition, and 94% α-glucosidase inhibition, likely due to the enhanced release of bioactive molecules. This study provides a comprehensive perspective on the processing conditions of *Macrocystis pyrifera* using MAE and MEAE techniques, aiming to produce functional and bioactive compounds that hold potential for utilization across various industries.

Keywords

Macroalgae; microwave-assisted aqueous extraction; microwave-enzyme assisted aqueous extraction; biological properties; FTIR; fucoidan

Highlights

- MAE enhanced extraction kinetics, with optimum extraction times as short as 15 minutes.
- MAE at acidic pH resulted in the highest fucose yields.
- The incorporation of enzymes in MAE processing increased laminarin and peptide extractability.

- MEAE produced extracts with the highest bioactivities.

3.1 Introduction

The global utilization of sustainable biomass is essential for supporting the increasing world population and alleviating the stress on traditional feedstocks and production systems caused by climate change impacts (1). Giant Kelp, scientifically known as *Macrocystis pyrifera*, is a brown macroalgae containing a diverse array of functional and bioactive compounds such as phenolics, carbohydrates (e.g., alginate, fucoidan, and laminarin), and proteins. Due to its rich composition, *M. pyrifera* holds great potential as a versatile feedstock with applications across various industries (2,3).

Brown macroalgae contain a class of phenolic compounds known as phlorotannins, which are renowned for their health-promoting properties and bioactivity, including bacterial growth inhibition and antioxidant activity (4,5). Among the carbohydrates found in macroalgae, fucoidans, laminarin, and alginates exhibit interesting functional and bioactive properties. Fucoidans, exclusive to brown macroalgae, are sulfated polysaccharides composed of 1-2 and 1-3-linked α-fucose residues along with other monomers (6). They have been associated with antiviral, anti-inflammatory, immunomodulatory, antithrombotic, and anticoagulant properties (7,8). Laminarin, a storage molecule found within macroalgal cells, is comprised of β-1-3-glucose units with β-1-6-linkages (1), and have been tied to antitumor, antioxidant, and anti-inflammatory properties (9,10). Alginates, which contribute to the flexibility of brown macroalgae, are composed of alternating units of mannuronic and guluronic acids. These compounds exhibit gelling and emulsifying capacities (11–14) and have been associated to antibacterial, antioxidative, and anticoagulant

properties (8). Proteins, while less abundant than carbohydrates in brown macroalgae, also demonstrate bioactivity, including antioxidant and antihypertensive properties (15).

Some of these compounds found in brown macroalgae are already being utilized by various industries (15). Their bioactive properties make them sought-after ingredients in supplements, and extracts are incorporated into cosmetic products due to their excellent bioavailability (16). The health industry has leveraged these compounds for various applications such as pharmaceuticals, drug delivery encapsulation, and bioadhesive bandages (15,16). Beyond their use in products targeting human wellbeing, these compounds have multiple applications in agriculture and animal husbandry. They serve as biostimulants, enhancing plant growth, and are incorporated into animal feed to improve the quality of animal products. Furthermore, the residues remaining from industrial applications hold potential for use in bioenergy production (15) and could be used as fertilizers as well (17). The versatility of these brown macroalgae compounds highlights their significance in various industries and their potential to contribute to sustainable practices in bioenergy and agriculture.

Extracting these compounds while maintaining the sustainability of these eco-friendly giants poses a challenge. Conventional extraction methods employed to release their functional and bioactive compounds involve the use of hazardous and flammable extraction solvents like formaldehyde, methanol, ethanol, and acetone (18–20). These solvents pose environmental, health, and safety risks and require additional downstream processing to be adequately removed from the product prior to consumption, leading to increased operational costs (18–20). In the pursuit of maintaining the integrity of this sustainable biomass, emerging research in the field of downstream processing of macroalgae should focus on exploring greener, alternative methods that do not rely on hazardous solvents for the extraction of biologically and functionally active compounds. Such

environmentally friendly approaches will be crucial for ensuring the continued sustainability of these valuable resources.

Microwave-assisted (MAE) and microwave-enzyme-assisted aqueous extractions (MEAE) are advancements built upon AEP and EAEP. Microwaves work by using dipole-dipole interactions of polar solvents, generating friction that leads to increased intracellular temperatures and pressures. This process weakens the cell wall and assists in the overall disruption of the cellular matrix (8). The disruption of the algal matrix represents a critical bottleneck in macroalgae processing where MAE and MEAE exhibit strong potential to overcome this limitation and enhance extractability beyond AEP and EAEP by effectively breaking apart the cell matrix. Recent research has shown that microwave processing, when combined with conventional solvents such as methanol and ethanol, can enhance the extractability of cell wall-bound polyphenol molecules in the brown macroalgae *Carpophyllum flexuosum* by up to 70% (8,21). Moreover, MAE extractions of fucoidan from the brown macroalgae *Ascophyllum nodosum* demonstrate the remarkable capability of microwaves to significantly shorten extraction times for fucoidan from 9 h to just 15 min and reduce solvent use threefold compared to conventional extraction methods (18).

Despite the potential of microwave processing, there remains limited knowledge about its impact, including the role of key extraction conditions such as pH, extraction time, temperature, water to biomass ratio (BWR), and type and amount of enzyme, on the extractability of the plentiful *M. pyrifera* compounds and their functional and biological properties. Currently, there are only a few studies that target the extraction of multiple macroalgae compounds or explore microwave extractions involving *M. pyrifera*. In that view, the major goal of this study was to gain a deeper understanding of how pH, reaction time, BWR, temperature, and use of enzymes

influence the extractability, structure, and functional/biological properties of major compounds found in *Macrocystis pyrifera* using MAE and MEAE processing. To achieve this goal, the role of extraction pH (4, 7 & 10), time (15, 30, 45, and 60 min), BWR (1:30 & 1:50), and temperature (60, 70, 80 °C) on the extractability and composition (total phenolic, fucose, sulfate, laminarin, and alginate contents and total protein extractability) of *M. pyrifera* extracts produced by the MAE was evaluated. Based on the best BWR and extraction time from the MAE experiments, the use of carbohydrases and proteases was evaluated, alone and in combination, with respect to the extractability and composition (total phenolic, fucose, sulfate, laminarin, and alginate contents, and total protein extractability) of the extracts produced by the MEAE process. Subsequently, to gain a more comprehensive understanding of the effects of these processes, selected MAE and MEAE extracts were further analyzed through various techniques. Carbohydrate profiling and quantification were conducted by liquid chromatography - mass spectrometry (LC-MS) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The composition of several other small molecules was assessed by untargeted metabolomics and amino acid compositional analysis was performed. Additionally, alginate characterization was carried out by Fourier-transform infrared spectroscopy (FTIR). These analyses aimed to determine the impact of extraction parameters on the composition of the extracts and gain deeper insights into the biological properties (antioxidant, antihypertensive, antidiabetic) of selected MAE and MEAE extracts.

While this knowledge is necessary for the development of a sustainable and effective circular downstream processes, it is currently limited due to the lack of comprehensive throughput analytical methods that can fully reveal the impact of these unit operations on the structural composition of these compounds (2,8,22–24), which will in turn determine their functional and

biological properties. Therefore, it is crucial to explore microwave processing strategies in conjunction with comprehensive analytical methods to better understand the impact of processing conditions on the extract composition and functionality. Such an approach will pave the way for the development of more effective and sustainable extraction processes for these valuable compounds.

3.2 Materials and methods

3.2.1 Starting material preparation and characterization

Macrocystis pyrifera from the Catalina Sea was harvested in the summer of 2021 by Primary Ocean (Catalina, CA, USA). Samples were frozen and shipped to Davis, CA, where they were processed as detailed in Chapter 2 and stored at -18 °C until use. The proximate composition of the starting material (freeze-dried *M. pyrifera* powder), including moisture, lipid, protein, ash content, amino acid composition, fatty acid composition, and total and free oxylipins, was analyzed in detail following the methods detailed in Chapter 2.

3.2.2 Extraction processes

The stepwise optimization of microwave processing was carried out as described in Figure 3.1. Extractions were carried out in the CEM MARS 6™ Microwave Digestion and Extraction System (CEM Corporation, Matthews, NC, USA). The microwave consists of a power system (0–1800 W), a magnetron (2450 MHz), and a Teflon®-coated microwave cavity holding the vessel turntable. The turntable is designed to accommodate up to 24 vessels and is equipped with magnetic stirring for each vessel. Stir bars were added to enable efficient stirring within the vessels. The glass vessels are sealed with Teflon® PFA caps and nested in composite sleeves within the

turntable. Sample temperatures were measured in real time using a fiber optic temperature probe (MTS-300, CEM Corporation, Matthews, NC, USA) inserted in a control vessel filled with water.

For each extraction, freeze-dried (FD) *M. pyrifera* powder was weighed and mixed with 10 g of deionized (DI) water in 20 mL glass extraction vessels (GlassChem, CEM Corporation, Matthews, NC, USA) to achieve the desired BWR. The slurry pH was then adjusted to the desired pH with by gradually adding 0.5 M HCl and 0.5 M NaOH. Additionality, a stir bar was added to facilitate the mixing during the extraction. If used for the extraction, enzymes were added once the required pH was reached. Prepared vessels were placed in the microwave, which automatically ramped to the specified temperature, with a come-up time of 5 min. After reaching the desired temperature, samples were kept at the specified temperature for the prescribed reaction time. Following the extraction, the solids were separated from the liquid phase (extract) through centrifugation using an Allegra X-14R centrifuge (Beckman Coulter, Brea, CA, USA) at 4,000 x g for 30 min at 4 °C.

The resulting extract was then transferred into a new falcon tube and 2% $CaCl_2$ (w/w) was added to the extract to induce the precipitation of alginate as calcium alginate. The mixture was vortexed for 1 min to homogenize and then left at 4 °C overnight to enable the precipitation of the calcium alginate. Calcium alginate was separated by centrifugation (Allegra X-14R centrifuge, Beckman Coulter, Brea, CA, USA) at 4,000 x g for 30 min at 4 °C.

Calcium alginate of selected extracts was mixed with 10 mL of a 3% (w/v) sodium carbonate for 2 h at 60 °C and the slurry was centrifuged to obtain a sodium alginate extract (supernatant). The sodium alginate extract was then freeze-dried (FreeZone 6 Liter Benchtop Freeze Dry, 77520 Series, Labconco, Kansas City, MO, USA) and resuspended in reverse osmosis (RO) water. Then, the alginate was precipitated out with 70% ethanol overnight and freeze-dried

again for 48 h following ethanol evaporation with a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA). To ensure sufficient extract volume for subsequent analyses, each extraction condition was replicated six times and the extracts were combined in 50 mL falcon tubes to create three reaction replicates. All extracts were frozen and stored at -18 °C or freeze-dried (FreeZone 6 Liter Benchtop Freeze Dry, 77520 Series, Labconco, Kansas City, MO, USA), depending on the analysis performed.

The initial phase of the optimization process involved assessing the impact of extraction time. This was accomplished by conducting extractions for 15, 30, 45, and 60 min at pH values 4, 7, and 10, at 60 °C, and 1:30 BWR. Starting temperature and BWR were determined based upon results from Chapter 2. Following the initial selection of reaction time, BWRs of 1:50 and 1:30 were compared at pH 4, 7, and 10 at the selected reaction time at 60 °C. The next step was to investigate the potential use of elevated temperatures. At the optimum extraction time and BWR, extractions were performed at 60, 70, and 80 °C at pH 4, 7, and 10. Following the MAE optimization, the implementation of enzymes to assist the extraction in the MEAE was evaluated. MEAE conditions were investigated upon findings from the MAE optimization (extraction time and BWR) and from the EAEP optimization results from Chapter 2 (enzyme type and concentration).

Based on MAE results, the MEAE process was evaluated at 15 and 30 min. The goal of the latter time was to allow for additional time for the enzymes to catalyze the breakdown of the *M. pyrifera* biomass. The individual impacts of using 5% of FoodPro's CBL, an acidic carbohydrase provided by the Genecor Division of DuPont™ Danisco® (Rochester, NY, USA), (w/w) at pH 4 and 2.5% of BioCat's (Troy, VA, USA) Neutral Protease L (NP) (w/w) at pH 7 were evaluated at 15 and 30 min. To explore their combined impact, a sequential approach was

employed, where 5.0% CBL was used at pH 4 for 15 min followed by extraction with 2.5% NP at pH 7 for an additional 15 min. Lastly, to determine the impact of switching the slurry pH from 4 to 7, extractions were conducted without the use of enzymes at 70 °C (as determined by the MAE optimization) for 15 min at pH 4, followed by 15 min at pH 7.

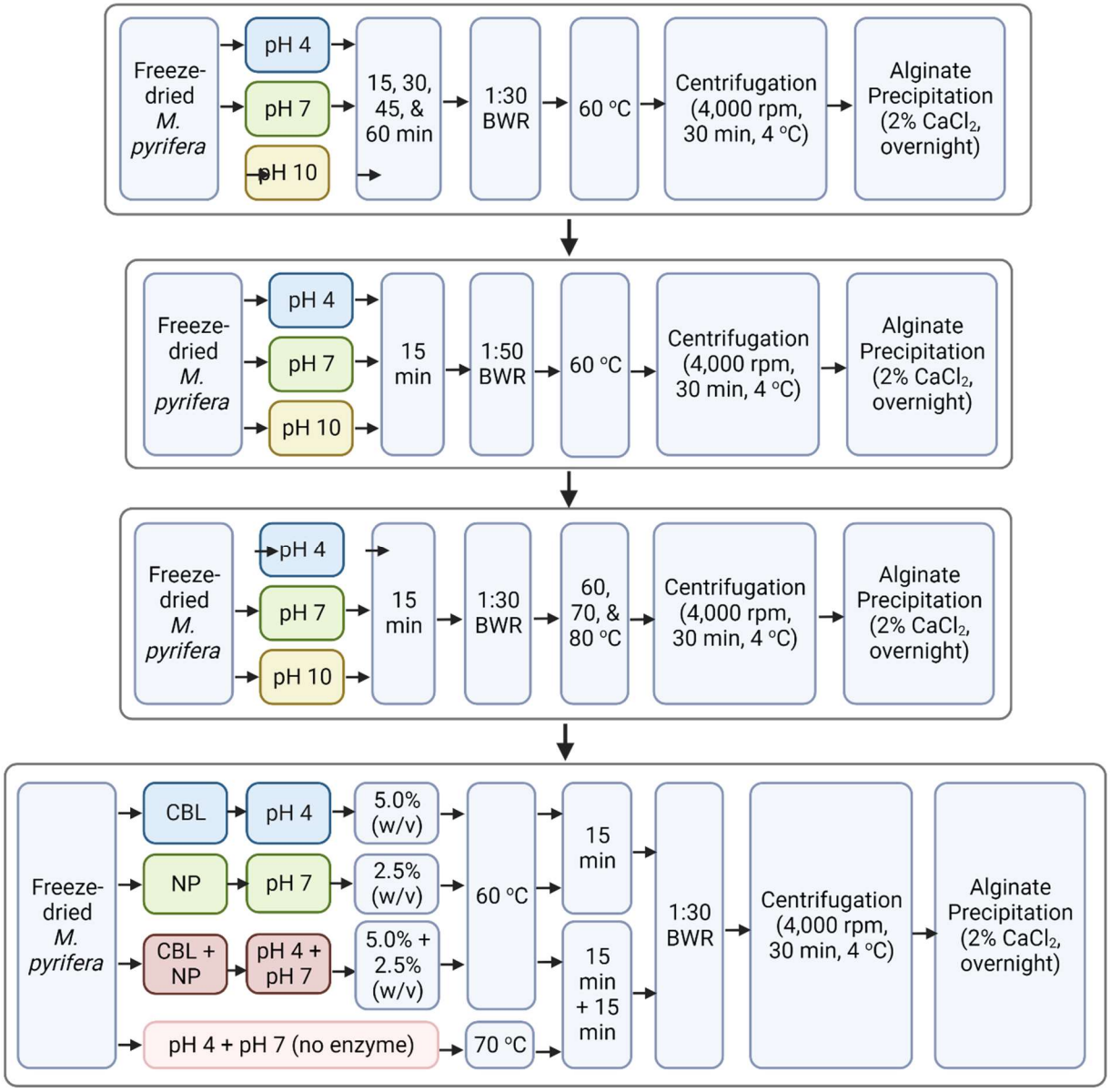


Figure 3.1: Flow chart for the stepwise optimization of the MAE process. Created with BioRender.com.

Extracts from all experimental conditions described in Figure 3.1 were analyzed as described in section 3.2.4, “Characterization of extracts from all extraction conditions”. Selected extracts were chosen for further analysis based on reaching their maximum yields or where less resource intensive conditions (i.e., lower temperature, higher BWR, shorter extraction time) demonstrate no significant differences from maximum yields, as described in section 3.2.5, “Characterization of selected extracts”.

3.2.3 Characterization of extracts from all extraction conditions

3.2.3.1 Total phenolic content

Total phenolic content (TPC) of the extracts was determined according to the Folin-Ciocalteu method as described by Singleton et al. (26). A 25 µL aliquot of diluted extract and 125 µL of 10% Folin-Ciocalteu reagent were transferred to a clear 96-well microplate, and agitated for 5 min at 300 rpm in the dark at 37 °C. After which, 100 µL of a 7.5% (w/v) sodium carbonate solution was added and the plate was agitated for another 30 min, and then allowed to rest (no agitation) for an additional 90 min. The absorbance was read at 760 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). TPC results were calculated using a standard curve of gallic acid with concentrations of 5–95 µg/mL and were presented as mg gallic acid equivalent (GAE)/ g FD *M. pyrifera*. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

3.2.3.2 Fucose content

Fucose content, used to approximate the fucoidan content in the extracts, was determined based on the method of Dische and Shettles (27), with modifications as described by Rajauria et al. (10). A 40 µL aliquot of diluted extract, followed by 180 µL of cold 6:1 sulfuric acid: RO water, was transferred to a 96-well microplate. The mixture was then left at room temp for 3 min,

followed by incubation at 90 °C for 10 min, then put in an ice bath for 5 min to stop the reaction. Next, 10 µL of 3% (w/v) L-cystine hydrochloride solution was added and the plate was left in the dark for 60 min at room temperature. The absorbance was measured at 396 and 430 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). Fucose content results were determined by absorbance difference based on a standard curve created with L-fucose with concentrations of 0–0.2 µg/µL and expressed as fucose equivalents (Eq). Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

3.2.3.3 Sulfate content

Sulfate content determination of the extracts was based on the method developed by Dodgson (28) with modifications from Torres et al. (29). Freeze-dried extracts and 0.5 M HCl were added to 2 mL Eppendorf tubes at 1 mg FD extract/50 µL. The mixture was incubated at 200 °C for 3.5 h with agitation at 300 rpm followed by a brief centrifugation for 2 min at 15,000 x g (accuSpin Micro 17, Fisher Scientific, Hampton, NH, USA). 20 µL aliquots of the supernatant and 140 µL of 0.5 M HCl were added to a clear 96-well microplate and absorbance was read at 405 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). Next, 40 µL of barium-chloride gelatin reagent (75 mg gelatin, 25 mL RO water, 250 mg $BaCl_2$) was added to the plate and left to sit for 20 min, after which the absorbance was read a second time at 405 nm. The first absorbance reading was subtracted from the second and the sulfate content was determined by a standard curve created with potassium sulfate with concentrations of 0-2 µg/µL. The sulfate content was expressed as SO_4^{2-} equivalents (Eq). Extraction triplicates were analyzed in duplicate, and the results were presented as an average of six measurements.

3.2.3.4 Laminarin content

Laminarin content was determined by measuring reducing sugars produced after sample hydrolysis with a method based on that of Breda et al. (30), with slight modifications. A 400 µL aliquot of diluted extract and 100 µL of an endo-1-3(4)-β-glucanase enzyme solution (150 U/mL), purchased from Novozymes, was transferred to 2 mL Eppendorf tubes. The tubes were incubated for 2 h at 37 °C. Controls for each condition were prepared in the same way but with the addition of RO water in place of enzyme. After hydrolysis, 500 mL of a 0.75% (w/w) dinitrosalicylic acid reagent, 0.75% (w/w) sodium hydroxide, 0.04% (w/w) sodium sulfate, and 10% (w/w) potassium tartrate tetrahydrate solution were added. Next, the mixture was incubated at 90 °C for 5 min followed by cooling in an ice bath for 3 min. 200 µL from each extract and control were added to a 96-well plate and the absorbance was read at 590 nm using a microplate reader (SpectraMax iD5, Molecular Devices LLC, San Jose CA, USA). Laminarin concentration was determined based on a standard curve prepared with laminarin concentrations ranging from 0–10 µg/µL and glucose concentrations ranging from 0–5 µg/µL. Extraction triplicates were analyzed in duplicate, and the results were presented as an average of six measurements.

3.2.3.5 Alginate content

Alginate content determination was based on the methods of Mohd Fauziee et al. (31) with the modifications described in Chapter 2. Mass of freeze-dried (FD) precipitated calcium alginate was determined and alginate yield was calculated using Equation 3.1. Results were presented as an average of three measurements.

$$Alginate\ yield\ (\%)\ (w/w)\ = \frac{freeze\ dried\ calcium\ alginate\ weight}{initial\ freeze\ dried\ kelp\ powder\ weight} * 100 \qquad \textbf{(Equation 3.1)}$$

3.2.3.6 Protein extractability

Protein content of the freeze-dried extracts was determined via the Dumas combustion method (32), as described in Chapter 2. Protein extractability was calculated according to Equation 3.2 and the results were presented as an average of three measurements.

$$Protein\ extraction\ yeild\ (\%\ w/w) = \frac{protien\ (g)\ in\ FD\ extract}{protien\ (g)\ in\ FD\ kelp\ powder} \times 100 \qquad \textbf{(Equation 3.2)}$$

3.2.4 Characterization of selected extracts

Extraction conditions for more in-depth characterization were selected based on extraction yields of key components (phenolics, fucose, sulfate, laminarin, alginate, and protein) and reduction in resource utilization. When yields were not statistically different from the maximum, the condition that was less resource intensive (i.e., higher BWR, less time, lower temperature, less enzyme) was selected to enhance the economic and environmental sustainability of the process.

3.2.4.1 Metabolomic profiling

Untargeted metabolomic profiling of phenolics and other small molecules such as dipeptides was carried out by the West Coast Metabolomics Center Central Services Core (UC Davis, Davis, CA, USA) (33) as described in Chapter 2 using LC-MS. Freeze-dried extracts were prepped for phenolic analysis by mixing 1:50 (v/w) in 50:50 methanol: water + 0.01% HCl, and sonicated (Branson 2800, Branson Ultrasonics, Brookfield, CT, USA) for 1 h. Samples were then centrifuged at 13,000 rpm for 5 min and the supernatants were used for analysis. Data was interpreted based on the International Chemical Identifier and presented by peak height. Metabolites were divided into four categories: phenolics, peptides, lipid derived compounds, and other metabolites. They were reported based on the number of unique identified metabolites per extraction condition.

3.2.4.2 Monosaccharide quantification

Monosaccharide analysis for fucose and galactose, which serves as an indicator of the presence of fucoidans in the extracts, was performed by HPAEC-PAD (34), as described in Chapter 2. Polysaccharides were first precipitated with ethanol and hydrolyzed with trifluoroacetic acid hydrolysis at 100 °C for 2 h. A linear range from 0.0001-0.01 mg/mL was established and analyzed on an ion chromatography system equipped with a detector consisting of an electrochemical cell with a disposable, gold, working electrode and a pH-Ag/AgCl reference electrode (Thermo Fisher Scientific, Waltham, MA).

3.2.4.3 Oligosaccharide analysis

Oligosaccharide analysis was performed by liquid chromatography mass spectrometry (LC-MS/MS) (34), as described in Chapter 2. LC-MS/MS analysis was performed with an Agilent 6520 Accurate-Mass Q-TOF instrument equipped with a Chip Cube coupled to an Agilent 1200 Series high performance liquid chromatography interface (Agilent Technologies, Santa Clara, CA) as described by Huang et al. (35). Data was annotated by Glyconote (https://github.com/MingqiLiu/GlycoNote) and manually verified using Agilent Mass Hunter Qualitative Analysis (B.07.00, Agilent Technologies, Santa Clara, CA, USA).

3.2.4.4 Characterization of sodium alginate structure by Fourier transform infrared spectrometry

A FTIR (Shimadzu IR Prestige-21) equipped with a quest single-balanced attenuated total reflectance accessory was used to characterize the functional groups present in the sodium alginate samples, as described in Chapter 2. Briefly, the freeze-dried sodium alginate was suspended in a 10% w/v solution of 0.1 M PBS buffer at pH 7.4 and was scanned from 4000-400 cm^{-1} at a resolution of 4 cm^{-1} (14,31) with 25 scans using the absorbance mode. A background scan of the

buffer was subtracted for the samples. Scans were analyzed with LabSolutions IR software and absorbance bands corresponding to units of mannuronic and guluronic acids were used to estimate the M/G ratio (14). Each extraction triplicate was analyzed and the values of absorbance for each wavelength were averaged. Microsoft Excel was used to graph the data.

3.2.4.5 Laminarin quantification by β-glucan analysis

The β-glucan content, which serves as an indicator of the presence of laminarin in the extracts, was determined using the K-YBGL enzymatic-assay kit from Megazyme International Ltd., Bray, Ireland (36), as described in Chapter 2. Since laminarins are composed primarily of β-glucans (10), this enzymatic assay is useful for assessing the amount of laminarin in the extracts. Total glucan content and α-glucans were measured, and the β-glucan content was determined by difference. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

3.2.4.6 Amino acid profiling

Amino acid analysis of freeze-dried extracts was performed by the UC Davis Proteomics Core Facility (37), as described in Chapter 2, using Hitachi amino acid analyzers (Models 8900 and 8800). All samples were analyzed in triplicate and results were presented as an average of nine measurements.

3.2.4.7 Antioxidant activity

The 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay for radical cation scavenging activity was performed as described by Al-Duais et al. (38), with modifications as described in Chapter 2. Additionally, the Oxygen Radical Absorbance Capacity (ORAC) assay was performed according to the method described by Zulueta et al. (39), as described in Chapter

2. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

3.2.4.8 Angiotensin-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of the chosen *M. pyrifera* extracts was determined according to a method previously developed (40), as described in Chapter 2. The method is based on the ability of ACE to hydrolyze the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO_2)-Pro). ACE inhibitory activities of the samples are expressed as the percentages of total activity obtained when no inhibitor is present (Equation 3.3).

$$ACE\ inhibition\ (\%) = \frac{ABS_C - ABS_S}{ABS_C} * 100 \quad \textbf{(Equation 3.3)}$$

where ABS_C is the absorbance of the control reaction (no sample extract addition: enzyme + substrate + water), and ABS_S corresponds to the absorbance of the sample reaction (enzyme + substrate +extract). Results were compared to a 1 μM solution of Captopril to see how inhibition of extracts compared to current treatment options. Extraction triplicates were analyzed in duplicate, and results were presented as an average of six measurements.

3.2.4.9 α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of the extracts was evaluated according to the method described by Ibrahim et al. (41), with modifications as described in Chapter 2. A control reaction and a sample background were used to calculate the inhibitory activity (Equation 3.4).

$$\alpha - glucosidase\ inhibition\ (\%) = \frac{ABS_C - ABS_S}{ABS_C} * 100 \quad \textbf{(Equation 3.4)}$$

where ABS_C is the absorbance of the control reaction (no sample extract addition: enzyme + substrate + water), and ABS_S corresponds to the absorbance of the sample reaction (enzyme +

substrate +extract). Results were compared to a 1 μM solution of Acarbose to see how inhibition of extracts compared to current treatment options. Extraction triplicates were analyzed in duplicate, and results are presented as an average of six measurements.

3.2.5 Statistical Analysis

All extractions were performed in triplicate. The results are expressed as the mean ± one standard deviation (SD). A one- or two-way Analysis of Variance (ANOVA), depending on the number of variables compared (1-way for characterizations of selected extracts and 2-way for extraction analysis of all conditions), was performed, followed by the HSD Tukey test to determine significant differences among the experiments at $p < 0.05$. All statistical analyses were performed using JMP Statistical Discovery LLC (Cary, NC, USA).

3.3 Results and discussion

The impact of key extraction parameters on the extractability of *M. pyrifera* and other brown macroalgae compounds have been minimally investigated. To the best of our knowledge, there have been no studies focusing on the use of microwave assisted extraction for *M. pyrifera*. The following results and discussion explore the impact of important extraction conditions (BWR, pH, time, temperature, and amount and type of enzyme) on the extractability (yield), structure/composition, and functional/biological properties of the extracts. While it is not possible to compare the results to other MAE extractions of *M. pyrifera*, results will be compared to the AEP and EAEP results described in Chapter 2, and to the results from other brown macroalgae extractions when applicable. Additionally, studies involving microwave processing focusing on the simultaneous extraction of multiple compounds from macroalgae are lacking.

3.3.1 MAE stepwise optimization

3.3.1.1 Impact of extraction time on the extractability of MAE

The impact of extraction pH (4, 7, and 10) and time (15, 30, 45, and 60 min) on the extractability of major *M. pyrifera* compounds (phenolics, fucoidan, laminarin, alginate, proteins) is shown in Figure 3.2. Highest TPC of 13.54 ± 0.30 mg GAE/ g FD *M. pyrifera* was achieved at 30 min and pH 10 (Fig. 3.2a). Although at pH 4 and 7, TPC values reached their maximum values after only 15 min, these values (9.38 ± 0.28 and 12.35 ± 0.36 mg GAE/ g FD *M. pyrifera*, respectively) were significantly lower than the TPC achieved at pH 10. In plant matrices, the pH of the slurry plays a crucial role in the phenolic extraction mechanism. Alkaline conditions have been shown to facilitate the cleavage of ester and ether linkages between phenolics and other carbohydrates and proteins in the plant material, leading to the release of bound phenolics and higher TPC values (42,43). Overall, alkaline conditions led to the highest TPC, with 15 to 30 min being sufficient processing time.

In Chapter 2, we demonstrated that AEP at neutral pH resulted in the highest TPC values, while lowest values were observed at pH 4 and 10. During microwave extractions, unlike the aqueous extractions discussed in Chapter 2 where the pH can be adjusted and maintained at a constant value throughout the process, the pH cannot be monitored during the microwave process. As a result, the initial alkaline pH of 10, used to start the extraction, dropped to around 8 to 9, at the end of the extraction. While the use of pH 10 may have been too harsh for the sensitive algal phenolics (44) when evaluating the AEP, as it could lead to their chemical degradation, the use of a more moderate alkaline pH, reached by the pH dropping in the MAE, might have been sufficient to cleave the ester and ether linkages without causing damage to sensitive phenolics. Additionally, when comparing these results to the AEP results presented in Chapter 2, 30 min in the MAE

produced comparable, albeit slightly lower, TPC values to the maximum TPC value obtained for the AEP at pH 7, 6 h (13.54 mg GAE/ g FD *M. pyrifera* vs. 15.56 mg GAE/ g FD *M. pyrifera*). This rapid extractability can be attributed to the ability of the microwave processing to rapidly disrupt the algal matrix through dipole interactions (8), thus offering a significant reduction in extraction time while still yielding comparable TPC results.

MAE of the brown macroalgae *A. nodosoum* (70% methanol, 110 °C, 15 min, 1:10 BWR) resulted in an increase in TPC when compared to conventional solid-liquid extraction (70% ethanol, room temp, 4 h, 1:10 BWR), with MAE yielding up to 1.40 mg GAE / g dry seaweed (compared to 0.51 mg GAE/ g for the conventional method) (24). These results are lower than those reported in the current study, possibly due to the different compositions across macroalgae species and/or the extraction temperature and solvents used, which could lead to both phenolic degradation and low extractability. It is important to note that TPC, while commonly used to estimate phenolic extractability, is a colorimetric assay that is not highly specific for phenolics and can be impacted by the presence of other molecules. The reagents used in the TPC assay react with other oxidizable compounds like aromatic amines and amino acids, and reducing sugars (45,46). Therefore, the potential impact of other compounds extracted from *M. pyrifera* on TPC readings must be carefully considered.

The impact of MAE conditions on fucoidan extractability, commonly assessed by measuring the fucose and sulfate content of the extracts are shown in Figures 3.2b and 3.2c, respectively. It is important to note that the reported fucose content does not directly translate to fucoidan content. While fucose is the most common monomer in fucoidan, around 50% of fucoidan molecules are composed of other monosaccharides including galactose, glucose, xylose, mannose, and rhamnose (47,48). Fucose extractability reached its maximum after just 15 min of

extraction in acidic conditions (9.81 ± 0.71 mg fucose Eq/ g FD *M. pyrifera* at 15 min, pH 4), while lowest extractability was observed at alkaline conditions (7.91 ± 0.89 mg fucose Eq/ g FD *M. pyrifera* at 15 min, pH 10). It is worth noting that the sulfate content of the extracts, as depicted in Figure 3.2c, serves as an additional surrogate measurement to estimate fucose extractability. Notably, the sulfate content showed relatively little variation across extraction pH and time conditions. Highest sulfate yields were achieved at 45 min at pH 4 (19.79 ± 1.34 mg SO_4^{2-} E/ g FD *M. pyrifera*). However, these values were not statistically different from the ones achieved at 30 min at pH 4 (17.63 ± 0.81 mg SO_4^{2-} E/ g FD *M. pyrifera*). Similar high sulfate yields were achieved at 15 min at pH 7 and 10 (16.59 ± 1.48 and 16.87 ± 1.39 mg SO_4^{2-} E/ g FD *M. pyrifera*, respectively).

Fucoidans are located within the cell wall. Therefore, breaking apart the algal matrix is crucial for extraction of these compounds (49). In general, acidic conditions aid in the extraction of carbohydrates by breaking glycosidic bonds present in carbohydrates (11,50,51), explaining why acidic extractions are often used in fucoidan extraction (52,53). Overall, the findings presented herein indicate that fucoidan extraction is greatest at acidic conditions and does not require extended microwave processing time. When compared to the results of the AEP (12.16–13.49 mg fucose Eq/ g FD *M. pyrifera*) performed at 6 h, 60 °C, across all pH values (described in Chapter 2), MAE resulted in lower fucose yields (maximum of 9.81 mg fucose/ g FD *M. pyrifera*). However, the sulfate content of the *M. pyrfieria* MAE extracts (16.15-19.79 mg SO_4^{2-}/g FD) was comparable to the ones achieved by AEP extracts at 6 h, at 60 °C, across all pH values (17.23–20.44 mg SO_4^{2-}/g FD), indicating that, while fucose yields may be lower for the MAE extracts, since the ratio of sulfate to fucose was higher for the MAE than AEP the extracted MAE fucoidans potentially have higher bioactivity as the sulfate content of fucoidan has been positively

correlated with its bioactivity (7,8). Similarly, lower fucoidan extractability (16.08 vs. 20.08% yield) has been observed when comparing MAE (0.1 M HCl, 15 min, 120 °C, 1:17.65 BWR) and conventional solvent extraction (0.1 M HCl, 3 h, 70 °C, 1:17.65 BWR) of *A. nodosum* (18). Similarly to the findings observed in the current study, the fucoidan content of MAE extracts from *A. nodosum* also had more sulfate than fucose, being composed of 18.19 mg fucose/ g dry seaweed and 23.65 mg sulfate/ g dry seaweed (18).

The laminarin content of the extracts can be an essential parameter in understanding the nutritional value and potential applications of the extracts in various industries. The laminarin content of the extracts (Fig. 3.2d) showed little variation (not statistically different at $p<0.05$) across pH and time, with yields ranging from 9.21 ± 0.35 to 9.98 ± 1.26 mg laminarin Eq/ g FD *M. pyrifera*. Laminarin are water-soluble polysaccharides found in vacuoles within brown algae cells (9) and their extractability is highly dependent on the extraction process's ability to break through the algal cell. These results illustrate that for MAE performed at 60 °C and 1:30 BWR, the disruptive dipole-dipole interaction of the microwaves plays a larger role in breaking through the cell wall compared with other parameters such as pH and time. Although extraction pH and time are important parameters in traditional extraction methods (54,55), their influence on the cell wall integrity may not be as pronounced as the effects of microwaves during MAE. MAE results are comparable to the AEP (6 h, 1:30 BWR) results described in Chapter 2, which ranged from 9.08–12.58 mg laminarin Eq/ g FD *M. pyrifera*. However, the significant reduction in extraction time with MAE (6 h vs. 15 min) highlights the potential of this technology to shorten the extraction process of laminarin and other bioactive compounds in brown macroalgae. To the best of our

knowledge, this study represents the only reported investigation into the use of MAE for laminarin extraction from *M. pyrifera.*

Understanding the effects of extraction parameters on alginate, a compound used in a variety of industries, holds significant commercial relevance. The impact of extraction pH and time on alginate extractability is shown in Figure 3.2e. Overall, alginate extractability was slightly favored when extraction pH increased from 4 to 10, with extraction time having a less pronounced effect. Maximum alginate extractability was achieved at 15 min, with alkaline conditions resulting in greatest yields (67 ± 5.27 mg FD calcium alginate/ g FD *M. pyrifera* at pH 10) (Fig. 3.2e). Higher alginate extractability at alkaline pH has been linked to the deprotonation of carboxylic acids molecules in the alginate, which enhances electrostatic repulsion between the negatively charged molecules and subsequently their solubility in the extraction medium (56). Furthermore, alkaline pH can weaken inter and intramolecular interactions within the cell wall (57), which facilitates the release of alginate and other bioactive compounds from the macroalgae matrix. Because MAE at alkaline pH values led to higher extractability of alginate and other compounds such as proteins (see discussion below), it is important to note that during the precipitation of alginate with calcium chloride, additional compounds such as proteins may have co-precipitated with the alginate (31), potentially leading to an overestimation of the alginate content of the extracts. Further structural analysis of alginate samples from selected extraction conditions will be discussed in the section describing the sodium alginate structure characterization by FTIR. MAE alginate yields (67 mg FD calcium alginate/ g FD *M. pyrifera* at pH 10) were much lower than the 159 mg calcium alginate/ g FD *M. pyrifera* achieved by the AEP at 6 h, 1:30 BWR, and pH 10, as described in Chapter 2. Reduced alginate extractability in MAE at pH 10 could be due to the lack of pH maintenance throughout the extraction process, which in turn led to reduced extraction pH

values. Extraction times as short as 15 min was found to be sufficient for extracting alginate from *M. pyrifera*. This finding is in agreement with the optimum extraction time reported for the extraction of sodium alginate from *N. zanardini* (67 °C, 1:29 BWR), which was 19 min.

While the alginate yield reported by Torabi et al. (310 mg sodium alginate/ g dry weight algae) (58) was much higher than the ones reported herein, it is worth mentioning potential differences in alginate composition and concentration in the different macroalgae species used in both studies and the fact that the aforementioned study focused on alginate extractability optimization, compared to our process where extractability was optimized to maximize the extraction of a wider number of compounds from the algae biomass.

Total protein extractability from *M. pyrifera* (Fig. 3.2 f) reached its maximum after 15 min and increased slightly with increasing the extraction pH from 4 to 10. The highest TPE (26.37 ± 1.47%) was achieved after 15 min of extraction at pH 10, and 1:30 BWR. Protein extractability is enhanced by furthering conditions from the protein's isoelectric point (PI), thereby increasing its solubility (54,59). Although the PI of *M. pyrifera* proteins is unknown, the PI for other seaweeds is reported to be between pH 3 and 4 (60), explaining the slightly lower protein extractability of *M. pyrifera* proteins at pH 4. Additionally, this study focused on providing a comprehensive understanding of the impact of processing conditions on the simultaneous extraction of several compounds form *M. pyrifera.* As previously mentioned, co-precipitation of alginate and proteins may have occurred, which could have resulted in lower protein yields in the extracts. Overall, in just 15 min, MAE achieved TPE yields ranging from 17.52 to 26.37%, compared to 26.43 to 36.10% for the AEP at 6 h, as discussed in Chapter 2. These results highlight the ability of the microwave process to effectively disrupt the macroalgae matrix through dipole-dipole interactions, resulting in the faster release of proteins and other macroalgae compounds.

Sasaki et al. reported a slight increase in protein extraction yields from *U. pinnatifida*, a brown macroalgae, as the extraction time increased from 5 to 40 min. They observed yields of approximately 27% for 5 min and the highest yields of 32.6% at 40 min using conditions of 120 °C and a BWR of 1:15 (61). While yields were higher with increased time for the aforementioned study, the current study shows that achieving high protein yields from *M. pyrifera* is possible with short extraction times.

Given the high yields achieved for most of the extracted compounds at 15 min of extraction, 15 min was selected as the extraction time for the following steps in the optimization process. High yields of fucose, laminarin, alginate, and proteins were reached at 15 min, with TPC requiring 30 min to reach maximum values at pH 10, and sulfate content requiring 30 min at pH 4. Increased extractability at 30 min was deemed not significant enough to warrant doubling the extraction time when considering the extractability of most biomass compounds. This optimum time of 15 min is significantly lower than the 6 h for the AEP, described in Chapter 2, demonstrating the ability of the microwave to provide similar results in a fraction of the time.

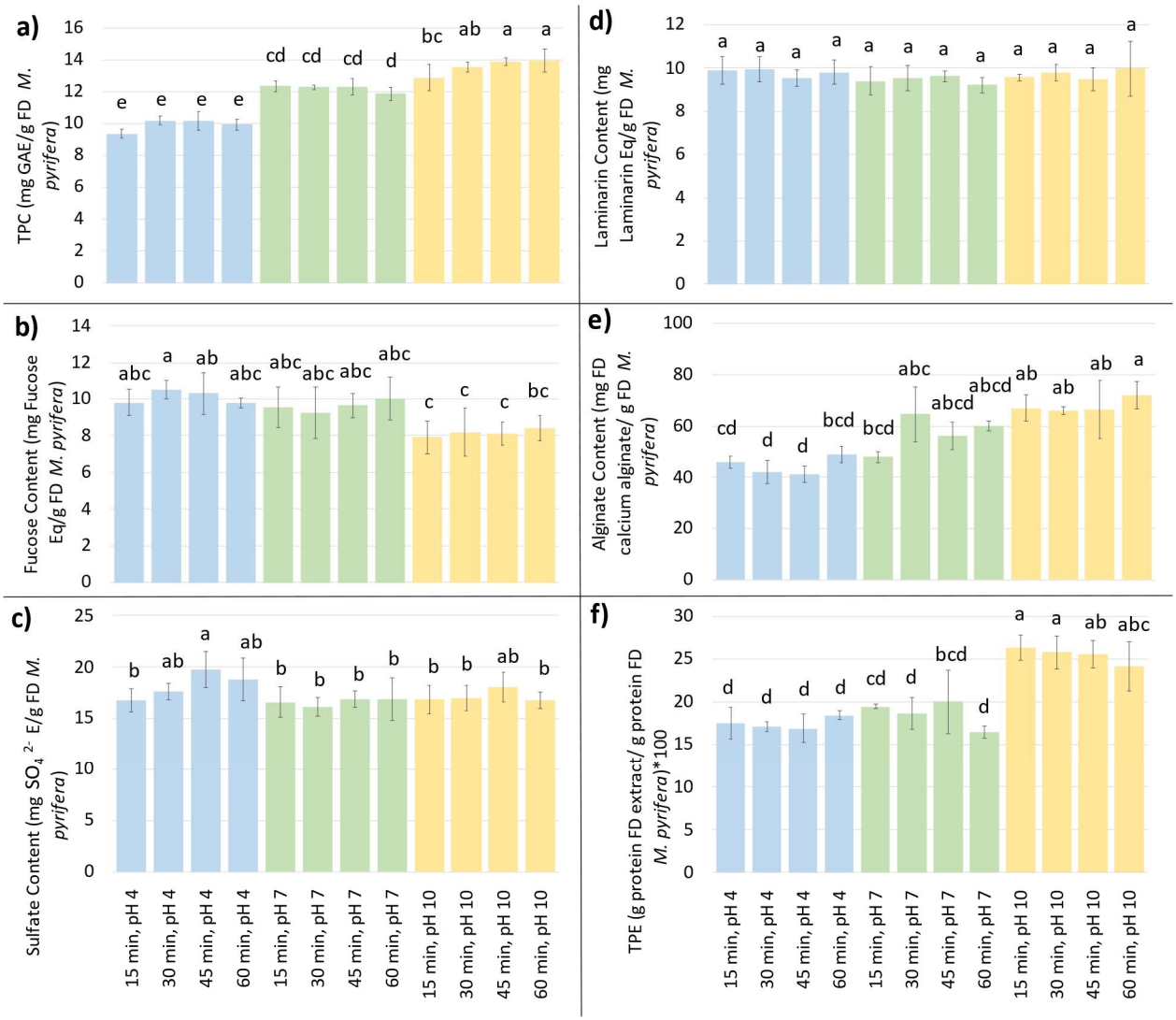


Figure 3.2: Impact of pH, extraction time, and 1:30 BWR at 60 °C on: a) Total phenolic content (TPC) via the Folin-Ciocalteu assay, b) Fucose content via the Dische and Shettles method, c) Sulfate content via the barium-chloride assay, d) Laminarin content via the reducing sugar assay, e) Alginate content of the freeze-dried calcium alginate, and f) Total protein extractability (TPE) by the Dumas combustion method.

3.3.1.2 Impact of biomass to water ratio on the extractability of MAE

The next step in the microwave optimization process was to determine the integrated impact of BWR (1:30 and 1:50) and extraction pH (4, 7, and 10) on compound extractability. As shown in Figure 3.3a, TPC did not increase at lower BWR (Fig. 3.3a), with TPC at pH 10, dropping from 12.89 ± 0.83 mg GAE/ g FD *M. pyrifera* at 1:30 BWR to 9.76 ± 0.75 mg GAE/ g FD *M.*

pyrifera at 1:50 BWR. Typically microwave processing uses higher BWR than other solid-liquid extraction processes, due to the ability of microwaves to enhance extractability without the addition of more solvent (62). Yield reductions at lower BWR during MAE have been observed for other matrices and compounds, such as the case of flavonoid extractability from *Osmanthus fragrans* flowers, where yields dropped with decreasing BWR (63).

A similar trend was observed for the AEP of *M. pyrifera*, described in Chapter 2, where the use of a lower BWR (1:50) did not increase the TPC compared to 1:30 BWR. While unable to find a study evaluating the impact BWR on TPC of extracts from *M. pyrifera*, our results are comparable to the TPC of extracts produced from *A. nodosum* with varying microwave power levels (250, 600, and 100W) (64) using a BWR of 1:10 and pH 1, which generated TPC values ranging from 4 to 18 mg GAE/ g FD *M. pyrifera*, highlighting that a lower BWR, within the range evaluated, was not necessarily beneficial for phenolic extraction.

When assessing fucose and sulfate contents (Figure 3.3b and 3.3c, respectively) of the extracts as markers for fucoidan and their bioactivity, lower BWR resulted in increased fucoidan extractability at pH 7 and 10. Fucose content increased significantly at pH 7 (9.56 ± 1.12 up to 10.86 ± 0.38 mg fucose Eq/ g FD *M. pyrifera*) and pH 10 (7.91 ± 0.89 to 11.71 ± 0.52 mg fucose Eq/ g FD *M. pyrifera*) when BWR decreased from 1:30 to 1:50. At pH 4, where fucose extractability is greatest, lowering the BWR did not significantly increase fucose content in the extract. As observed with the AEP results described in Chapter 2, this indicates that acidic pH is more influential in fucose extractability than BWR, which has been attributed to the crucial role of acidic conditions on cleaving glycosidic bonds within the fucoidan molecules (11,50,51). Conversely, the sulfate content of the extracts decreased when BWR increased from 1:30 (16.59 ± 1.48 to 16.87 ± 1.39 mg SO_4^{2-}/ g FD *M. pyrifera*) to 1:50 (12.16 ± 1.10 to 14.93 ± 1.66 mg SO_4^{2-}

/ g FD *M. pyrifera*). Although extracts obtained using 1:50 BWR had a greater fucose content, indicating a higher fucoidan content in the extracts, the extracted fucoidan are less sulfated. This means that while less abundant, fucoidan extracted at 1:30 BWR could be more bioactive (7,8). Due to the potential higher bioactivity of the 1:30 BWR extracts, in addition to water savings, the 1:30 BWR was selected as a favorable extraction condition. As for phenolics, there is a lack of studies comparing the role of BWR in MAE with respect to fucose extractability. However, Yuan et al. reported 18.19 mg fucose/ g dry seaweed and 23.65 mg sulfate/ g dry seaweed for *A. nodosum* when using a 1:10 BWR, indicating that low BWR is not necessary for microwave extraction of fucoidan (18).

For laminarin content (Figure 3.3d), higher BWR led to slightly higher yields, with yields being statistically higher for MAE performed at pH 7 and 1:30 BWR (9.40 ± 0.66 mg laminarin Eq/ g FD *M. pyrifera*) vs. at 1:50 BWR (12.80 ± 2.16 mg laminarin Eq/ g FD *M. pyrifera*). As discussed previously, pH did not play a significant role in extractability. These findings are similar to those of the AEP of *M. pyrifera*, described Chapter 2, where reducing BWR from 1:30 to 1:50 did not significantly enhance laminarin extractability. However, comparing our results with the literature is challenging due to the lack of studies evaluating the impact of MAE on the extractability of laminarin from *M. pyrifera*, underscoring that further investigations are needed to better understand the potential of MAE for laminarin extraction.

The impact of extraction time and pH on alginate yields is presented in Figure 3.3e. Although lower BWR often leads to increased extractability, in contrast to the results described in Chapter 2, alginate extractability decreased when BWR decreased from 1:30 to 1:50 at pH 4 and 7. Maximum alginate yields achieved varied from 21 ± 2.12 to 68.5 ± 3.77 mg calcium alginate/ g FD *M. pyrifera* at 1:50 BWR and from 46 ± 2.29 to 67 ± 5.27 mg calcium alginate/g FD *M. pyrifera*

at 1:30 BWR. As forementioned, in conventional extraction techniques a higher volume of solvent often leads to increased extractability and subsequently higher recovery, but in MAE a higher solvent volume may lead to lower recoveries (65,66), highlighting the variation in optimum slurry dilution among different extraction methods. However, this effect was not observed at pH 10, where alginate extractability was similar for both BWRs, which result from the ability of alkaline conditions to deprotonate carboxylic acids molecules in the alginate, enhancing alginate solubility and therefore its extractability. The maximum alginate yield at lower BWR (1:30) in this study aligns with the findings of Torabi et al., who reported alginate yields of 313.9 mg sodium alginate/ g dry seaweed from *N. zanardini,* at an optimum BWR of 1:29 when evaluating the impact of decreasing BWR from 1:10 to 1:30 (58).

TPE increased from 17.52 ± 1.83 to 25.84 ± 3.91% when the BWR decreased from 1:30 to 1:50 at pH 4. However, similar TPE was achieved at higher pH values (7 and 10), which often favor protein extractability, regardless of the BWR used (Fig 3.3f). A small, but not statistically significant, increase in TPE was observed at pH 7 when using lower BWR. These results show the synergistic impact of different extraction parameters on TPE, where at pH 4, which does not favor TPE due to the proximity to the isoelectric point of macroalgae proteins (60), the increased concentration gradient between solutes and extraction media (67) provided by the lower BWR of 1:50 led to increased extractability. However, at pH 10, where protein extraction is already high, the use of lower BWR was not necessary to increase yields. Our results are in agreement with those presented by Sasaki et al., who reported no increase in TPE (120 °C, 20 min), which varied from 29.8 to 30.7%, when BWR decreased from 1:15 to 1:25 (61), indicating that lower BWR was not necessary to increase protein yields.

One of the benefits of using microwave processing is the ability of using lower BWR, resulting in the use of less extraction solvent (in this case water) (44). While a lower BWR (1:50) led to increased yields for several compounds at specific pH values, these differences were not always statistically significant (only for TPE at pH 4, fucose at pH 7 and pH 10, and laminarin at pH 7). Lower BWR resulted in lower sulfate content in the extracts, indicating potential lower bioactivity of the extracted fucoidans. With a BWR of 1:50 being more resource intensive than 1:30, while resulting in similar yields for most conditions tested, the BWR of 1:30 was selected for the remaining steps in the optimization process.

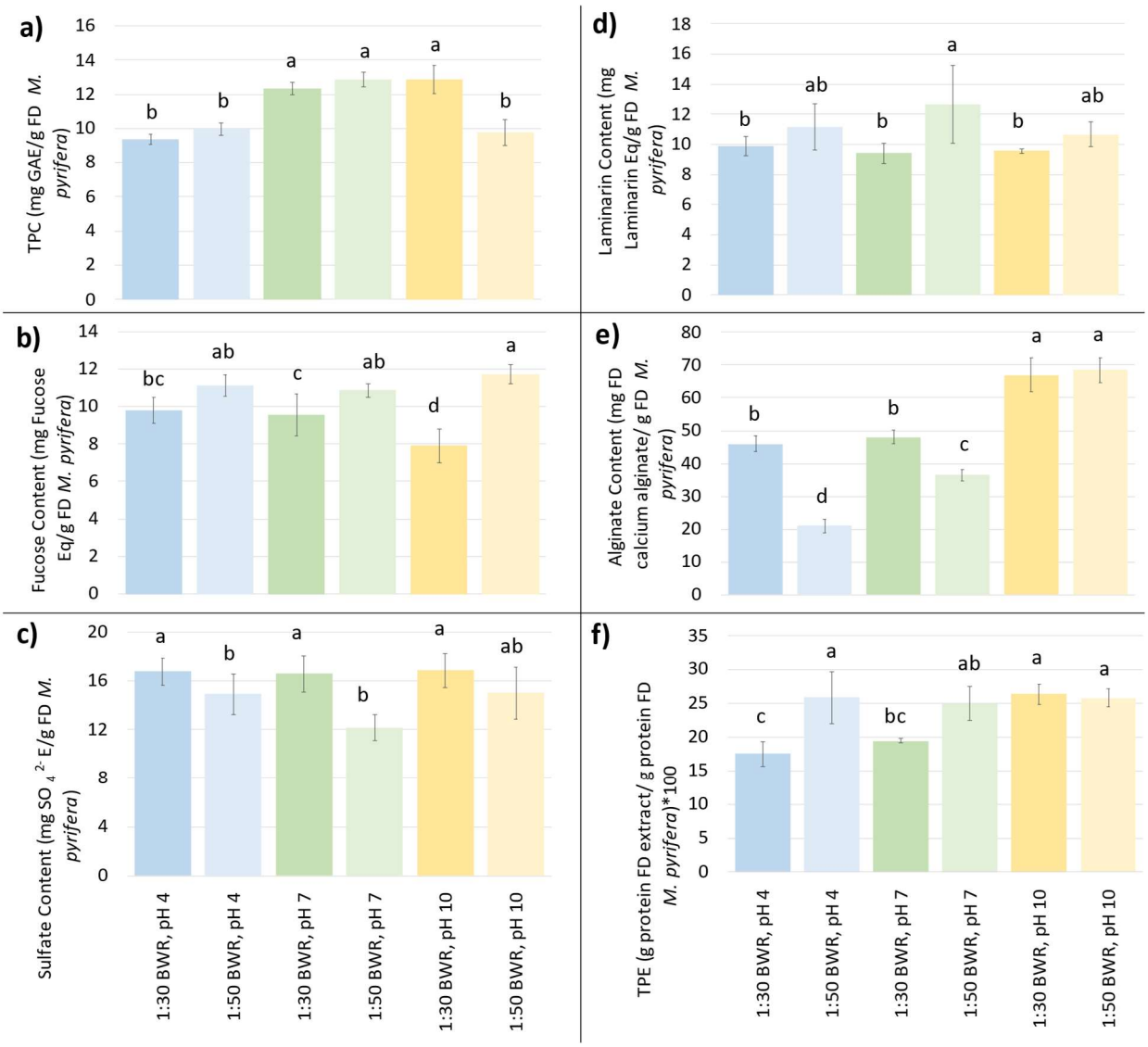


Figure 3.3: Impact of biomass-to-water ratio (BWR) and pH at 15 min and 60 °C on: a) Total phenolic content (TPC) via the Folin-Ciocalteu assay, b) Fucose content via the Dische and Shettles method, c) Sulfate content via the barium-chloride assay, d) Laminarin content via the reducing sugar assay, e) Alginate content of freeze-dried calcium alginate, and f) Total protein extractability (TPE) by the Dumas combustion method.

3.3.1.3 Impact of extraction temperature on the extractability of MAE

The next step in the optimization process evaluated the integrated impact of temperature (60, 70, and 80 °C) and extraction pH (4, 7, & 10) at 15 min, and 1:30 BWR on the overall MAE extractability (Figure 3.4).

Increasing extraction temperature from 60 to 70 °C significantly increased the TPC of the extracts from 9.38 ± 0.28 to 10.89 ± 0.40 mg GAE/ g FD *M. pyrifera* at pH 4, likely due to the reduced viscosity of the slurry at higher temperatures and enhanced diffusion of the compounds (Figure 3.4a). However, this effect was not statistically significant for extractions at pH 7 and 10. Increased TPC at higher temperatures at pH 4 is likely due to enhanced extractability from decreased viscosity at elevated temperatures that aids in increasing yields at acidic conditions where phenolic extraction is not typically favored. At the same temperature, increasing extraction pH from 4 to 7 and 10 led to higher TPC values, with TPC yields at pH 7 not being statistically different from the ones at pH 10. Our results are higher than those reported by Yuan et al., where TPC values ranging from 0.73 to 1.40 mg GAE/ g FD *M. pyrifera* were achieved when evaluating the impact of MAE at 110 °C (15 min, 70% methanol, 1:10 BWR) on phenolic extractability from different brown macroalgae (*Ascophyllum nodosum, Laminaria japonica, Lessonia trabeculate* and *Lessonia nigrecens*) (24). Differences in extractability between both studies are likely due to differences in the macroalgae species, pretreatment effects (drying at 105 °C vs. freeze-drying), and extraction conditions (temperature,110 vs. 70 °C, and extraction solvent, 70% methanol vs. water), which could impact extraction efficiency and stability of the extracted phenolics.

The integrated impact of extraction pH and temperature on fucose extractability is shown in Figure 3.4b. The fucose content (Fig. 3.4b) of the extracts obtained at pH 4 and 10 increased with increasing temperature, reaching its statistically significant maximum of 15.33 ± 1.62 mg fucose Eq/ g FD *M. pyrifera* at pH 4, and 70 °C. This is an example of how careful selection of optimum processing conditions can lead to a continuous increase in extractability and further

maximize yields of key components. In this case, interactions from microwaves, acidic pH, and increased temperature are compounding to increase fucose extractability.

Overall, the sulfate content (Fig. 3.4c) of the extracts underwent a small increase when extractions were performed at higher temperatures. The use of pH 4 and 7 resulted in maximum sulfate yields of 19.11 ± 0.53 and 18.40 ± 1.85 mg SO_4^{2-}/ g FD *M. pyrifera*, respectively, while pH 10 reached a maximum sulfate yield of 22.17 ± 1.99 mg SO_4^{2-}/ g FD *M. pyrifera* at 80 °C. Higher sulfate yields at elevated temperatures are likely the result of increased fucoidan extraction, which could, in turn, lead to a higher sulfate content in the extracts. These results show the benefits of enhanced extractability provided by the higher temperature's ability to lower viscosity and enhance diffusion, in agreement with the findings of Yuan and Macquerrie, who reported 16.08% fucoidan extractability (18.19 mg fucose/ g dry seaweed and 23.65 mg sulfate/ g dry seaweed) from *A. nodosum* at 120 °C, 15 min, 1:17.67 BWR, when evaluating the impact of temperatures varying from 90–150 °C (18). Similarly, Sasaki et al. found highest fucoidan yields of 12.3%, with extracts containing 40.22 mg fucose/ g dry seaweed and 25.71 mg sulfate/ g dry seaweed, when extractions were performed at 150 °C, for 30 min, at 1:66.67 BWR. However, yields decreased when testing temperatures exceeding this range (61), likely due to degradation of fucoidan above 160 °C (68). While is possible that testing higher temperatures could have revealed a different optimum temperature for fucoidan yields, this project focused on a holistic view of compound extraction from *M. pyrifera*, and temperatures above 80 °C were not tested to avoid potential degradation of temperature sensitive compounds such as phenolics.

Laminarin extractability (Figure 3.4d) exhibited a remarkable increase from 9.40 ± 0.66 up to 14.81 ± 1.07 mg laminarin Eq/ g FD *M. pyrifera* when the extraction temperature increased from 60 to 70 °C at pH 7. However, at pH 4 and 10, no significant increases were observed in

laminarin extractability under similar temperature conditions. Laminarin is often extracted using hot water and is soluble at any pH (69). In general, improvements in laminarin extractability have been correlated with higher temperatures (9). As mentioned previously, it is difficult to compare these results to the existing literature due to the lack of studies reporting the impact of MAE on laminarin extractability. Nevertheless, it appears that at neutral pH and elevated temperature, laminarin solubility is further enhanced when compared to acidic and alkaline conditions.

Overall, alginate extractability (Figure 3.4e) was favored by the use of higher temperatures (80 °C), with yields ranging from 71.24 ± 9.55 to 90 ± 10.61 mg FD calcium alginate/ g FD *M. pyrifera* across pH 4, 7, and 10. These results are within the range, albeit near the lower end, of the ones achieved by the AEP at 6 h, 1:30 BWR, 60 °C, where alginate yields ranging from 63.5 to 159 mg FD calcium alginate/ g FD *M. pyrifera* were achieved. This demonstrates the ability of microwaves and higher temperatures to increase yields, allowing for comparable results at a fraction of the time. Our results demonstrate that alginate extractability is enhanced at alkaline conditions, or at neutral conditions at higher temperatures, in agreement with the findings reported in the literature, which reported higher yields at alkaline conditions (70). As mentioned previously, it is important to remember that in the calcium chloride precipitation of alginate, additional compounds, including proteins, may have precipitated along with the alginate (31). At pH 10, where TPE is higher, it is likely that some proteins may have precipitated along with the alginate leading to an overestimation of alginate content compared to that of other pH values.

The enhanced alginate extractability at 80 °C observed in this study differs from the findings of Torabi et al. who determined the optimum temperature to be 67 °C (1:29 BWR, 19 min, 400 Wm pH 1) for sodium alginate extraction when testing temperatures from 45 to 75 °C for *N. zanardini*, resulting in 31% extraction yield (58). These variations could stem from inherent

differences among species or be attributed to the highly acidic extraction pH used in their study (pH 1.0), as alkaline conditions have been tied to increased alginate solubility (56). Protein extractability did not increase with statistical significance with increasing temperature, except for MAE conducted at pH 4 and 80 °C (31.59 ± 1.18% TPE) (Fig. 3.4f). While pH 4.0 is near the protein isoelectric point, the synergistic combination of acidic conditions, enabling the breaking of glycosidic bonds, and elevated temperatures could have collectively played a role in disrupting the cellular matrix, allowing for the increased release of intracellular proteins.

Sasaki et al. reported higher protein extractability from *U. pinnatifida* (30.7%) at higher temperatures (120 °C, 1:25 BWR, no pH adjustment) (61), illustrating the benefits of higher temperatures on protein extractability, as the trends in Figure 3.4g suggest.

Because phenolic, fucose, sulfate (except for pH 10), laminarin, and protein (except for pH 4) contents all reached maximum values at 70 °C, this temperature was selected as the most desirable temperature and was used for further in-depth analysis across the pH values. While alginate extractability was not maximized at 70 °C, avoiding the use of higher temperatures such as 80 °C helps preserve sensitive phenolics and saves energy by requiring less heating.

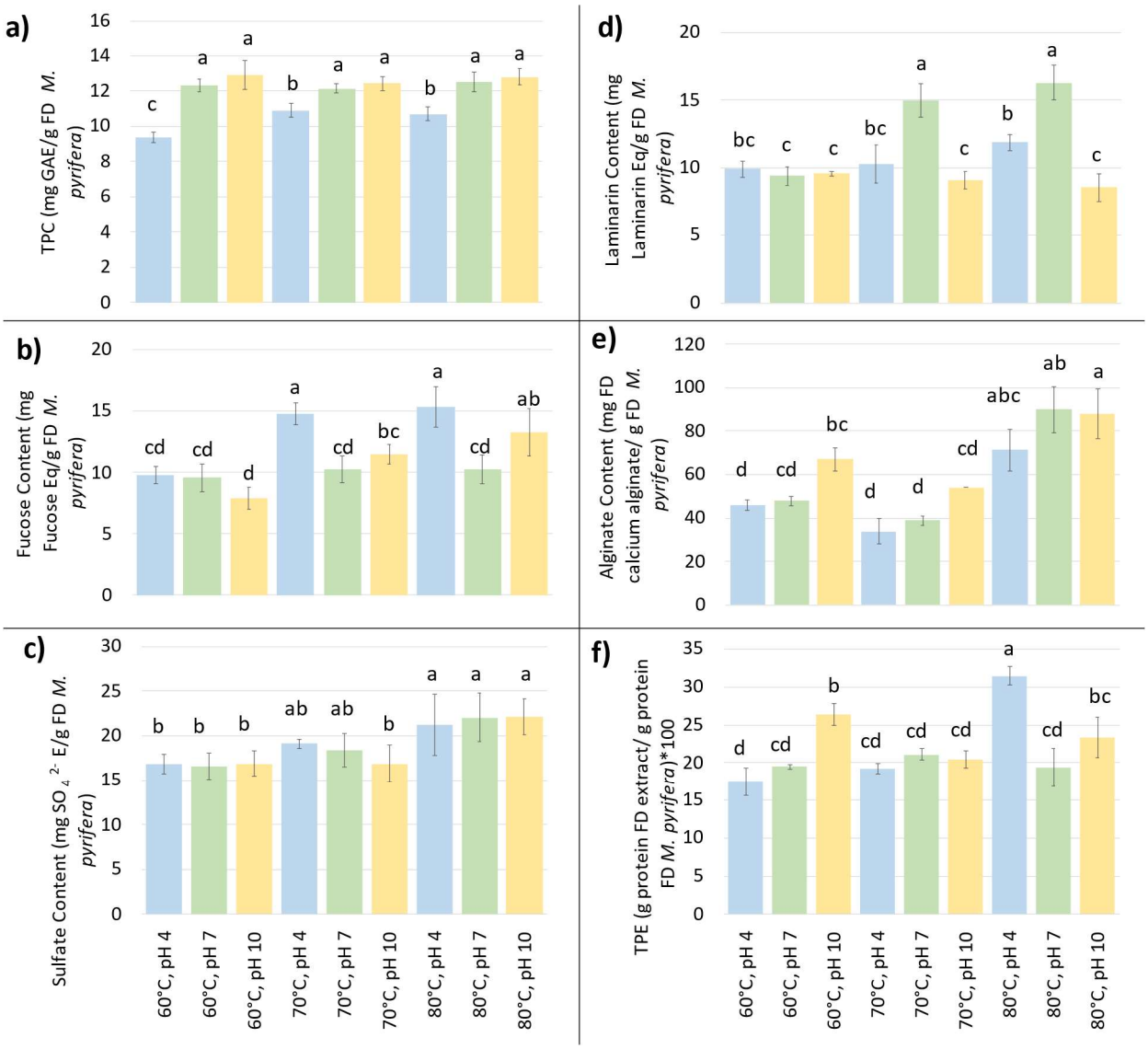


Figure 3. 4: Impact of temperature (60, 70, and 80 °C) and pH (4, 7, and 10) at 15 min and 1:30 BWR on: a) Total phenolic content (TPC) via Folin-Ciocalteu assay, b) Fucose content via Dische and Shettles method, c) Sulfate content via barium-chloride assay, d) Laminarin content via reducing sugar assay, e) Alginate content by freeze-dried calcium alginate, and f) Total protein extractability (TPE) by Dumas combustion.

3.3.1.4 Impact of enzyme use and time on the extractability of MEAE

The last phase of the microwave optimization was to incorporate enzymes into the microwave-enzyme-assisted extractions (Figure 3.5). As described in the extraction processes above, CBL and NP were evaluated alone and then, once the optimum enzymatic extraction time

was determined, the sequential use of a CBL pretreatment (15 min) followed by NP (15 min) extraction was evaluated. Enzymatic extractions were performed at 60 °C due to the optimum temperature for the enzymes. Additionally, a sequential extraction with a pH shift from 4 to 7 was conducted at 70 °C with 15 min at pH 4 followed by 15 min at pH 7, mimicking the pH change in the carbohydrase pretreatment followed by protease extraction. Very few macroalgae MEAE processes have been reported in literature and, to the best of our knowledge, there are no MEAE examining the use of multiple enzymes or pH values in the same study, highlighting the importance of the current research to fill this knowledge gap.

When evaluating the individual impact of enzymes in the TPC of the extracts (Figure 3.5a), a maximum TPC value of 12.92 ± 0.61 mg GAE/ g FD *M. pyrifera* was achieved when 2.5% NP (w/w) was used to assist the extraction at 15 min. However, this value is similar to those achieved by MAE (without enzymes) (12.15 ± 0.27 mg GAE/ g FD *M. pyrifera* at pH 7, 70 °C, 15 min), indicating that the enzymes did not enhance TPC. Additionally, the sequential use of enzymes resulted in TPC of 11.64 ± 0.47 mg GAE/ g FD *M. pyrifera*, and the pH switch from 4 to 7 resulted in TPC of 11.68 ± 0.12 mg GAE/ g FD *M. pyrifera* (Fig. 3.5a), indicating that the enzymes are not leading to changes in TPC values. Consistent with the trend above described, MEAE results closely align with the maximum found for MAE at pH 10, 70 °C, 15 min (12.43 ± 0.40 mg GAE/ g FD *M. pyrifera*), demonstrating that alkaline conditions, by breaking ester bonds, can effectively enhance the release phenolics, being comparable to the role of enzymes in facilitating phenolic release. It is possible that the reduced time in MEAE might not have been sufficient for enzymes to break the bonds holding phenolics in the algal matrix. Comparison of our data with the literature is hindered by the scarcity of MAE for *M. pyrifera*. MEAE from the brown macroalgae *E. radiata* using 10% (v/w) Viscozyme at 50 °C, 30 min, pH 4.5 resulted in 46 mg GAE/ g dry extract (71).

While these numbers indicate the extraction of phenolics, they are not compared to a non-enzymatic extraction of phenolics from *E. radiata* hindering the ability to correlate the phenolic extractability to the addition of enzymes, or just as a result of the microwave processing alone.

Fucose content (Figure 3.5b) increased slightly with the addition of NP, reaching 11.38 ± 0.71 mg fucose Eq/ g FD *M. pyrifera* at 15 min compared to 10.27 ± 1.10 mg fucose Eq/ g FD *M. pyrifera* at MAE pH 7, and 70 °C. Fucose yields (Figure 3.5b) of 12.76 ± 1.55 and 15.75 ± 0.38 mg fucose Eq/ g FD *M. pyrifera* were achieved for the combined enzyme use and pH shift (without enzyme), respectively. These results show that the pH shift was more beneficial than the use of enzymes for increasing fucose yields. This outcome is likely due the addition of NP, an enzyme in powder form, which could have increased viscosity and hindered extractability. Additionally, the pH shift involved the use of higher temperature (60 vs. 70 °C), potentially contributing to a reduction in slurry viscosity. This is interesting when comparing with the AEP results reported in Chapter 2, where CBL exhibited a greater impact on fucose yields than NP. It is possible that the mechanism by which microwaves induce cellular disruption could potentially interfere with the action of the enzymes. Microwaves are likely disrupting the bonds between fucoidans and cellulose and hemicellulose prior to the enzymes' action, rendering the use of CBL unnecessary for the extraction process.

With respect to the sulfate content (Figure 3.5c), CBL did increase the sulfate content of the extracts, reaching 21.55 ± 1.65 mg SO_4^{2-}/ g FD *M. pyrifera* after just 15 min compared to 19.11 ± 0.53 mg SO_4^{2-}/ g FD *M. pyrifera* for MAE alone at pH 4, 15 min, and 70 °C. While the use of CBL did not aid in fucose release, it did appear to allow for the release of fucoidan with higher degrees of sulfation, and therefore higher bioactive potential. The sequential use of enzymes resulted in extracts with a sulfate content of 17.69 ± 2.42 mg SO_4^{2-}/ g FD *M. pyrifera*, while the

pH shift resulted in extracts with 18.88 ± 1.89 mg SO_4^{2-}/ g FD *M. pyrifera*. Explaining the reduced results observed with the sequential enzyme use, compared to CBL alone is challenging. However, this discrepancy could potentially rise from the diminished solubility of fucoidan at a neutral pH. MEAE of *E. radiata* (Viscozyme 10% (v/w) at 50 °C, 30 min, pH 4.5) resulted in extracts containing 18 mg fucose/ g dry extract (71). Unfortunately, the above study did not compare microwave extraction without the use of enzymes, making it difficult to evaluate the role of enzyme use on extractability.

The impact of MEAE on laminarin extractability is shown in Figure 3.5d. When comparing the effectiveness of using CBL and NP alone, or in tandem, the use of 5% CBL at 15 min at pH 4 reached maximum yields of 16.10 ± 0.88 mg laminarin Eq/ g FD *M. pyrifera*, being comparable to the use of 2.5% NP for 30 min at pH 7, which resulted in yields of 14.39 ± 1.89 mg laminarin Eq/ g FD *M. pyrifera*. The sequential use of CBL (15 min, pH 4) and NP (15 min, pH 7) resulted in yields of 17.17 ± 2.49 mg laminarin Eq/ g FD *M. pyrifera*, not being significantly higher than the ones using CBL or NP alone. Although laminarin yields obtained with the use of CBL (16.10 ± 0.88 mg laminarin Eq/ g FD *M. pyrifera)* were greater than those achieved by MAE at pH 4 at 15 min, and 70 °C (10.29 ± 1.43 mg laminarin Eq/ g FD *M. pyrifera*), NP yields were similar to yields achieved by MAE at 15 min, pH 7, 70 °C (14.81 ± 1.07 mg laminarin Eq/ g FD *M. pyrifera*).

The lack of MEAE data for macroalgae in the literature makes comparing these results difficult. However, the same trend of increasing laminarin extractability with the addition of CBL was seen in Chapter 2, where the EAEP process with 5% CBL at pH 4 over 6 h resulted in significantly higher laminarin content (13.20 mg laminarin Eq/ g FD *M. pyrifera*) compared to the AEP alone at pH 4 over 6 h (11.00 mg laminarin/ g FD *M. pyrifera*). However, it is worth noting that the pH shift from 4 to 7 achieved similar laminarin yields (16.22 ± 1.24 mg laminarin Eq/g

FD) to the one using enzymes, demonstrating that pH shift had similar impact on laminarin extractability as using enzymes alone or in tandem.

Alginate content (Figure 3.5e), as with the EAEP experiments described in Chapter 2, was not affected by the addition of CBL and/or NP, with results ranging from 27 ± 2.12 to 66 ± 4.24 mg FD calcium alginate/ g FD *M. pyrifera* for the MEAE compared to 34 ± 6.06 to 70.5 ± 2.12 mg calcium alginate/ g FD *M. pyrifera* for the optimum selected MAE conditions and pH shift extractions without enzymes. This aligns with what we have seen previously where the addition of enzymes did not lead to an increase in calcium alginate content. However, these results cannot be compared with literature as there are no reports on microwave-enzyme assisted extractions for alginate.

Total protein extractability increased up to 28.85± 3.48% TPE with the addition of CBL after just 15 min, whereas the use of NP did not increase yields (Figure 3.5f). This is interesting considering the EAEP results presented in Chapter 2, where an increase in TPE was observed with the addition of NP, not CBL. Given the absence of pH maintenance during the MEAE process in comparison to the EAEP, is possible that the pH shift throughout the extraction process may have resulted in pH values further way from the protein isoelectric point, possibly contributing to the observed increased protein extractability. Additionally, in microwave processing involving enzyme combination, which involved a pH shift from 4 to 7, TPE reached the highest values among all tested conditions, reaching 33.93 ± 1.96%. The pH shift (no enzymes) experiments resulted in TPE of 19.56 ± 0.52%, falling in the range of the MAE extractions for pH 4, 7, and 10 at 70 °C, 15 min (19.21–21.11% TPE). The findings from both MEAE and EAEP results presented in Chapter 2 demonstrate the ability of enzymes to enhance breakdown of the algal matrix, resulting in greater extractability of proteins from within the brown macroalgae matrix, including the

intracellular proteins. To the best of our knowledge, there are no existing MEAE studies focusing on protein extractability from *M. pyrifera*, which limits the comparability of these results.

When comparing the impact of 15 and 30 min in the MEAE, the results discussed above indicate that 15 min was sufficient to reach yields not statistically different from 30 min, except for TPC. Additionally, CBL and NP enhanced the extractability of different compounds (sulfate, laminarin and protein with CBL and fucose with NP). Therefore, the integrated use of enzymes was deemed the most effective MEAE due to its ability to reach high yields for a wider range of compounds compared with the use of CBL or NP alone. Thus, to maximize the overall process extractability, the use of CBL for 15 min followed by NP for an additional 15 min was selected for the enzymatic approach. Extracts from the integrated enzyme use and pH switch, which led to similar extractability of many compounds, were both subjected to further analysis to identify potential differences in composition and bioactivity at the extracts.

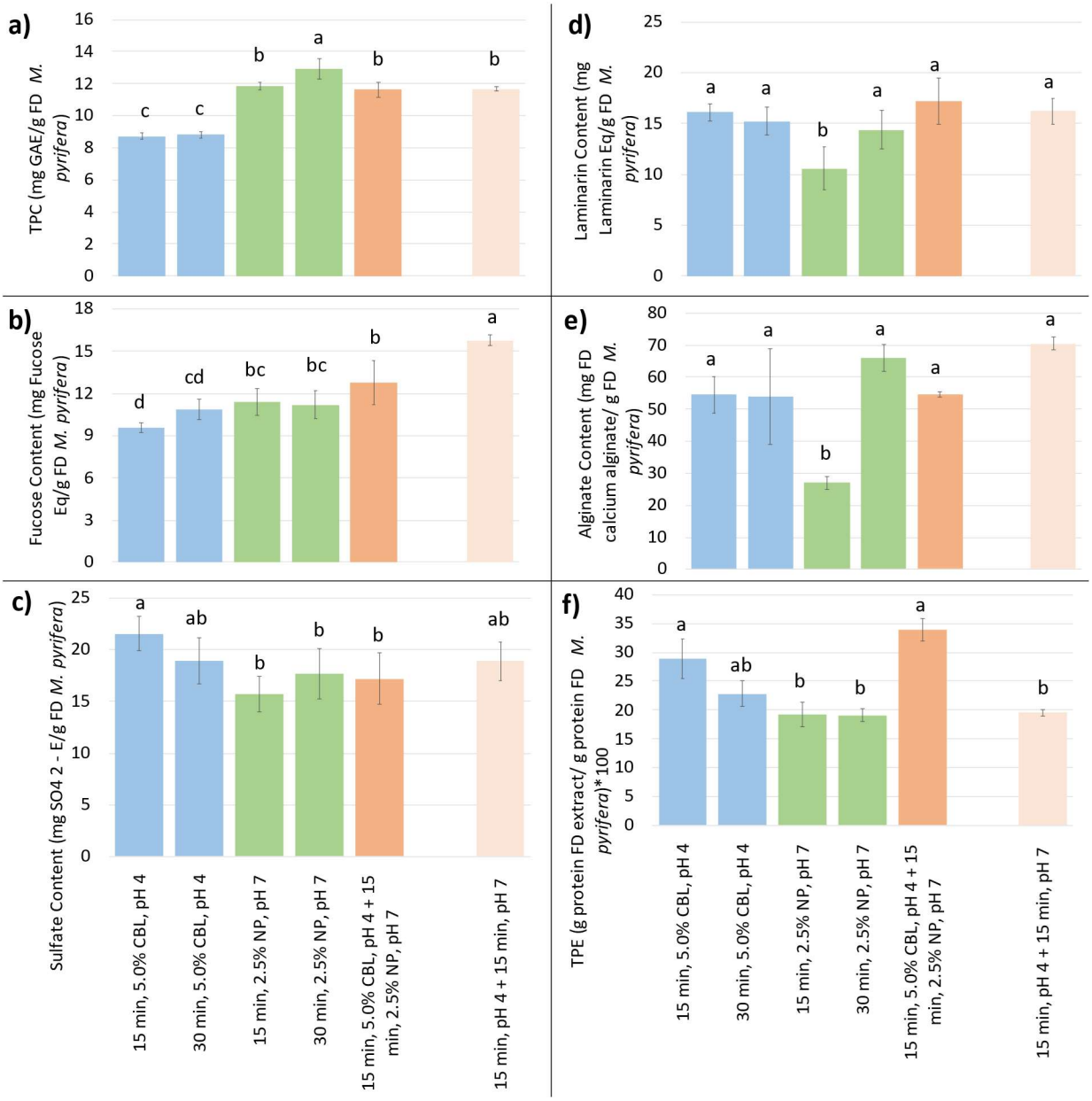


Figure 3.5: Impact of enzyme usage and pH change at 60 °C and 1:30 BWR on: a) Total phenolic content (TPC) via the Folin-Ciocalteu assay, b) Fucose content via the Dische and Shettles method, c) Sulfate content via the barium-chloride assay, d) Laminarin content via the reducing sugar assay, e) Alginate content of freeze-dried calcium alginate, and f) Total protein extractability (TPE) by the Dumas combustion method.

3.3.2 Selecting extracts from specific extraction conditions for comprehensive analytic characterization and determination of biological properties

Extracts from MAE and MEAE conditions that maximized yields of key components (phenolics, fucose, sulfate, laminarin, alginate, protein) and minimized resource use were selected for further, in-depth analysis (Table 3.1).

Table 3.1: Selection of extracts from optimum MAE and MEAE conditions for comprehensive analysis

Process	pH (enzyme)	Temperature	BWR	Total Time	Abbreviated title
MAE	pH 4	70 °C	1:30	15 min	MAE pH 4
MAE	pH 7	70 °C	1:30	15 min	MAE pH 7
MAE	pH 10	70 °C	1:30	15 min	MAE pH 10
MAE	pH 4 followed by pH 7	70 °C	1:30	30 min	MAE pH 4+7
MEAE	pH 4 followed by pH 7 (carbohydrase followed by protease)	60 °C	1:30	30 min	MEAE

3.3.2.1 Metabolomic profiling

Phenolic profiling and quantification were determined by untargeted metabolomics. As previously reported in Chapter 2, no phlorotannins and very few phenolic compounds were identified in the extracts from *M. pyrifera*. In addition to phenolic compounds, as with the AEP and EAEP results, this untargeted analysis confirmed the presence of peptides, lipid derivatives, and other metabolites. The number of identified metabolites is shown in Figure 3.6, reported as the number of unique identified metabolites in each category (phenols, peptides, lipid derivatives, and other metabolites). Apart from peptides, extraction conditions contained nearly identical amounts of each metabolite category (phenols, lipid derivatives, and other metabolites). When looking at the peptides, the MEAE resulted in the release of a greater number of peptides compared to the other conditions. Figure 3.7 shows the peak heights for the 10 most abundant peptides

illustrating how, in addition to having more identified peptides than other conditions, the MEAE also has many peptides released in much greater abundance. It is important to note that the untargeted metabolomics approach used has limitations when it comes to peptide identification. The results reported are of interest but are an underestimation of the peptides in the extracts as the library used for comparison does not contain all possible peptides and hydrophilic peptides could not be identified with the methods employed. To the best of our knowledge, this is the only metabolomic profiling describing the composition of the extracts obtained by MAE and MEAE of *M. pyrifera*. These results are similar to the ones presented in Chapter 2, where EAEP extracts had significantly more peptides than the AEP extracts.

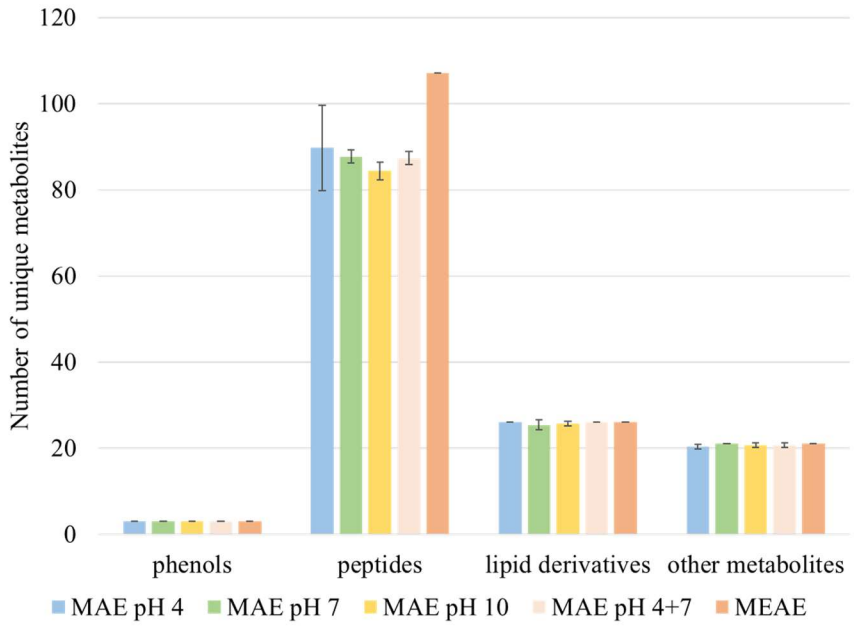


Figure 3.6: Impact of MAE and MEAE on the metabolomic profile of the extracts using a LC-MS untargeted metabolomic approach.

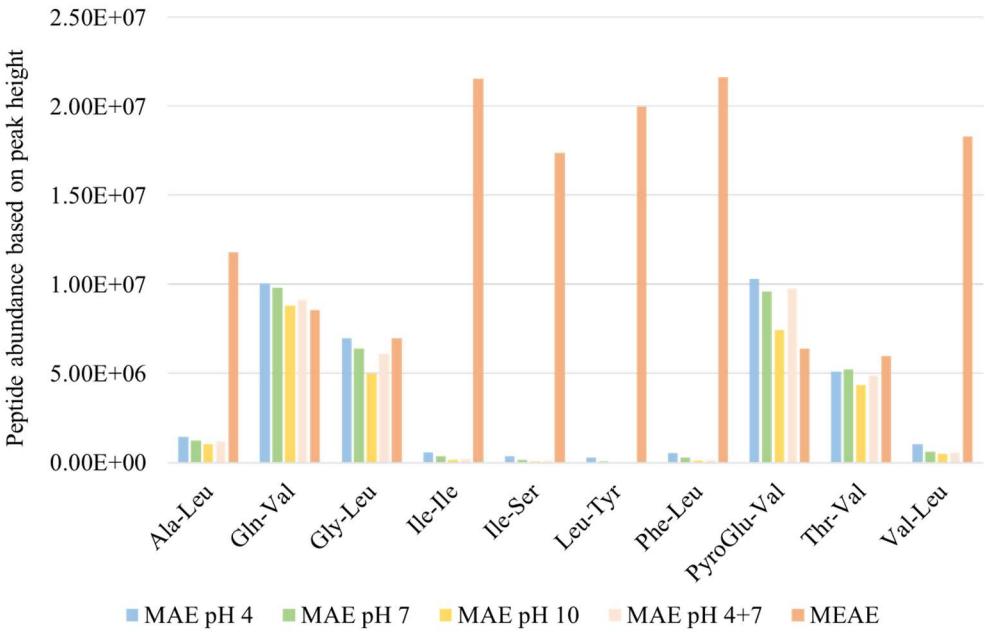


Figure 3.7: Impact of MAE and MEAE on the relative abundance of the 10 most prevalent peptides using a LC-MS untargeted metabolomic approach.

3.3.2.2 Monosaccharide quantification

Table 3.2 shows the results of monosaccharide quantification for fucose and galactose, the two most common monomers in fucoidan. Extracts generated by MAE at pH 4 and MAE with the pH shift from 4 to 7, had the highest concentrations of fucose (8.27 ± 0.14 and 8.32 ± 0.10 mg fucose /g freeze dried *M. pyrifera*) and galactose (4.27 ± 0.08 and 4.39 ± 0.11 mg galactose /g freeze dried *M. pyrifera*), respectively. These results highlight the ability of acidic conditions to cleave glycosidic bonds, aiding in the release of fucose (11,50, 51). Fucose and galactose yields for extracts generated by MAE at pH 7 and 10, and MEAE at pH 4 with 5% CBL followed by pH 7 with 2.5% NP very similar. It should be noted that the commercial enzymes used in the process contain notable quantities of carbohydrates and these carbohydrate amounts have been conservatively subtracted from the results reported, meaning that the actual MEAE yields presented may be higher than the values reported.

In comparison, MAE of *E. radiata* at 60 °C produced nearly 10 times as much fucose as galactose, with around 10 mg fucose/ g dry seaweed and 1.2 mg galactose/ g dry seaweed after a 20 min extraction with a 1:30 BWR (72). This difference is likely due to the high variability of fucoidan composition amoung species (8).

Table 3.2: Impact of extraction conditions on fucose and galactose yields obtained by HPAEC-PAD

Samples	mg fucose/ g FD *M. pyrifera*	mg galactose/ g FD *M. pyrifera*
MAE pH 4	8.27 ± 0.14	4.27 ± 0.08
MAE pH 7	6.66 ± 0.08	3.62 ± 0.01
MAE pH 10	6.35 ± 0.06	3.78 ± 0.05
MAE pH 4+7	8.32 ± 0.10	4.39 ± 0.11
MEAE	6.13 ± 0.07	4.12 ± 0.14

3.3.2.3 Oligosaccharide analysis

Oligosaccharide composition of selected extracts are shown in the heatmap below (Figure 3.8). Oligosaccharides are reported as a code along the y-axis, corresponding to the number of constituent monosaccharides. The number of identified oligosaccharides for the MAE samples is uniform across pH values, ranging from 37 to 41 identified oligosaccharides. When comparing these results to the AEP/EAEP results presented in Chapter 2, where the number of identified oligosaccharides ranged from 34 to 46, it becomes evident that changes in pH have a smaller impact on MAE compared to the AEP/EAEP. This indicates that MAE may be a more robust extraction process compared to traditional AEP/EAEP, an important consideration, especially for high throughput processing operations. For MEAE extracts, conservative estimates of the remaining compounds after enzyme subtraction (CBL and NP) are reported. Enzyme contributions could not be quantitatively subtracted from the total amount detected. Thus, identified compounds were fully removed from EAEP extracts if they were also detected in the enzyme blank. As such,

true values of the number of identified oligosaccharides for EAEP samples are likely higher than the ones reported.

Of the 60 oligosaccharides that remain after enzyme blank subtraction, 16 were identified as potentially containing fucose. Full identification of the oligosaccharides was not possible due to lack of cohesive fragmentation data. The neutral masses of these compounds are shown along the y-axis in Figure 3.8. It is likely that these unknowns contain fucose units that are linked to other molecules aside from the five monosaccharides (fucose, galactose, glucose, xylose, and mannose) used in the data search. Due to the novelty of this research, there are no similar findings in the existing literature, which hinders the comparison of the results with the existing literature.

Among all oligosaccharides, a fucose containing compound with neutral mass of 473.223 Da and its dimerized form (neutral mass 946.446 Da) were detected in much greater concentration compared to other identified structures. These structures were found in high abundance in extracts from all conditions, but with the greatest abundance in extracts from pH 4 and 7. Other abundant oligosaccharides include 0_0_3_0_0 (an oligosaccharide consisting of 3 fucose units, likely a fucoidan fragment) and 0_0_3_0_0 dimer, which were detected among all extraction conditions with comparable abundances. The 0_0_3_0_0 dimer is in lower concentration than reported for the AEP extracts described in Chapter 2. Additionally, in contrast to AEP and EAEP findings reported in Chapter 2, fucose containing oligosaccharide compounds with neutral mass of 514.214 Da and 711.257 were identified in higher abundance for MAE extracts at pH 4 compared to other conditions. MEAE extracts also contained several fucose containing compounds not detected in the non-enzymatic extractions and had a structure with neutral mass 527.231 Da found in relatively high abundance, indicating that the enzymes enhanced the breakdown of the algal matrix.

It is expected that fucoidan would contain primarily sulfated oligosaccharides with many fucose subunits (73). However, none were detected through the methods described here, likely due to the sulfate groups preventing the production of comprehensive fragmentation data that can be used to deduce the structure of the parent molecule. Therefore, it is likely that all features reported in Figure 3.8 as “containing fucose” are unsulfated oligosaccharides.

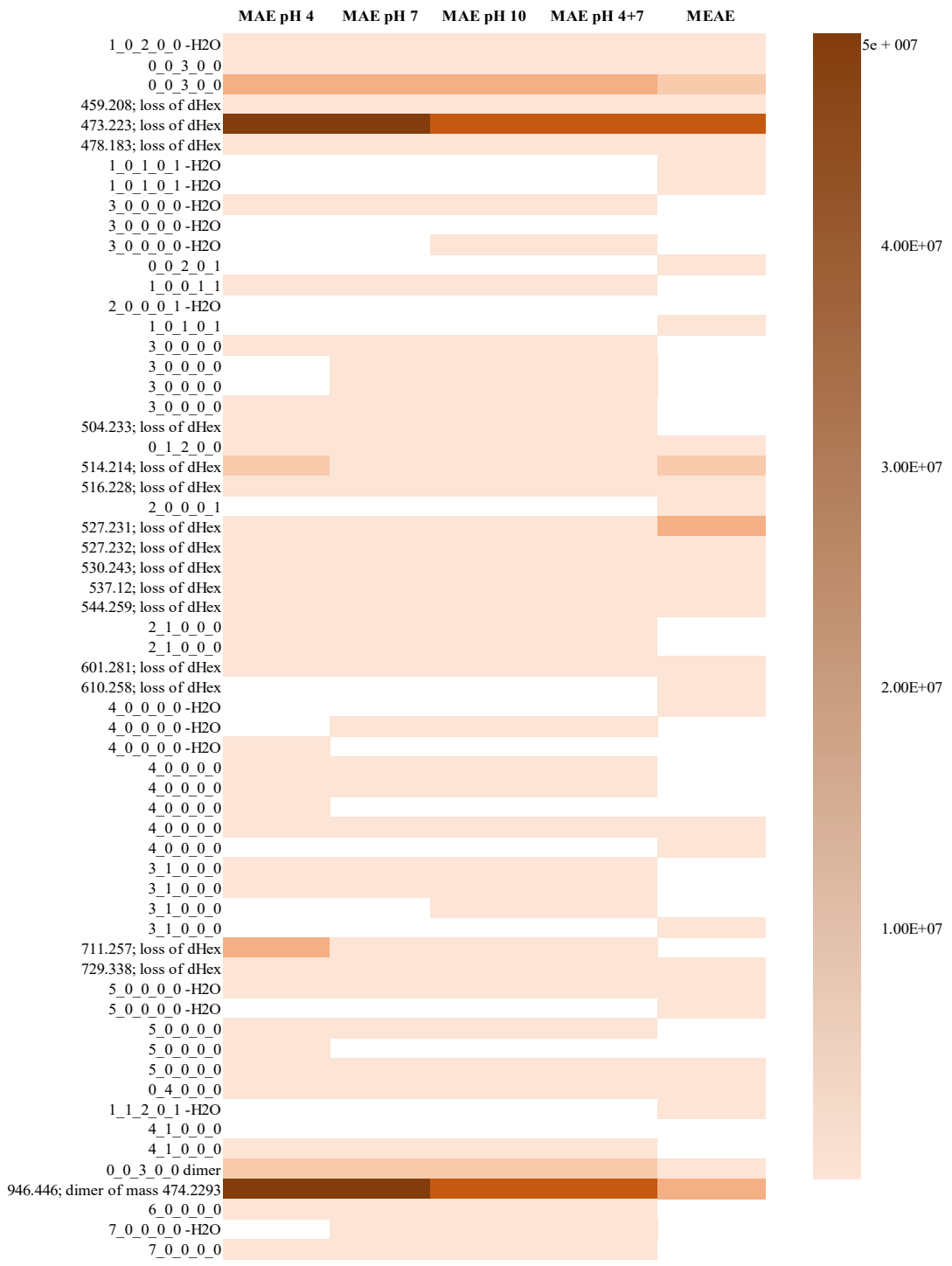


Figure 3.8: Glycoprofile results obtained by nanoLC-QToF following enzyme blank subtraction for all extraction conditions. Identified oligosaccharides are reported by a 5-digit code, corresponding to the number of constituent monosaccharides shown along the y-axis. Unknown oligosaccharides containing fucose are reported by their neutral mass.

3.3.2.4 Characterization of sodium alginate structure by FTIR

FTIR spectra of sodium alginate for the selected extraction conditions is shown in Figure 3.9.

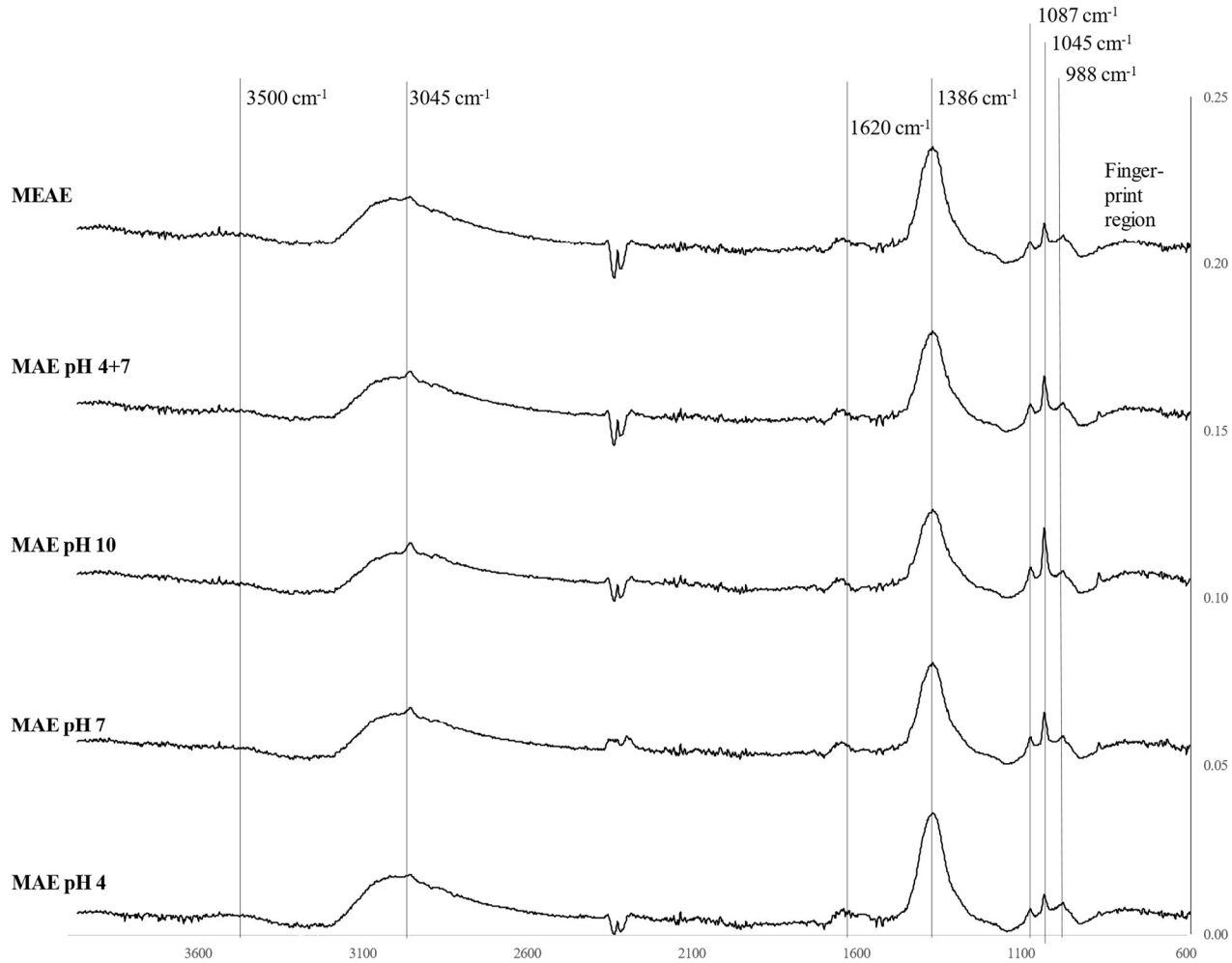


Figure 3.9: Impact of extraction conditions on FTIR spectra of sodium alginate.

Overall, the MAE and MEAE spectra shown in Figure 3.9 and the AEP and EAEP sodium alginate spectra reported in Chapter 2 are very similar. The peaks around 3500 cm^{-1} relate to O-H stretching vibrations (31,58,74) and the band centered at 3045 cm^{-1} has been assigned to C-H stretching vibrations of uronic acids (31). The small band around 1620 cm^{-1} corresponds to asymmetric stretching of carboxylate vibrations (58,74). The peak at 1386 cm^{-1} can be attributed

to the symmetric carboxylate group stretching vibration (COO−) of the mannuronate and guluronate moieties (14,31,58,75). The peaks at 1088 cm^{-1} and 1045 cm^{-1} indicate C-O stretching vibration of mannuronic acids and C-O or C-C stretching of pyranose rings of guluronic acids, respectively (14,31). The guluronic acid peak is more pronounced in the MAE extract at pH 7 and 10, and MAE extract at pH 4+7, indicating a lower mannuronic to guluronic acid (M/G) ratio in these extracts. Using these peaks' heights to estimate the M/G ratio, the ratios are shown in Table 3.3. The lower the M/G ratio, the more rigid is the gel formed by the alginate. Conversely, the higher the M/G ratio, the more flexible is the gel formed by the alginate (14). Alginate extracted by MAE at pH 10 presented the lowest ratio of 0.46 while alginate extracted by MAE at pH 4.0 had a ratio of 0.66. As shown in chapter 2, the M/G ratio for MAE and MEAE extracts were all below 1, indicating the potential formation of more rigid, stronger gels.

Overall, the sodium alginate extracted by microwave processing presented in this study had more guluronic acid than the sodium alginate extracted (0.1 M HCl at 90 °C, 15 min, 1:66.67 BWR) from *A. nodosum*, with a M/G ratio of 1.5 (76), indicating the potential of forming more flexible gels than those of *M. pyrifera* extracts.

Table 3.3: Impact of extraction conditions on sodium alginate M/G ratio by FTIR

Condition	Ratio
MAE pH 4	0.66^{a}
MAE pH 7	0.52^{ab}
MAE pH 10	0.46^{b}
MAE pH 4+7	0.59^{ab}
MEAE	0.66^{a}

3.3.2.5 Laminarin quantification by β-glucan analysis

β-glucan content of the extracts (mg β-glucan/ g of FD extract) are shown in Table 3.4 and were used as an alternative to the laminarin assay results reported in the MAE stepwise optimization results and discussion sections. This test has higher specificity than the one used

previously. Laminarin are made of linear β-1-3-glucose units with β-1-6-linkages which control their solubility (1). This β-glucan assay uses enzymes that can cleave the laminarin molecules at their 1-6 and 1-3 glycosidic bonds and provides the results as β-glucan content.

Results of β-glucan quantification, for identification of conditions resulting in greater laminarin extractability, are shown in Table 3.4. Extractability was significantly higher with the use of enzymes. MEAE extracted 5.3 ± 1.06 mg β-glucan/ g of FD extract compared to 0.12 ± 0.02 to 0.86 ± 0.10 mg β-glucan/ g of FD extract for the non-enzymatic extracts. As described in chapter 2, these results show the ability of the enzymes to aid in breakdown of the cellular matrix allowing for release of the storage molecules within the algal cells (9). It should be noted that the carbohydrates present in the commercial enzymes have not been subtracted from the results reported in table 3.4, meaning that MEAE yields, while likely still higher than those found in the other conditions, may be lower than shown. It is difficult to compare these results to the current literature, as there is a lack of microwave extractions with focus on laminarin extractability. Although other studies have used similar methods to characterize their starting material (10), there is a lack of laminarin quantification of extracts based upon the β-glucan content.

Table 3.4: Impact of extraction conditions on the β-glucan content of extracts obtained by MAE and MEAE processing

Condition	β-glucan (mg β-glucan/ g FD extract)
MAE pH 4	0.12 ± 0.02^{b}
MAE pH 7	0.30 ± 0.05^{b}
MAE pH 10	0.16 ± 0.02^{b}
MAE pH 4+7	0.86 ± 0.10^{b}
MEAE	5.30 ± 1.06^{a}

3.3.2.6 Amino acid profile

The amino acid (AA) profile in Table 3.5 show that *M. pyrifera* microwave extracts contain all nine essential amino acids. The first column reports the guidelines from the Food and

Agriculture Organization of the UN (FAO) for a complete protein based upon the individual amino acid mass contribution to the total protein mass (77). While the extracts do not meet the essential amino acid values in the ratios specified by the FAO, an optimized extraction process targeting protein from *M. pyrifera* has the potential to meet these requirements. As described in Chapter 2, the AA profile of enzymatic extracts approaches the FAO requirements for a complete protein, likely due to the enhanced breakdown of the cellular matrix by enzymes, which led to the release of proteins previously trapped. Literature focusing on the AA profile of microwave extracts of *M. pyrifera* is lacking. The information provided here is crucial for achieving a comprehensive understanding of how extraction conditions influence the composition and quality of the extracts.

Table 3.5: Amino acids composition of MAE and MEAE extracts (g amino acid/100 g total protein)

Amino Acid	FAO guide	MAE pH 4	MAE pH 7	MAE pH 10	MAE pH 4+7	MEAE pH 4+7
Asx		10.17 ± 0.13	10.27 ± 0.15	10.36 ± 0.06	10.35 ± 0.06	11.84 ± 0.13
Thr	2.3	4.31 ± 0.08	4.41 ± 0.10	4.64 ± 0.04	4.38 ± 0.03	5.85 ± 0.07
Ser		3.53 ± 0.05	3.70 ± 0.09	3.99 ± 0.03	3.66 ± 0.04	5.12 ± 0.07
Glx		32.61 ± 0.77	28.68 ± 0.76	25.61 ± 0.60	31.95 ± 0.13	22.51 ± 0.66
Pro		2.40 ± 0.21	2.85 ± 0.43	3.47 ± 0.55	2.94 ± 0.09	3.56 ± 0.01
Gly		3.91 ± 0.05	4.16 ± 0.12	4.52 ± 0.04	4.25 ± 0.08	4.86 ± 0.05
Ala		22.27 ± 0.31	19.01 ± 0.70	16.38 ± 0.50	21.16 ± 0.06	14.15 ± 0.51
Val	3.9	3.19 ± 0.09	3.86 ± 0.15	4.40 ± 0.06	3.38 ± 0.02	4.64 ± 0.05
Ile	3	1.72 ± 0.05	2.32 ± 0.14	2.80 ± 0.05	1.83 ± 0.02	2.85 ± 0.06
Leu	5.9	2.29 ± 0.09	3.60 ± 0.27	4.61 ± 0.08	2.50 ± 0.03	4.93 ± 0.12
Tyr	3.8 (total Tyr + Phe)	1.88 ± 0.06	2.30 ± 0.10	2.63 ± 0.01	1.87 ± 0.03	3.88 ± 0.13
Phe		1.58 ± 0.08	2.32 ± 0.15	2.90 ± 0.04	1.71 ± 0.03	3.18 ± 0.10
His	1.5	0.79 ± 0.03	1.09 ± 0.06	1.34 ± 0.03	0.88 ± 0.01	1.30 ± 0.06
Lys	4.5	2.63 ± 0.40	3.20 ± 0.15	3.21 ± 0.07	2.42 ± 0.04	3.85 ± 0.13
Arg		1.92 ± 0.07	2.93 ± 0.18	3.75 ± 0.10	2.19 ± 0.01	3.21 ± 0.09
Taurine		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cys acid	0.6	2.29 ± 0.05	2.33 ± 0.07	2.25 ± 0.27	2.38 ± 0.08	1.96 ± 0.04
Met sulfone	1.6	0.69 ± 0.03	0.89 ± 0.06	1.11 ± 0.03	0.65 ± 0.03	0.91 ± 0.03
Trp	0.6	1.84 ± 0.71	2.07 ± 0.25	1.99 ± 0.16	1.49 ± 0.05	1.38 ± 0.20

Abbreviations: Methionine (Met), Tryptophan (Trp), Asparagine (Asx), Threonine (Thr), Serine (Ser), Glutamine (Glx), Proline (Pro), Glycine (Gly), Alanine (Ala), Valine (Val), Isoleucine (Ile), Leucine (Leu), Tyrosine (Tyr), Phenylalanine (Phe), Histidine (His), Lysine (Lys), Arginine (Arg), Cystine (Cys). FAO guidelines are based upon the individual amino acid mass contribution to the total protein mass (77).

3.3.2.7 Antioxidant activity

ABTS and ORAC antioxidant activities are reported in μmol Trolox Equivalent per gram of freeze-dried extract and are shown in Figure 3.10a and 3.10b, respectively. Similar to the

findings observed in Chapter 2 with AEP and EAEP, ABTS activities of MAE and MEAE extracts were similar across extraction conditions, ranging from 48.02 ± 0.14 to 48.47 ± 0.39 μmol Trolox Eq/ per g FD extract. ORAC antioxidant capacity, which indicates oxygen radical absorbance capacity, shows that the maximum antioxidant capacity observed for the MEAE extracts (210.67 ± 43.81 μmol TE/ g FD extract) was not statically different from the antioxidant activity of MAE extracts obtained at pH 4, 7, & 10, demonstrating the ability of the microwave processing to produce extracts with high bioactivity even without the addition of enzymes.

In comparison, the study by Yuan et al. focusing on MAE of phenolics from four species of brown macroalgae (70% methanol, 110 °C, 15 min, 1:10 BWR) found between 0.45 (*L. japonica*) and 0.98 (*A. nodosum*) mg Trolox Eq/ gram of dry seaweed for ABTS scavenging activity (24). The results, while not from an aqueous-based extraction method and lower than those detected for the current study with *M. pyrifiera,* highlight how differences among species can result in varying bioactivities.

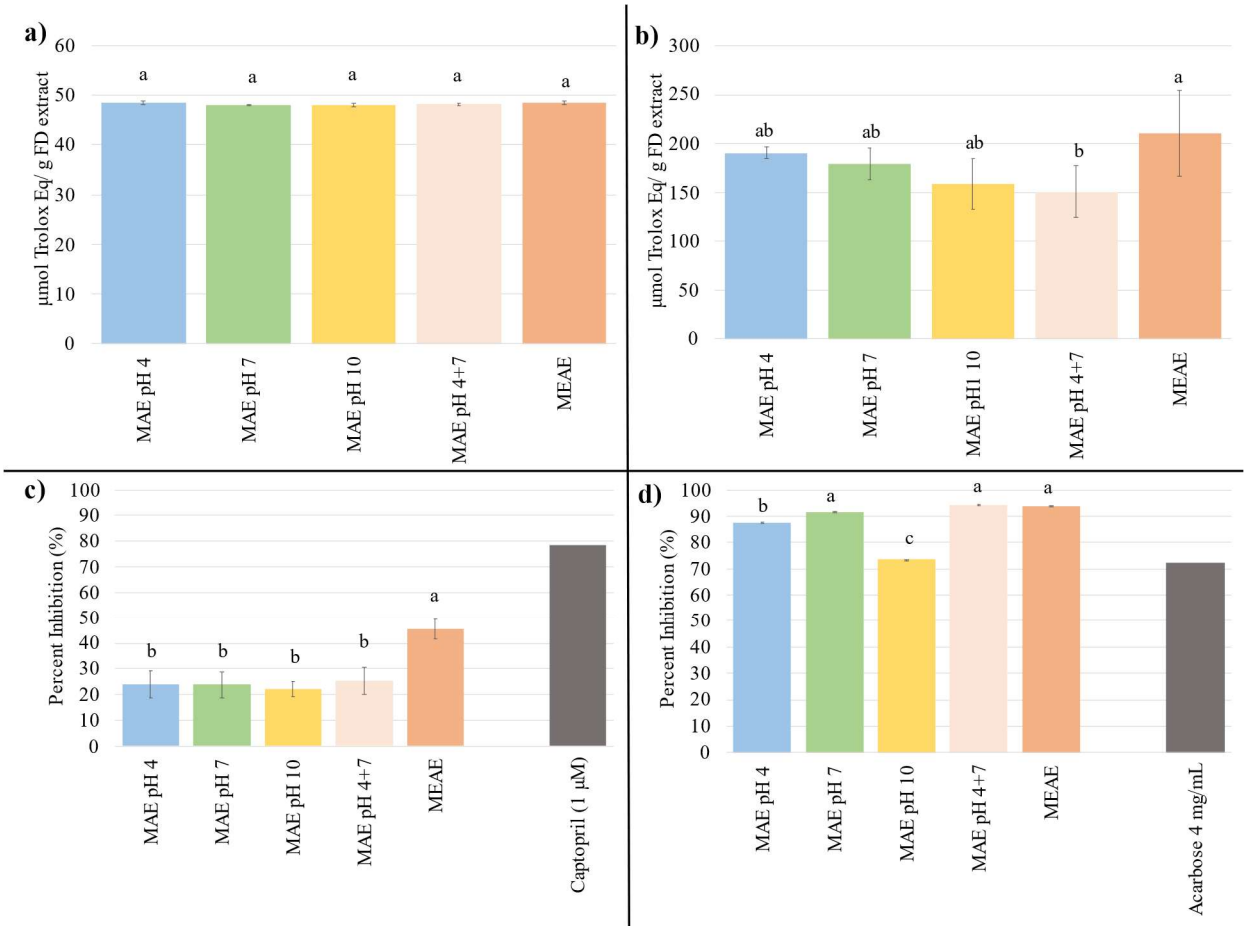


Figure 3.10: Impact of extraction conditions on radical cation scavenging (ABTS) activity (a), oxygen radical absorbance capacity (ORAC) (b), ACE inhibition (c), and α- glucosidase inhibition (d) of selected extracts.

3.3.2.8 Angiotensin-converting enzyme (ACE) inhibitory activity

The ability of *M. pyrifera* extracts to inhibit ACE, which can be exploited as a strategy to relieve arterial hypertension, is shown in Figure 3.10c. Results are shown as percentage inhibition for a 10 mg FD extract/ per mL of solution. Highest inhibition (45.70 ± 3.90%) was observed for the MEAE extracts. As discussed in the metabolomic profiling section previously, MEAE resulted in the greatest release of peptides amongst the extraction conditions evaluated, which is of great importance since ACE inhibition is tied to the presence of bioactive peptides (80).

Like in Chapter 2, and in agreement with previous studies, inhibition results are lower than those exhibited by the commercial drug used to manage hypertension, captopril (78,81). This research demonstrates the potential use of these extracts as a functional food to aid in ACE inhibition and the opportunity for further optimization to maximize this inhibitory activity. In comparison, MAE extraction (100 °C, 20 min, 1:66.67 BWR) of the brown macroalgae *U. pinnatifida* achieved 50% inhibition with 0.03 mg extract/mL (61), much higher than the results found for MAE extracts from *M. pyriferia*. These differences may be tied to differences among peptides from species (different species have been shown to have different properties (24,82)), and differences in extraction procedure (the high temperature used by Sasaki et al. may have led to the greater release of bioactive peptides).

3.3.2.9 α-glucosidase inhibitory activity

Type 2 diabetes is a massive global health problem defined by failure of the body to effectively control blood glucose levels as a result of impaired insulin secretion (83). α-glucosidase inhibition is one way to manage diabetes and the inhibitory activity of the extracts is shown in Figure 3.10d as percent inhibition for a 0.125 mg FD extract/ mL of solution. MAE at pH 7 and pH 4+7 and MEAE processes all resulted in extracts with high inhibition, with MEAE resulting in the highest inhibition at 94.28 ± 0.66% inhibition. As observed for AEP/EAEP experiments in Chapter 2, the inhibition displayed by these extracts is much higher than that of the market-available drug to manage diabetes, acarbose. A 4 mg/mL solution (comparable to a 0.125 mg/mL solution of the extracts) of acarbose resulted in around 72.27 ± 0.71% inhibition, demonstrating the potential of these extracts as a therapy for diabetes. When comparing these results to the ones presented in Chapter 2, extracts inhibition increased with microwave processing. The most

dramatic increase was observed at pH 4, where inhibition went from 67% for the AEP, at pH 4, and 6 h, up to 88% for MAE extracts obtained at pH 4, and 15 min, demonstrating the potential of microwave processing to produce extracts with higher bioactivity compared to traditional aqueous processing.

Previous studies have found antidiabetic potential in *Macrocystis pyrifera* (24,84). Antidiabetic effects can be tied to algal polysaccharides, like fucoidans and laminarin, as well as bioactive peptides (24,84). A study by Yuan et al. (24), focusing on MAE (70% methanol, 110 °C, 15 min, 1:10 BWR) of phenolics from four species of brown macroalgae showed widely varying α-glucosidase inhibition, based on 10 mg of extract/mL, for extracts generated by MAE and their respective solvent extracted controls (70% methanol, 4 h, 1:10 BWR, room temp). They reported extracts from *L. nigrecens* showing 0% inhibition (for both MAE and control), *A. nodosum* extracts demonstrating around 85% inhibition (compared to 75% for the control), and *L. trabedulate* extracts exhibiting 100% inhibition (for both MAE and the control). These findings highlight the differences for antidiabetic potential and the effects of microwave processing on antidiabetic activity among species.

3.4 Conclusions

The successful design of sustainable and fast microwave-assisted and microwave-enzyme-assisted aqueous extraction processes has resulted in high yields of a variety of functional bioactive compounds (fucoidan, alginate, laminarin, proteins, phenolics) from *Macrocystis pyrifera*. In comparison to the aqueous process discussed in Chapter 2, extraction times in MAE decreased from 6 h down to 15 min, while still achieving comparable yields for most compounds. The dipole interactions introduced by microwave-processing were effective in breaking apart the algal matrix,

allowing for uniform extraction of many compounds, regardless of the extraction pH, as shown by the oligosaccharide and metabolomic profiling. The addition of enzymes during MAE allowed for increased extractability of some compounds, including proteins and laminarin. Furthermore, the resulting MEAE extracts demonstrated high bioactivity (antioxidant, ACE inhibitory, and alpha-glucosidase inhibitory activities). Overall, this research plays a crucial role in bridging gaps in our understanding of the impact of sustainable downstream processing unit operations such as pH, extraction time, temperature, water to biomass ratio, and type of enzyme used, in the context of microwave-assisted aqueous processes for *Macrocystis pyrifera*. Future research should evaluate the economic feasibility of larger-scale microwave and microwave-enzyme processing to mass produce *Macrocystis pyrifera* compounds for introduction into a wide array of industries.

3.5 Supplementary materials

Table S3.1: Average peak heights of phenolic compounds in freeze-dried *M. pyrifera* extracts for selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (min)	Average peak heights for extraction conditions				
						MAE pH 4	MAE pH 7	MAE pH 10	MAE pH 4+7	MEAE
4-Hydroxyphenyllactic acid	[M-H]-	2	181.0508	neg	1.456	3361609	3386054	3049794	2961522	2826227
5,7-Dihydroxy-4-methylcoumarin-3-acetic acid	[M-H]-	2	249.0403	neg	0.309	473483.3	890863	276623.7	928325.7	334050.7
Homovanillic acid sulfate	[M-H]-	2	261.0076	neg	0.936	4675906	5077402	5318259	5137295	4221490

Table S3.3: Average peak heights of peptides in freeze-dried *M. pyrifera* extracts for selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (min)	Average peak heights for extraction conditions				
						MAE pH 4	MAE pH 7	MAE pH 10	MAE pH 4+7	MEAE
Ala-Ala	[M+H]+	2	161.0922	Pos	0.279	1854106.528	1599594.041	1302658.266	1535919.12	3942127.732
Ala-Ile	[M+H]+	2	203.1391	Pos	1.254	703659.3284	452466.7664	533140.1158	663011.3267	1060260.323

Ala-Leu	[M+H]+	2	203.1391	Pos	1.415	1450770.036	1249427.134	1012221.524	1166816.108	11807584.99
Ala-Leu-Ala-Leu	[M+H]+	2	387.2601	Pos	2.421		57301.66294	3212.400647		653262.9588
Ala-Met	[M-H]-	2	219.0808	Neg	0.565	45121	21098.33333	22457.66667	24135.66667	649371.6667
Ala-Phe	[M+H]+	2	237.1233	Pos	1.671	448409.614	341195.5279	271311.1463	321889.3758	4819363.902
Ala-Pro	[M+H]+	2	187.1077	Pos	0.435	1989452.661	1690272.806	1470301.997	1617856.443	1898942.102
Ala-Trp	[M+H]+	2	276.1343	Pos	1.906	2145.42037				373029.6622
Ala-Tyr	[M+H]+	2	253.1187	Pos	0.658	138132.6059	54160.76346	37229.45304	42281.41989	3555481.413
Ala-Val	[M+H]+	2	189.1234	Pos	0.508	1978458.567	1990571.711	1657681.638	1905686.865	4224747.594
Arg-Leu	[M+H]+	2	288.2034	Pos	0.851	9968.1884	13614.5269	12767.41145		75944.51576
Asn-Ile	[M+H]+	2	246.1452	Pos	1.174		126233.9589			139512.8438
Asn-Leu	[M+H]+	2	246.1449	Pos	1.329	728908.4853	664549.4528	554510.7982	662333.3845	1059765.248
Asn-Phe	[M+H]+	2	280.1292	Pos	1.583	96426.9794	78459.54116	59627.22816	74524.19432	707412.3316
Asn-Tyr	[M+H]+	2	296.124	Pos	0.653					593080.3505
Asn-Val	[M+H]+	2	232.1295	Pos	0.487	1932157.769	1968599.297	1660438.872	1784603.218	1721787.108
Asp-Leu	[M+H]+	2	247.1287	Pos	1.402	1167214.265	1081526.833	874217.7791	1074586.526	1278615.607
Asp-Tyr	[M-H]-	2	295.0933	Neg	0.698	16449	2475.333333	4295.5		584667
gamma-glutamyl-alanine	[M-H-H2O]-	2	199.0723	Neg	0.481	35295489.33	35221921.33	33766268.67	34762011.33	28562585.33
Gln-Ala	[M-H]-	2	216.0992	Neg	0.277	1353352.667	1420821.667	1581308.333	1311708	1271513.333
Gln-Phe	[M-H]-	2	292.1304	Neg	1.597	198533	206100	228156.3333	181593	348596.6667
Gln-Val	[M+H]+	2	246.1452	Pos	0.505	10052925.91	9795919.807	8831843.703	9157478.725	8569944.095
Glu-Ala	[M+H]+	2	219.0977	Pos	0.284	2261185.212	2016948.177	1382837.068	1488430.28	1788788.493
Glu-Ile	[M+H]+	2	261.1447	Pos	1.324	1411973.502	1253188.957	985185.126	1138455.098	989026.2958
Glu-Leu	[M+H]+	2	261.1444	Pos	1.464	1814573.739	1719220.431	1280054.051	1498636.257	1875605.734
Glu-Tyr	[M-H]-	2	309.1091	Neg	0.774	33619.33333	42788.33333	39171.33333	47971	236015.3333
Glutathione, oxidized	[M-H]-	2	611.145	Neg	0.437	699374.3333	708690.3333	399582.6667	572233.6667	574349.6667
Gly-Leu	[M+H]+	2	189.1234	Pos	1.423	6996946.96	6375378.822	4964417.37	6090998.14	6959215.354
Gly-Phe	[M+H]+	2	223.1077	Pos	1.621	673527.9245	563047.9744	469061.7956	603821.9414	2000256.894

Gly-Val	[M+H]+	2	175.1082	Pos	0.529	2724585.572	2669231.008	2129072.995	2395691.07	2691678.572
Ile-Ala	[M+H]+	2	203.1392	Pos	0.704	389162.1732	323088.3445	242912.1479	277404.9072	5699292.248
Ile-Asn	[M+H]+	2	246.1448	Pos	0.445	206475.4391	62778.13888	48094.72031	56444.28974	11712520.58
Ile-Glu	[M+H]+	2	261.1444	Pos	0.581	80704.5459	56555.43669	25705.36847	44800.20753	2080669.698
Ile-Ile	[M+H]+	2	245.1858	Pos	2.238	565180.1684	348713.5264	157159.8336	204039.2453	21548286.74
Ile-Leu	[M+H]+	2	245.1859	Pos	2.768					348258.1044
Ile-Met	[M+H]+	2	263.1423	Pos	1.737	77800.19437	36445.20653	19513.63563	26574.12	3650659.452
Ile-Ser	[M+H]+	2	219.134	Pos	0.475	343950.8078	159778.4946	59583.432551	83653.78351	17406866.11
Ile-Thr	[M+H]+	2	233.1496	Pos	0.494	312569.3249	194451.5611	161779.0154	148143.7927	7705911.222
Ile-Trp	[M+H]+	2	318.1813	Pos	2.401					418196.9336
Ile-Tyr	[M+H]+	2	295.1651	Pos	1.66	103241.3747	54901.06547	30899.7997	37019.31689	4142249.125
Ile-Val	[M+H]+	2	231.1703	Pos	1.559	472045.8598	407513.9615	309701.7344	334335.1602	7462770.108
Leu-Arg	[M+H]+	2	288.203	Pos	0.462	149405.8364			60350.67377	4769728.881
Leu-Asp	[M+H]+	2	247.1288	Pos	0.529	2410579.328	2303041.065	1688317.785	1803875.595	6044970.047
Leu-Gln	[M+H]+	2	260.1604	Pos	0.497	187533.9257	109458.6914	84211.52048	102758.2976	3573385.724
Leu-Gly	[M+H]+	2	189.1234	Pos	0.739	165482.7521	70286.46852	45287.87898	59570.4935	4203423.539
Leu-Gly-Leu	[M+H]+	2	302.2074	Pos	2.457	29014.6158	22093.61713	12761.10179	19955.97193	448621.0645
Leu-Gly-Lys	[M+H]+	2	317.2181	Pos	0.482				21277.05512	3082240.536
Leu-Leu-Lys	[M+2H]2+	2	187.1442	Pos	1.562	26655.76549	7403.49958	8585.62133	13864.87694	422218.8907
Leu-Lys	[M+H]+	2	260.1968	Pos	0.399					3177921.689
Leu-Phe	[M+H]+	2	279.1703	Pos	2.342	227063.0916	234588.2961	63886.08895	82867.99693	7308645.736
Leu-Pro	[M+H]+	2	229.1546	Pos	1.739	557246.6839	540976.6229	437988.3334	514031.7127	1311627.691
Leu-Thr-Lys	[M+H]+	2	361.2444	Pos	0.447					1708824.54
Leu-Trp	[M+H]+	2	318.1812	Pos	2.503					1255283.359
Leu-Tyr	[M+H]+	2	295.1652	Pos	1.741	266738.2908	68581.6265	22736.55076	39581.56009	19986087.67
Leu-Val	[M+H]+	2	231.1703	Pos	1.694	409267.3369	324479.574	282458.5133	328839.7608	5140597.213
Leucyl-leucine methyl ester	[M+H]+	2	259.2015	Pos	2.842	31938.59828			7496.508348	216647.336
Met-Ala	[M+H]+	2	221.0954	Pos	0.484	119683.9231		22163.93627	25187.6156	3052228.077
Met-Ile	[M+H]+	2	263.1424	Pos	2.017	100415.4924	44526.25159	24718.52088	36399.47276	3564705.861

Met-Met	[M-H]-	2	279.0841	Neg	1.544	3232.666667	27691.33333	76089.66667	23713.66667	211911.3333
Met-Phe	[M+H]+	2	297.1266	Pos	2.241	16870.57533	3840.740961	2024.015022	4940.790843	865609.6292
Met-Tyr	[M+H]+	2	313.1214	Pos	1.565	28301.94271				1257280.541
Met-Val	[M-H]-	2	247.1121	Neg	1.454	16503.5	3534.333333	2680.5	1754	573633
N-Acetyl-valine	[M-H]-	2	158.0822	Neg	1.612	120195.3333	118174	121283.3333	112655.3333	151139.3333
Phe-Ala	[M+H]+	2	237.1233	Pos	1.439	137821.3915	84653.99784	33698.29571	45249.9673	5643926.999
Phe-Asn	[M-H]-	2	278.1145	Neg	0.675			4294.5	3794	846242.3333
Phe-Asp	[M+H]+	2	281.1131	Pos	0.963	39898.22968	10180.98719	8754.850212	5097.535587	1832247.12
Phe-Gln	[M+H]+	2	294.1448	Pos	0.942					1021605.83
Phe-Glu	[M+H]+	2	295.1288	Pos	1.299	46764.24008	25619.2429	15952.96432	24190.41202	905199.9396
Phe-Gly	[M-H]-	2	221.0931	Neg	1.401	50058.33333	23342	22931.33333	20858.66667	1338010
Phe-Gly-Phe	[M+H]+	2	370.176	Pos	2.739					237182.3255
Phe-Ile	[M+H]+	2	279.1702	Pos	2.277	16581.88244	19204.37838	14534.08968	16098.37017	359503.2899
Phe-Leu	[M+H]+	2	279.1703	Pos	2.457	534938.058	261426.5442	118817.4736	133869.3905	21649436.87
Phe-Met	[M+H]+	2	297.1267	Pos	2.092		4183.735071			1619553.394
Phe-Phe	[M+H]+	2	313.1545	Pos	2.567	87312.73223	43549.51252	20887.729325	25033.98211	4131988.883
Phe-Ser	[M-H]-	2	251.1036	Neg	0.81	35253	6050	3582.333333	4159	2154599.333
Phe-Thr	[M-H]-	2	265.1193	Neg	0.983	78703.5	5886	3379	3343.333333	4274771
Phe-Trp	[M+H]+	2	352.1654	Pos	2.645					123326.4463
Phe-Tyr	[M+H]+	2	329.1494	Pos	1.957		9027.066042	2032.930193	2437.209141	2131353.059
Phe-Val	[M+H]+	2	265.1546	Pos	1.912	42671.8889	42959.17184	21928.991695	27393.30999	1033423.146
Pro-Ile	[M+H]+	2	229.1552	Pos	1.442	452285.4307	435241.4153	332184.0265	432178.6551	443443.5672
PyroGlu-Met	[M+H]+	2	261.0903	Pos	1.6	217474.5188	191318.2567	117587.9055	187266.9243	225113.1384
PyroGlu-Val	[M+H]+	2	229.1187	Pos	1.589	10327204.36	9623143.907	7432186.245	9778073.098	6379218.204
Ser-Leu	[M+H]+	2	219.134	Pos	1.305	2280212.682	2087458.637	1761511.268	2039273.919	3865911.912
Ser-Phe	[M+H]+	2	253.1181	Pos	1.562	208529.5055	165705.8956	150470.6562	169561.1379	2033403.684
Ser-Tyr	[M-H-CH2O]-	2	237.0881	Neg	0.646	33036.66667	21936.33333	18686	12747	543133
Ser-Val	[M+H]+	2	205.1185	Pos	0.485	1824940.468	1729996.5	1494161.375	1623487.924	1657555.335
Thr-Leu	[M+H]+	2	233.1496	Pos	1.442	2346737.171	2194959.967	1825070.814	2194091.109	3197891.425

Thr-Phe	[M-H]-	2	265.1193	Neg	1.663	95756.66667	86022.66667	83857.66667	83590	588972.6667
Thr-Tyr	[M+H]+	2	283.1288	Pos	0.765					881831.1396
Thr-Val	[M+H]+	2	219.134	Pos	0.532	5105150.568	5213924.934	4348244.622	4900488.721	5976310.028
Trp-Asn	[M+H]+	2	319.1401	Pos	1.218					294979.0262
Trp-Leu	[M+H]+	2	318.1812	Pos	2.649					277083.6429
Tyr-Leu	[M+H]+	2	295.1651	Pos	1.977	38575.41919	32429.70237	22704.99776	27348.30374	670814.5697
Tyr-Phe	[M+H]+	2	329.1495	Pos	2.149	12353.052	5116.014094	2784.653949	6061.308883	616274.6438
Tyr-Pro	[M+H]+	2	279.1375	Pos	1.463	185087.9785	170994.5909	138542.2158	174549.8351	325539.4646
Tyr-Tyr	[M+H]+	2	345.1443	Pos	1.548	75479.08886	51422.46594	32258.71336	51242.36451	914696.1113
Tyr-Val	[M-H]-	2	279.1349	Neg	1.38	11319.66667		1900.333333	3825	375704.3333
Val-Ala	[M-H]-	2	187.1088	Neg	0.427	124505.6667	123227.3333	113854.3333	102415.6667	1196278
Val-Gly	[M+H]+	2	175.1077	Pos	0.431	103386.661	55333.03007	38656.67959	53165.0827	2059321.893
Val-Gly-Val	[M-H]-	2	272.1616	Neg	1.726	34472	33270	29711.66667	29807.33333	346806.3333
Val-Leu	[M+H]+	2	231.1704	Pos	1.82	1003034.397	602983.0661	458825.6427	563620.7165	18296478.92
Val-Met	[M-H]-	2	247.1121	Neg	1.306	28374.33333	8377.333333	14361.33333	13564.33333	1093043
Val-Phe	[M-H]-	2	263.1399	Neg	2.013	107167.3333	63279	43696.33333	38528	3400468.667
Val-Thr	[M+H]+	2	219.134	Pos	0.32	53132.37813				4678850.368
Val-Trp	[M+H]+	2	304.1656	Pos	2.148		3095.136751			345547.3565
Val-Tyr	[M+H]+	2	281.1495	Pos	1.321	204877.9617	37254.13941	19413.943133	33105.0344	10656074.19
Val-Val	[M-H]-	2	215.1399	Neg	0.973	205773.6667	187275.6667	197409	188501.3333	1400526.667
Val-Val	[M+H]+	2	217.1547	Pos	1.014	782749.6395	789477.673	556074.0493	647464.5625	4584652.253

Table S3.3: Average peak heights of lipid derived metabolites in freeze-dried *M. pyrifera* extracts for selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (mins)	Average peak heights for extraction conditions				
						MAE pH 4	MAE pH 7	MAE pH 10	MAE pH 4+7	MEAE
LPA 16:0	[M+Na]+	2	507.2696	pos	5.28	195020	157320	43600	209483	202093.9
LPA 18:1	[M+H-H2O]+	2	493.2926	pos	5.4	271341	174034	45809	269705	252161.1
LPC 14:0	[M+Na]+	2	490.2908	pos	5.067	115153	85953	31573	127821	93374.17

LPC 15:0	[M+H]+	2	482.3247	pos	5.367	1176780	427575	22653	86268	1005469
LPC 18:1	[M+Na]+	2	544.3375	pos	5.809	144833	87165	23524	125622	101722.5
LPC 20:0	[M+H]+	2	552.4024	pos	6.863	35673	17079	1795	31615	34459.26
LPE 14:0	[M+H]+	2	426.2614	pos	5.041	819765	461621	145273	635173	579588.1
LPE 16:0	[M+H]+_[M+Na]+	2	454.2928_476.2749	pos	5.6385	1320099	743272	221980	1102195	975022.6
Palmitoyleicosapentaenoyl phosphatidylcholine	[M+H]+	2	780.5536	pos	7.025	129061	50392	49401	33506	87254.22
PC 18:1_14:0	[M+H]+	2	732.5532	pos	7.102	129124	50270	105098	28227	228093.6
PC 20:4_20:4	[M+H]+	2	830.5688	pos	9.532	341739	30834	162821	273047	198978.8
(3.beta.,5.alpha.)-4,4-Dimethylcholesta-8,14,24-trien-3-ol	[M+H-H2O]+	2	393.3516	pos	7.03	90162	60443	8393	73150	130838.4
1-(Benzoyloxy)propan-2-yl benzoate	[M+H-C7H6O2]+	2	163.0757	pos	3.784	10860	38624	9377	10665	5578.412
1,11-Undecanedicarboxylic acid	[M-H]-	2	243.1603	neg	4.105	196918.7	388469	243163.7	226578	189648
2-hydroxyisocaproic acid	[M-H]-	2	131.0715	neg	2.065	222439.3	212880.3	208981.3	206335.7	353489.3
6-Oxooctadecanoic acid	[M+NH4]+	2	316.2843	pos	4.712	41963	36094	52385	66784	40088.11
9-(2,3-Dihydroxypropoxy)-9-oxononanoic acid	[M-H]-	2	261.1345	neg	2.604	134780.7	167629.3	148771.3	174120.7	127007.7
Acetyl-carnitine	[M+H]+	2	204.1235	pos	0.382	6135209	6271576	2050211	4748765	4810776
Azelaic acid	[M+H-H2O]+	2	171.102	pos	2.707	45496	63705	53187	51177	34591.84
Dinorprostaglandin E1	[M-H-H2O]-	2	307.1917	neg	4.549	1786985	630099	1923167	633690.7	656274
Hymeglusin	[M-H-H2O]-	2	305.176	neg	4.423	398093.7	683818.7	464256	459647.7	467745.7
LPI-(1-oxo-9,12-octadecadienyl)	[M-H]-	2	595.2891	neg	4.913	325465.7	197590	81688.67	331984.3	331321
MG 16:0	[M+H]+	2	331.2842	pos	6.041	587241	373348	99182	502419	506661.8
Sebacic acid	[M-H]-	2	201.1135	neg	3.079	388876.7	472153.3	641606.3	616252.3	527751.3
Suberic acid	[M-H]-	2	173.0818	neg	2.287	1081038	1336503	1932167	1831092	1484269
Succinic acid	[M-H]-	2	117.0193	neg	0.443	9327027	10102788	11571586	10603583	7847928

Table S3.4: Average peak heights of other metabolites in freeze-dried *M. pyrifera* extracts for selected extracts as identified by untargeted metabolomics

Compound	Species	MSI level	m/z	ESI mode	RT (min)	Average peak heights for extraction conditions				
						MAE pH 4	MAE pH 7	MAE pH 10	MAE pH 4+7	MEAE
1-(2-Hydroxyethyl)-2,2,6,6-tetramethyl-4-piperidinol	[M+H]+	2	202.1801	pos	0.454	8341818	6656574	5666768	8833519	5108881
1,5-Pentanediamine	[M+H-NH3]+	2	86.0967	pos	0.503	1279895 3.98	1200817 9	1060673 0	11764031	14435775.86
13,14-Dihydro-15-ketoprostaglandin A2	[M+Cl]-	2	369.1836	neg	4.888	934842.3 333	1312264 .667	1980021	909838	1193416
2-Hydroxyglutaric acid	[M-H-H2O]-	2	129.0193	neg	0.32	940145.6 667	984286. 6667	991168.6 667	839739	852140.66 67
4-Oxododecanedioic acid	[M-H]-	2	243.124	neg	2.909	75880.33 333	90293	157695.6 667	112428.6 667	144560.33 33
5'-S-Methyl-5'-thioadenosine	[M+H]+	2	298.0973	pos	1.63	4070437. 936	4678383	1585904	5418652	3516279.971
Carnitine	[M+H]+	2	162.1126	pos	0.265	2871470 9.54	2454256 7	2178959 1	2172565 7	19322158
Dicyclohexylamine	[M+H]+	2	182.1907	pos	2.686		79137	118068	102360	
Flavine mononucleotide	[M+H]+	2	457.112	pos	1.802	606968.1 605	484397	307066	593251	337703.7902
Glyceric acid	[M-H]-	2	105.0193	neg	0.268	138100.3 333	409143	977732.6 667	503571.6 667	227611.33 33
Glycerol 1-myristate	[M+H-H2O]+	2	285.2423	pos	5.414	313022.1 966	217167	68201	299360	279861.198
Guanosine	[M-H]-	2	282.0842	neg	0.465	123655	137773	147281.6 667	125495	642871.33 33
N-Acetyltyramine	[M+H]+	2	180.1024	pos	1.811	222315.5 235	243720	240303	232260	181876.2018
Pantothenic acid	[M-H]-	2	218.1033	neg	1.283	204731	210024. 6667	211975.3 333	192443	421000
Pentadecylamine	[M+H]+	2	228.2686	pos	6.209	522646.2 246	335947	103869	487407	177442.3247
Phenylalanine, methyl ester	[M+H]+	2	180.1023	pos	1.918	185758.8 66	227230	168548	151615	251265.4148
Pheophorbide a	[M+H]+	2	593.2757	pos	6.927	1347991. 376	907499	376557	1107999	814613.3618
Phloroglucinocarboxaldehyde	[M-H]-	2	153.0193	neg	1.979	157507.3 333	266608	286556.6 667	350425.6 667	170179
Propionylcarnitine	[M+H]+	2	218.1391	pos	0.665	1302844. 558	1317258	573688	1218652	1077862.636
Rubinaphthin A	[M-H]-	2	365.0877	neg	2.464					
Undecanedioic acid	[M-H]-	2	215.129	neg	3.441	507742.6 667	621065. 6667	621249.3 333	580705.3 333	495687
Violaceol I	[M-H-C7H6O2]-	2	139.0402	neg	1.606	189080.6 667	153167	17944.66 667	77657.33 333	203089.3333

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