Analysis of Polyphenols from *Laminaria hyperborea* for application as Life Science ingredients

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Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

This thesis is submitted for the degree of Philosophiae Doctor (PhD) in Chemistry at the University of Bergen, Norway. The experimental work was conducted at the University of Bergen's Department of Chemistry and at Alginor ASA OEWA laboratories from 2019 to 2023. The project was supervised by Assoc. Professor Monica Jordheim and Assoc. Professor Jarl Underhaug at the University of Bergen, and by R&D Manager Georg Kopplin at Alginor ASA. Founding of the project was provided by Alginor ASA and partly by The Norwegian Research Council (NFR) as a Doctoral Project in industry, an industrial PhD project. The thesis comprises three papers (**I-III**) preceded by an abstract.

Chapter 1 provides the scientific background, chapter 2 outlines the project's aim and motivation, chapter 3 details the extraction and analytical methods used, and chapter 4 presents the results of papers **I-III**. The appendices contain (A) extraction optimization experiments, (B) a personal natural product database used for identifying polyphenols, and (C) characterization of non-phenolic compounds in *L. hyperborea*.

Acknowledgements

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Abstract

Macroalgae, or seaweed, has been recognized as a valuable source of bioactive compounds. Among these, polyphenols have gained particular attention. Polyphenols are well-known for their various bioactivities, such as antioxidant, anti-inflammatory, antidiabetic, and anticancer activities, and have been associated with a range of health benefits. This project focused on the investigation of two types of macroalgae, namely the leaf biomass of the brown algae *Laminaria hyperborea* (eng.: tangle/cuvie) and the green algae *Ulva intestinalis* (eng.: gut weed). The initial extractions of *L. hyperborea* leaves were challenging due to its high polysaccharide content. Therefore, an initial case study was conducted on *U. intestinalis* to explore the combination of analytical techniques employed for polyphenol quantification and identification in seaweeds. The techniques utilized in this investigation included DAD-HPLC, the total phenolic content (TPC) assay with the Folin–Ciocalteu (FC) reagent, total flavonoid content (TFC) assay with an $AlCl_3$ reagent, and ^1H qNMR – including 2D NMR. The analyses showed a relatively composed polyphenolic nature with lower individual concentrations. For the crude sample of *U. intestinalis*, the total phenolic content was found to be 11.3 ± 1.4 mg GAE/g using HPLC, 5.0 ± 1.0 mg GAE/g with TPC, and 27.3 ± 2.7 mg GAE/g with qNMR. However, the TFC assay detected no flavonoids in the crude sample. The case study strongly indicated the need for optimization of the analytical methods. In addition, characterization using LC-MS was performed on purified samples of *U. intestinalis*, which tentatively identified the presence of several phenolic acids and flavonoid aglycones. Some of these were confirmed using reference standards.

Following optimization of the TPC assay and the ^1H qNMR method, the quantification and comparison of *Laminaria hyperborea* to four other brown algae was conducted. The total polyphenolic content of *L. hyperborea* was found to be 5.51 ± 0.00 mg GAE/g (qNMR) and 5.72 ± 0.07 mg GAE/g (TPC). For seaweeds belonging to the Laminariaceae family, which grow in the sublittoral zone, the TPC assay and selective qNMR showed similar, lower polyphenol yields. Still, a larger difference was observed between the TPC and qNMR results for seaweed growing in the eulittoral zone, such as *Ascophyllum nodosum* (eng.: knotted kelp) and *Fucus vesiculosus* (eng.: bladder wrack).

Seaweed growing in more shallow waters will have higher light accessibility, possibly producing a polyphenolic pool with an increased portion of polyphenols containing an increased number of reacting groups compared to the reference standard (gallic acid or phloroglucinol) used, thus resulting in an overestimation when quantifying with the TPC assay. It is also possible that these species in more shallow waters have a higher occurrence of TPC-interfering compounds than seaweed species growing in the sublittoral zone. To characterize the polyphenolic content of *L. hyperborea*, flash chromatography and preparative HPLC were used to purify the seaweed sample, obtaining purified fractions for analysis. Results showed that increasing purification corresponded to higher polyphenolic content, as determined by both the TPC assay and selective qNMR. In addition, ORAC assay results revealed increasing antioxidant activity with increasing purification.

To identify the molecular structure of polyphenols in *L. hyperborea*, both low- and high-resolution LC-MS were utilized. Analysis showed that the polyphenolic matrix was mostly composed of low-molecular weight polyphenols, with 96% of the tentatively identified compounds having masses below 800 Da. Eleven polyphenolic compounds were confirmed, including phenolic acids and phlorotannins, and several were found to be sulfated, which is believed to be an ecological adaptation to the marine environment.

This project provides the first comprehensive characterization of the polyphenolic content of *L. hyperborea* leaves. These results provide a characterization of the polyphenolic content of *L. hyperborea*'s leaf biomass, important for the implementation of a "total utilization" strategy in commercial alginate production. Moreover, the project provides valuable molecular-level insights into the phenolic content of seaweed, yielding valuable implications for research across disciplines, such as in the seaweed biorefinery, chemical ecology, and ocean monitoring.

Sammendrag

Makroalger, også kjent som tang og tare, er anerkjent som en verdifull kilde til bioaktive forbindelser. Av disse har polyfenoler fått særlig oppmerksomhet. Polyfenoler har blitt assosiert med en rekke helsefordeler, og er mest kjent for sine ulike biologiske egenskaper, så som antioksidant-, antiinflammatorisk-, antidiabetisk-, og antikreftegenskaper. og. Dette prosjektet undersøkte to typer makroalger; den brune algen *Laminaria hyperborea* (nor.: stortare) og grønnalgen *Ulva intestinalis* (nor.: tarmgrønske). De første ekstraksjonene av *L. hyperborea* blader var utfordrerne grunnet dere høye polysakkarid innhold. Derfor ble det utført en innledende studie med grønn algen *U. intestinalis* for å utforske de ulike analyseteknikkene som brukes for kvantifisering og identifisering av polyfenoler. Teknikkene som ble benyttet i denne studien inkluderte tradisjonell DAD-HPLC, en totalt-fenolinnhold (TPC) test med Folin-Ciocalteu reagens, en totalt-flavonoidinnhold (TFC) test med en $AlCl_3$ reagens, samt ^1H kvantitativ NMR (qNMR) – inkludert 2D NMR. Resultatene viste at det polyfenoliske innholdet var komplekst, og med lav konsentrasjon. For råprøven (crude) av *U. intestinalis* ble det totale fenolinnholdet målt til 11.3 ± 1.4 mg GAE/g ved bruk av HPLC, 5.0 ± 1.0 mg GAE/g med TPC, og 27.3 ± 2.7 mg GAE/g med qNMR. TFC-testen oppdaget imidlertid ingen flavonoider i råprøven. Denne innledende studien avdekket et sterkt behov for å optimalisere de analytiske metodene som ble benyttet. I tillegg ble det utført LC-MS karakterisering på opprensede prøver av *U. intestinalis*. Disse identifiserte tentativt tilstedeværelsen av flere fenolsyrer og flavonoider. Noen av disse ble også bekreftet av referansestandarder.

Etter optimalisering av TPC-testen og ^1H qNMR-metoden, ble kvantifisering og sammenlikning av *L. hyperborea* med fire andre brunalger gjennomført. Det totale polyfenolinnholdet i *L. hyperborea* ble funnet å være 5.51 ± 0.00 mg GAE/g (qNMR) og 5.72 ± 0.07 mg GAE/g (TPC). For tang som tilhører Laminariaceae-familien, som vokser i sublitoralsone, viste TPC-testen og selektiv qNMR liknende, men lave polyfenolkonsentrasjoner. Samtidig ble det for tang som vokser i epilittoralsone, som *Ascophyllum nodosum* (nor.: grisetang) og *Fucus vesiculosus* (no.: blæretang), observert en større forskjell mellom TPC og qNMR resultatet. Tang som vokser i strand sonen vil

bli utsatt for mer lys, noe som kan føre til at det polyfenolske innholdet består av flere polyfenoler som inneholder flere reagerende grupper enn referanse standarden (gallesyre eller floroglucinol) som benyttes, noe som kan produsere overestimering av polyfenol innholdet ved bruk at TPC testen. Det er også mulig at disse artene som vokser nærmere havoverflaten inneholder en økt mengden TPC-forstyrrende forbindelser enn artene som vokser dypere i den sublitorale sonen. For å karakterisere det polyfenoliske innholdet i *L. hyperborea*, ble flash-kromatografi og preparativ HPLC benyttet til å rense opp tangprøven, for å opparbeide rensede fraksjoner til bruk i analysen. Resultatene viste at prøver som var mer opprenset korresponderte med høyere polyfenolinnhold både i TPC-testen og ved selektiv qNMR. I tillegg viste ORAC-testresultatene økende antioksidantaktivitet med økende renhet.

For å identifisere den molekylære strukturen til polyfenolene i *L. hyperborea*, ble både lavt- og høytoppløselig LC-MS benyttet. Analysene avdekket at polyfenolinnholdet hovedsakelig var sammensatt av lavmolekylære forbindelser, der 96% av de tentativt identifiserte forbindelsene hadde masser under 800 Da. 11 polyfenoliske forbindelser ble identifisert, inkludert fenolsyrer og florotanniner, og flere ble funnet å være sulfaterte, hvilket antas å skyldes en økologisk tilpasning til det marine miljøet.

Dette prosjektet gir den først omfattende beskrivelsen av stortares polyfenoliske innhold. Resultatene gir en grundig identifisering av polyfenolinnholdet i bladene av *L. hyperborea* og antyder potensialet for en «total utnyttelses»-strategi innen kommersiell alginatproduksjon. Videre gir prosjektet verdifull molekylær innsikt i det fenoliske innholdet i tang, noe som gir viktig informasjon til videre forskning på tang-bioraffineriet, kjemisk økologi, og havovervåkning.

Abbreviations

HPLC	High performance Liquid Chromatography
TPC	Total Phenolic Content
FC	Folin-Ciocalteu
GAE	Gallic Acid Equivalents
PGE	Phloroglucinol Equivalents
NMR	Nuclear Magnetic Resonance
UV	Ultraviolet
MS	Mass Spectrometry
qNMR	Quantitative Nuclear Magnetic Resonance
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
COSY	Correlated Spectroscopy
d_6-DMSO	Deuterated Dimethyl sulfoxide
$DMSO_2$	Dimethyl sulfone
TMS	Tetramethylsilane
TFA	Triflouroacetic Acid
DAD	Diode Array Detector
LOD	Limit of Detection
LOQ	Limit of Quantification
DW	Dry Weight
U. intestinalis	*Ulva intestinalis*
L. hyperborea	*Laminaria hyperborea*
L. digitata	*Laminaria digitata*
F. vesiculosus	*Fucus vesiculosus*
S. latissima	*Saccharina latissima*
A. nodosum	*Ascophyllum nodosum*
Da/ kDa	Dalton/ Kilodalton
HPV	Human papillomavirus
PEDV	Porcine epidemic diarrhea virus

ASE	Accelerated solvent extraction
UAE	Ultrasound assisted extraction
MeOH	Methanol
EtOH	Ethanol
EtOAc	Ethyl acetate
MS	Mass spectrometry
LR LC-MS	Low-resolution liquid chromatography with mass spectrometry detector (TOF)
HR LC-MS	High-resolution liquid chromatography with mass spectrometry detector (TOF)
MS/MS or MS^2 (or PI)	MS fragmentation experiments (Product Ion chromatogram)
TOF	Time-of-flight
TIC	Total Ion Chromatogram
SIM	Selected Ion Monitoring
m/z	Mass-to-charge ratio
CHS	Chalcone synthase
CHI	Chalcone isomerase
FNS	Flavone synthase
HIS	2-hydroxyiso-flavanone synthase
IFD	Isoflavone dehydratase
F3H	Flavanone-3-hydroxylase
FLS	Flavonol synthase
DFR	Dihydroflavonol reductase
ANS	Anthocyanidin synthase
PAL	Phenylalanine ammonia lyase
TAL	Tyrosine ammonia lyase
CAH	Cinnamic acid 5-hydroxylase
p-CAH	*p*-Coumaric acid 3-hydroxylase
BAH	Benzoic acid 4-hydroxylase
PhlD	Type III polyketide synthase

HCA	Hydroxycinnamic acid
HBA	Hydroxybenzoic acid
HT	Hydrolysable tannin
PAC	Proanthocyanidin
PT	Phlorotannin

List of publications

Paper I Wekre, M. E; Kåsin, K.; Underhaug, J.; Holmelid, B.; Jordheim, M. Quantification of Polyphenols in Seaweeds: A Case Study of *Ulva intestinalis*. *Antioxidants* **2019**, 8, 612 - 627.

Paper II Wekre, M. E.; Brunvoll, S. H.; Jordheim, M. Advancing Quantification methods for Polyphenols in Brown Seaweeds - applying a Selective qNMR method compared to the TPC Assay. *Phytochemical analysis*, **2022**, 33(7), 1099 - 1110.

Paper III Wekre, M. E.; Holmelid, B.; Underhaug, J.; Pedersen, B.; Kopplin, G.; Jordheim, M. Characterization of high-value products in the side stream of *Laminaria hyperborea* alginate production – Targeting the phenolic content. *Algal Research*, **2023**, 72, 103109.

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1. INTRODUCTION

1.1 Polyphenols

Polyphenols are a large, diverse group of natural products (Figure 1). In terrestrial plants, polyphenols play important roles in plant growth and development, as well as protection against biotic and abiotic stressors.[1,2] Additionally, certain polyphenols, such as flavonoids, have been found to be involved in pollination of flowering plans, UV-protection, and seed dispersal.[3–9] The evolution of terrestrial plants is a complex process that has been developing over millions of years. One of the most important adaptations of terrestrial plants has been the development of chemical defenses, which include polyphenols.[10] Polyphenols have evolved in response to selective pressures in the plant's environment, including pathogens, abiotic stress, and coevolutionary interactions with herbivory.[11] For instance, polyphenol's role as protection against UV-B radiation is thought to be an important factor in the initial plant evolution from aquatic- to terrestrial habitats.[8,10] Additionally, as herbivores adapted to feed on plants, plants developed new

and complex chemical defenses to deter them, resulting in an increased heterogeneity of polyphenols.[12]

Despite their diverse functions in terrestrial plants, research on marine polyphenols has been relatively limited compared to their terrestrial counterparts, although interest has increased in recent years. In macroalgae, polyphenols serve as secondary metabolites that can be synthesized during plant growth or in response to external conditions such as UV-radiation, wounding, salinity, and temperature, primarily functioning to protect against environmental stressors.[13–17] These compounds contain one or more aromatic rings with one or more hydroxyl substituent or functional derivatives, and can either be soluble or cell wall bound depending on their synthesis and function.[18]

It is important to differentiate between polyphenols and phenolic compounds, as both are referred to throughout the thesis. Phenolic compounds are organic molecules containing a phenol group, consisting of a hydroxyl (-OH) group bonded to an aromatic ring, such as a benzene ring. Polyphenols, on the other hand, are a subclass of phenolic compounds that contain multiple phenol groups and are synthesized only through the acetate-malonate and/or the shikimate pathway. However, the terms ‘polyphenols’ and ‘phenolics’ are often used interchangeably when referring to plant and algae polyphenols.

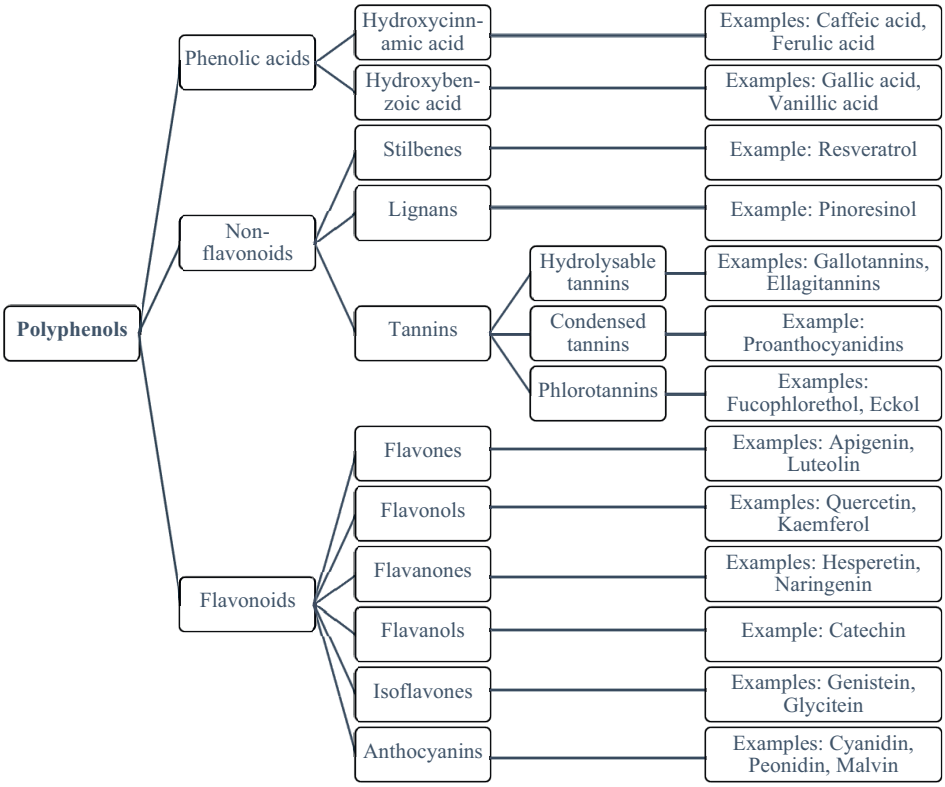


Figure 1: General scheme of polyphenol classification. Adapted from Harborne and Dey (1989)[19] and El Gharras (2009).[20]

1.2 Phlorotannins

Phlorotannins form a sub-group of polyphenols found exclusively in brown macroalgae, formed by the polymerization of two or more phloroglucinol units.[21–24] These metabolites serve both primary and secondary roles.[25]

1.2.1 Structure

The structure of phlorotannins can vary in size and can be classified into four different subgroups based on the linkage between phloroglucinol units, as shown in figure 2. Long polymeric chains with phloroglucinol units can form, with a molecular weight range from 126 Da to 650 kDa.[22] The chains connect using phenyl (C-C)- and/or ether linkages

(C-O-C), and coupling takes place at different positions on the phloroglucinol base unit, creating several structural- and conformational phlorotannin isomers.[26]

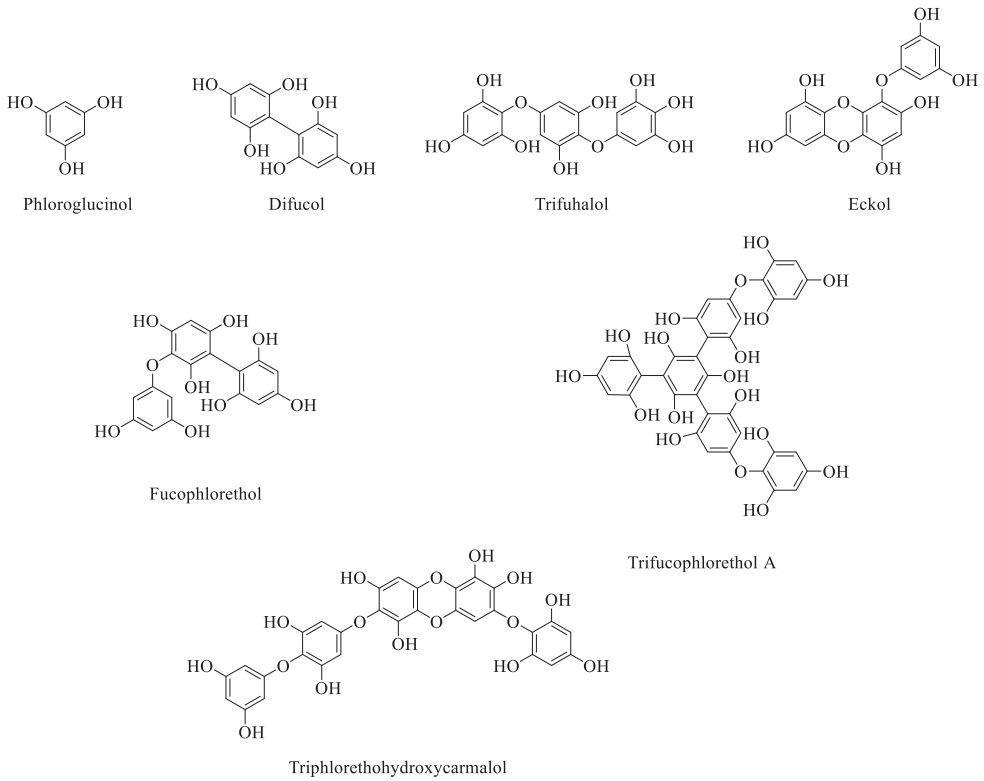


Figure 2: The base unit of all phlorotannins, phloroglucinol, and selected examples of phlorotannins with various structure- and linkage schemes.

1.2.2 Biosynthesis

The biosynthesis of phlorotannins is not yet fully understood, but is thought to occur via either the shikimate or the acetate-malonate pathways.[22,27–31] A study by Meslet-Cladière *et al.* (2013)[21] found indications that the phloroglucinol monomer is synthesized from malonyl-CoA via the acetate-malonate pathway, catalyzed by a type III polypetide synthase (PKS1), as shown in figure 3. Other studies also suggest that phlorotannins are synthesized via the acetate-malonate pathway (polyketide pathway).[32–37]

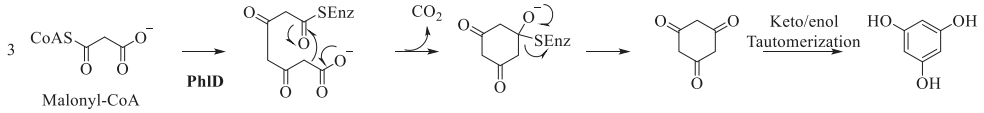


Figure 3: Biosynthesis of phloroglucinol. Adapted from Achkar *et al.* (2005)[35], Meslet-Cladière *et al* (2013)[21], and Biessy and Filion (2021)[36].

The synthesis of phlorotannins via the acetate-malonate pathway involves several steps. First, malonyl-CoA units undergo condensation, catalyzed by PhlD, a type III polyketide synthase, to create a polyketide chain.[37] Next, this chain undergoes a Claisen-type cyclization to form a hexacyclic triketide, which in turn undergoes keto/enol tautomerization to produce phloroglucinol.[21,36] The polymerization of phloroglucinol units occurs through the oxidative coupling of phloroglucinol radicals, as shown in figure 4.[27,38–42]

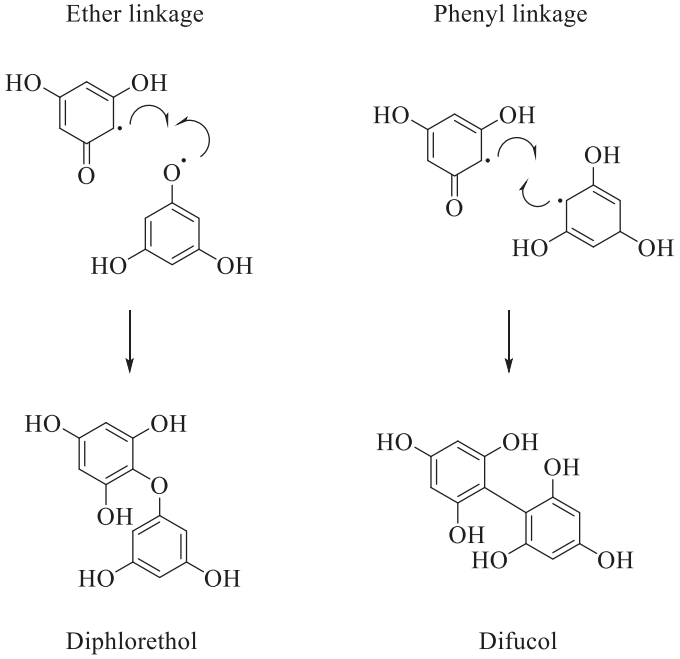


Figure 4: Formation of phlorotannins from phloroglucinol with ether- and phenyl linkage via oxidative couplings. Adapted from Fernando *et al*. (2021)[27].

1.2.3 Plant function

Phlorotannins can either be soluble or cell-wall bound in algae. Soluble phlorotannins are found in membrane bound vesicles (physodes) or in the cytoplasm of algal cells.[23,43–47] In contrast, cell-wall bound phlorotannins serve as important structural components of brown algal cell walls and can form complexes with alginate.[41,48,49] These compounds mainly function as a chemical defense mechanism against herbivory, UV-radiation, oxidative stress, and other external stressors, making them a crucial part of the algae's allelopathy.[22,43,50–52] For instance, Connan and Stengel (2011)[44] found that the concentration of cell-wall bound phlorotannins increased when brown algae were exposed to copper-contaminated water, illustrating their role in chemical defense. In addition to their protective functions, phlorotannins have been shown to contribute to wound healing in leaves, cell wall hardening, and reproductive functions in some algae species.[50,53,54]

1.2.4 Bioactivity

Antioxidant activity. Polyphenols are known for their wide range of biological activities, including their ability to act as antioxidants.[22,55,56] In humans, an imbalance between reactive oxygen species (ROS) and antioxidant defense mechanisms can lead to oxidative damage, causing lifestyle diseases and aging.[57,58] Phlorotannins could contribute to disease prevention through their antioxidative effect. The antioxidant capacity of phlorotannins could be due to specific scavenging of radicals formed during peroxidation, scavenging of oxygen-containing compounds, or metal-chelating ability.[59] As electron-rich compounds, phlorotannins are prone to enter into electron donation reactions, producing phenoxyl radical species intermediates, which are then stabilized by resonance delocalization of the unpaired electrons on the *ortho*- and *para* positions on the aromatic ring.[22,59]

Antiviral activity. Due to polyvalent biological activity of phlorotannins, viruses do not acquire resistance to these compounds, making them potential antiviral agents.[60] Phlorotannins react and bind to enveloped viruses, preventing the pathogen from interacting with the host cell. Furthermore, phlorotannins have also been found to have

an inhibitory effect on nonenveloped viruses such as human papillomavirus (HPV), porcine epidemic diarrhea virus (PEDV) and influenza A virus.[60–62] Specifically, Park *et al.* (2013)[63] isolated dieckol from the brown algae *Ecklonia cava*, which was found to inhibit SARS-CoV $3CL^{pro}$. Additionally, phlorotannins have been reported to have an inhibitory effect on human immunodeficiency virus (HIV). Ahn *et al.* (2004)[64] and Artan *et al.* (2008)[65] reported an inhibitory effect of both reverse transcriptase and protease of HIV-1 *in vitro* by phlorotannins isolated from *Ecklonia cava*.

Antibacterial activity. Several studies have found phlorotannins to have a bacteriostatic effect.[66–69] The effect on the bacteria has been reported to be due to inhibition of oxidative phosphorylation, and their ability to bind with bacterial proteins such as enzymes and cell membranes, causing cell lysis.[52] Lopes *et al.* (2012)[66] reported that extracts of species belonging to the *Cystoseira* genus and *Fucus spiralis* have an increased bactericidal activity related to the phlorotannin content, having a greater effect on Gram positive bacteria, which has also been found by others.[70] Nagayama *et al.* (2002)[71] also reported that the bactericidal effect of phlorotannins is proportional to the degree of polymerization, indicating that larger phlorotannins have higher antibacterial activity.

Anticancer activity. Cancer is an extensive health problem and one of the leading causes of mortality globally.[72] Cancer cell formation is associated with free radicals and inflammation.[56,59,73] Through their antioxidant activity, phlorotannins can indirectly reduce cancer formation. Additionally, Mansur *et al.* (2020)[74] suggested that phlorotannins may decrease angiogenesis, cell adhesion and invasion in cancer cells. In 2009, Kong *et al.*[75] reported that two phlorotannins extracted from *Ecklonia cava* inhibited the proliferation of human breast cancer cells *in vitro*, indicating the potential of phlorotannins as chemo preventative agent.

Antiallergic effect. Allergy and allergic reactions result from the immune system's overreaction to typically harmless substances, known as allergens. These allergens trigger the release of Immunoglobulin E (IgE), an antibody that induces the release various chemicals, such as histamine, which cause symptoms in the lungs, nose, throat, and other parts of the body.[76] Shim *et al.* (2009)[77] reported that phlorotannin extracts

from *Ecklonia cava* inhibited the expression of FcεRI, a high-affinity receptor for Ige that act as an effector cell in allergic reactions. As a result, the histamine release that often leads to allergic reactions was also suppressed. Li *et al.* (2008)[78] similarly demonstrated the inhibitory effect of phlorotannins from *Ecklonia cava* on FcεRI, with dieckol and 6,6'-bieckol displaying the strongest inhibitory effect.

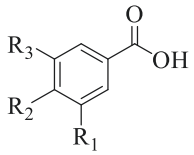
Antidiabetic activity. Diabetes mellitus is a prevalent chronic metabolomic disorder affecting approximately 5.1 % of the adult population globally.[79–81] The disease is characterized by high glucose levels in the plasma due to the activity of α-glucosidase and α-amylase enzymes in the body. Several studies have demonstrated the inhibitory effect phlorotannins on both α-glucosidase and α-amylase, indicating their potential antidiabetic activity.[81–83] Additionally, Kim *et al.* (2021)[84] fund that seven red-, green-, and brown seaweeds exhibited antidiabetic activity by inhibiting α-glucosidase.

1.3 Phenolic acids

Phenolic acids are the most widely distributed secondary metabolite in plants. They are the simplest of the polyphenol class and can be found in many different plant species.

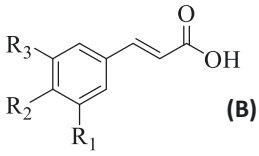
1.3.1 Structure

Phenolic acids are a type of polyphenol that can be divided into two sub-groups: hydroxybenzoic acids and hydroxycinnamic acids (see figure 1).[17,85,86] Hydroxybenzoic acids are derived from benzoic acid and have a general C6-C1 structure, as see for salicylic acid (syn.: *p*-hydroxybenzoic acid), vanillic acid, syringic acid, and protocatechuic acid (refer to figure 5). Hydroxycinnamic acids, on the other hand, generally have a C6-C3 structure and include esterified conjugates such as chlorogenic acid. Examples of hydroxycinnamic acids also include cinnamic acid, coumaric acid, ferulic acid, and caffeic acid (see figure 5).



(A)

Compound	R_1	R_2	R_3
Salicylic acid	H	OH	H
Vanillic acid	OH	OH	OH
Protocatechuic acid	OCH_3	OH	OCH_3
Gallic acid	OH	OH	H



(B)

Compound	R_1	R_2	R_3
Cinnamic acid	H	H	H
p-Coumaric acid	H	OH	H
Ferulic acid	OCH_3	OH	H
Sinapic acid	OCH_3	OH	OCH_3

Figure 5: General structure for phenolic acids showing examples of hydroxybenzoic acids (A) and hydroxycinnamic acids (B).

1.3.2 Biosynthesis

Phenolic acids are normally synthesized in the plant via the shikimate pathway from L-phenylalanine or L-tyrosine.[87,88] Phenylalanine is a precursor for the hydroxycinnamic acids, while tyrosine leads to the formation of hydroxybenzoic acids, as seen in figure 6. The amino acids undergo a series of enzymatic reactions, which results in the formation of cinnamic acid and *p*-coumaric acid. These initial phenolic acids can then be further modified to form various phenolic acids through methylation and hydroxylation reactions.

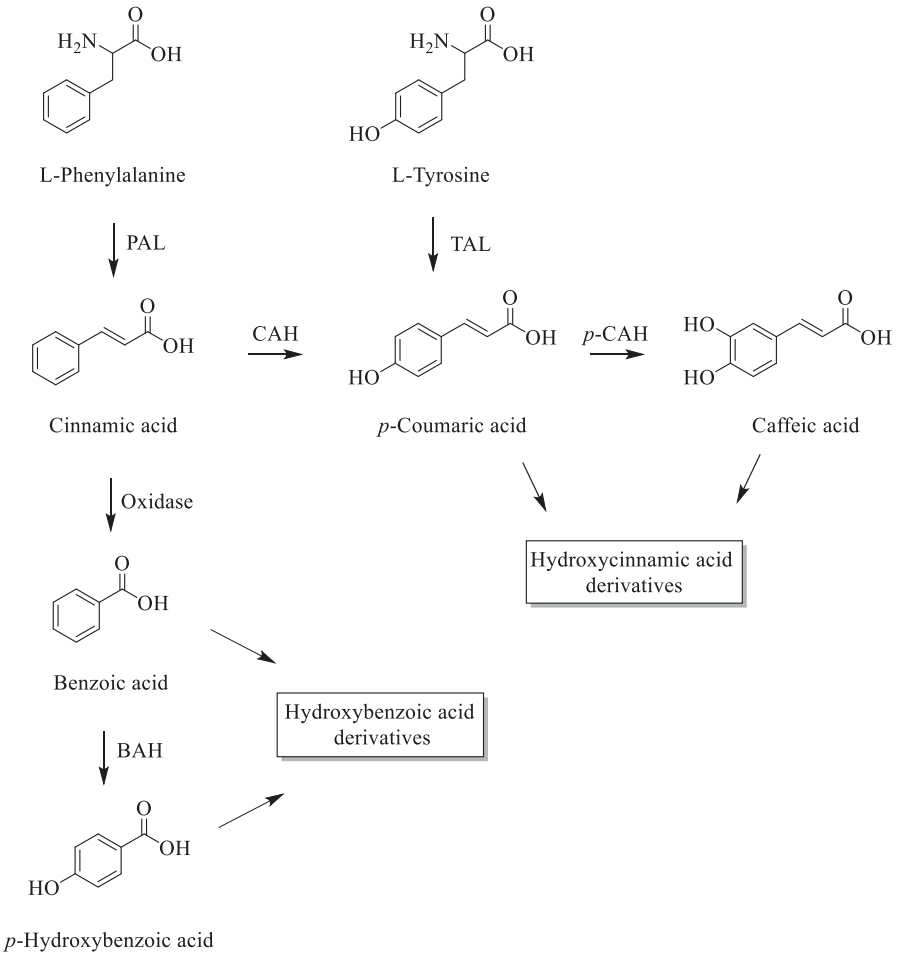


Figure 6: Biosynthesis of phenolic acids. Adapted from Heleno *et al.* (2015)[87] and Gross (1981)[89]. Enzyme abbreviations: PAL = Phenylalanine ammonia lyase, TAL = Tyrosine ammonia lyase, CAH = Cinnamic acid 5-hydroxylase, *p*-CAH = *p*-Coumaric acid 3-hydroxylase, BAH = Benzoic acid 4-hydroxylase.

1.3.3 Plant function

Similar to other polyphenols, phenolic acids have several functions in algae. These include acting as defense against herbivores and pathogens, protection against UV-radiation, nutrient uptake, various structural components, and act as antioxidants.[17,88]

1.3.4 Bioactivity

Phenolic acids have been studied from many plants and are found to exhibit several bioactivities. Many of the bioactivities are the same as described for phlorotannins (Section 1.2.4). Some of phenolic acids biological activities include anti-inflammatory, antioxidant, anti-tumor, and anti-diabetic activities.[87,90–93]

1.4 Flavonoids

Flavonoids are a large, heterogenous, polar group of secondary metabolites found in plants and algae. They can be divided into several subgroups and are associated with several health promoting effects.

1.4.1 Structure

The basic flavonoid fifteen-carbon skeleton consists of two benzene rings, namely A and B, connected by a heterocyclic pyrene ring (C) (as shown in figure 7).[6,94,95] Various substitutions on the C-ring and level of oxidation separates flavonoids into several subgroups, including flavones, isoflavones, flavonols, flavanones, and flavanols.[96,97] Differences in the substitution patterns on the A- and B-ring also distinguish between flavonoids within the same subgroup.

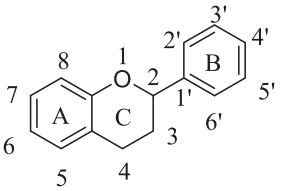


Figure 7: Basic flavonoid C_{15} backbone with carbon numbering.

1.4.2 Biosynthesis

Flavonoids, along with phenolic acids, are synthesized in the plant via the shikimate pathway from L-phenylalanine. The synthesis of the basic flavonoid C_{15} skeleton (Figure 7) begins with a series of condensation reactions between malonyl residues and 4-coumaroyl CoA, which are catalyzed by chalcone synthase (CHS).[98,99] This

results in the formation of a chalcone, which can subsequently form a variety of flavonoid subclasses through several enzymatic reactions, such as isomerases, reductases, hydroxylases, glycosyltransferases, and acyltransferases as (as described in figure 8).

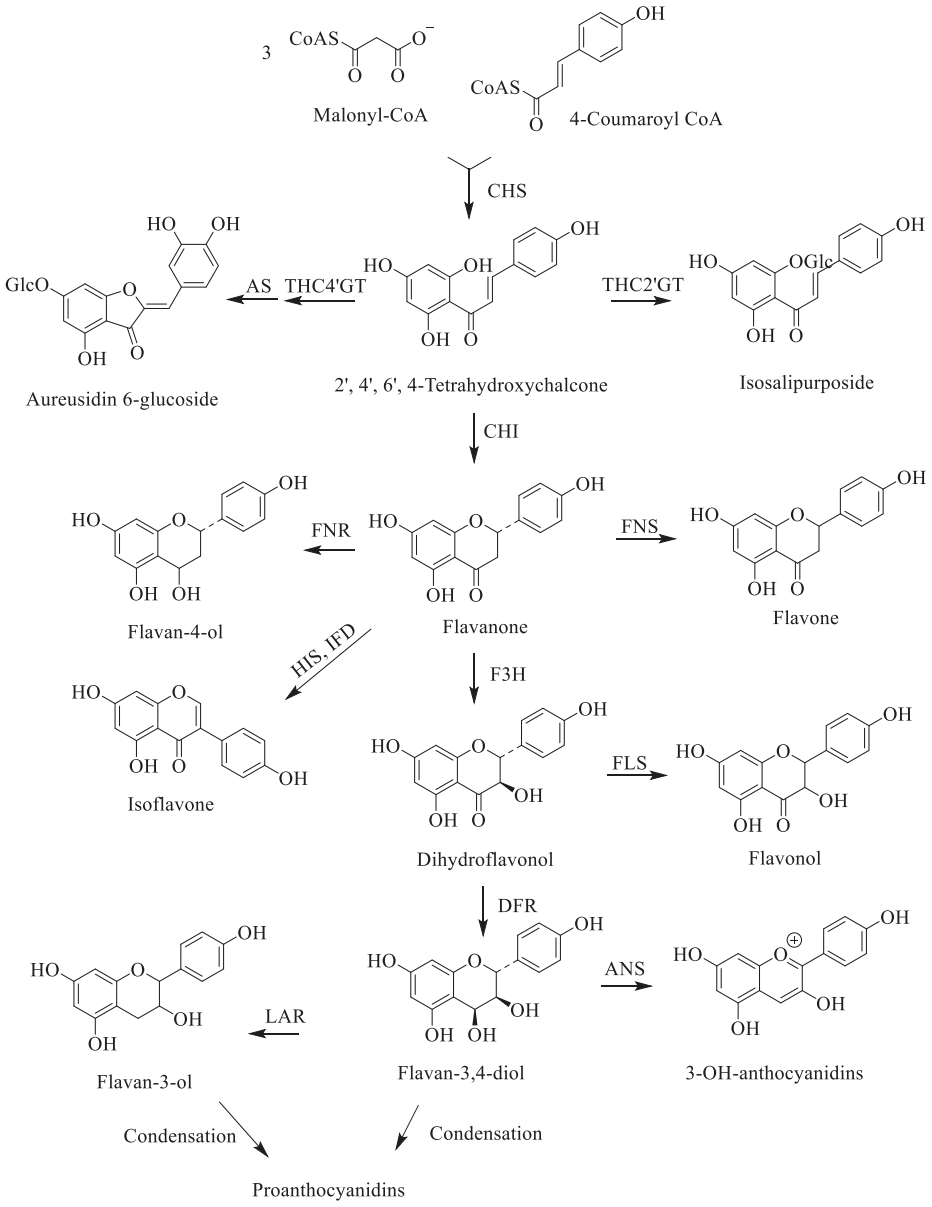


Figure 8: Biosynthesis of flavonoids in plants and algae. Adapted from Goiris *et al.* (2009)[100] and Andersen and Jordheim (2010)[98]. Enzyme abbreviations: CHS= Chalcone synthase, CHI= chalcone isomerase, FNS= flavone synthase, HIS= 2-hydroxyiso-flavanone synthase, IFD= isoflavone dehydratase, F3H= flavanone-3-hydroxylase, FLS= flavonol synthase, DFR= dihydroflavonol reductase, ANS= anthocyanidin synthase.

1.4.3 Plant function

Flavonoids serve various functions in plants. One of their key roles is to act as a secondary antioxidant defense system, which helps to prevent damage caused by free radicals. This has been extensively documented in previous studies.[6,94,101] In addition, flavonoids possess UV-absorbing properties, which enable them to protect plants from the harmful effects of UV radiation from the sun.[6,102] Apart from the antioxidant properties, flavonoids also function as signaling molecules in a range of biological processes, including reproduction, pathogenesis, and symbiosis. These molecules have been shown to play crucial roles in these processes in numerous studies.[6,97,100,102] For pollinating plants, the colors produced by flavonoids play a crucial role in attracting pollinating insects, which are essential for reproduction.[6,103] This is particularly important for terrestrial plants, which rely heavily on pollinators to ensure their survival. Therefore, the functions of flavonoids in plants are multifaced and play vital roles in various biological processes.

1.4.4 Bioactivity

Similar to the other polyphenols described (Sections 1.2 and 1.3), flavonoids are also reported to have diverse and extensive bioactivities. Flavonoids have been shown to possess anti-inflammatory, anticancer, cardiovascular, neuroprotective, antidiabetic, and antimicrobial activities, among others.[94,104–111] These biological activities are mostly attributed to their ability to modulate multiple signaling pathways and scavenge free radicals, thereby acting as antioxidants.[112–114]

1.5 Sulfated polyphenols

Sulfated polyphenols are observed in marine plants and some algae, with sulfated flavonoids being the most reported with over 150 compound.[96,115–118] Sulfation increases hydrophilicity and solubility of a molecule and is suggested to be an ecological adaption to the marine environment.[119–125] This increased solubility enhances the bioavailability of the compound and may also contribute to the color of the biomass by forming stable complexes with other polyphenols such as anthocyanins.[126–128] In plants, sulfation is

believed to influence the inactivation of toxic products and play an important part in plant growth regulation.[96,118,129,130] Sulfation of a polyphenols occur via a nucleophilic substitution, where sulfotransferases catalyze the reaction, transferring a sulfonate group (SO_3^-) from the sulfate donor, 3'-phodphoadenosine 5'-phosphosulfate (PAPS), to the hydroxyl groups in the phenolic compound.[131–133] However, sulfated polyphenols are susceptible to hydrolyzation, which poses challenges during both extraction and isolation.[96,125]

Polyphenols, including sulfated polyphenols, are known for their bioactivities, such as antioxidant, antiviral, anti-inflammatory, and anti-cancer activity as previously described for phlorotannins, phenolic acids, and flavonoids (Sections 1.2.4, 1.3.4, and 1.4.4). Studies on sulfated polyphenols have also revealed specific anticoagulant, antitumor, and antiviral activities.[118,134–140]

1.6 Polyphenols in macroalgae

1.6.1 Macroalgae

Algae are photosynthetic nonvascular plants that contain chlorophyll a and simple reproductive structures.[141] Macroalgae, also known as seaweeds, are a large group of marine organisms which emerged as a variety of independent lineages early in the evolution of eukaryotes.[142] Macroalgae are multicellular, marine organisms that lack true roots, stems, or leaves.[143] However, larger seaweeds such as Laminariaceae possess organs or "hold-fasts" and stipes which broadens to leaf-like portions. Macroalgae can be classified as Chlorophyta (green algae), Rhodophyta (red algae), and Phaeophyta (brown algae) based on their pigments and chemical composition.[144] This project looked at six species of macroalgae; the green alga *Ulva intestinalis* and five brown algae, *Laminaria hyperborea, Laminaria digitata*, *Fucus vesiculosus*, *Ascophyllum nodosum*, and *Saccharina latissima*. The six species studied exhibit unique morphological characteristics and are important components of marine ecosystems.

Ulva intestinalis (syn.: *Enteromorpha intestinalis*) is a green alga belonging to the order Ulvales. The seaweed grows on intertidal surfaces and slightly into the subtidal zone,

often attached to a stable substrate such as rocks.[145–147] It is characterized by its bright green color and consist of a tubular frond and unbranched thalli.[146,148]

Laminaria hyperborea, Laminaria digitata and *Saccharina latissima* (syn.: *Laminaria saccharina*) belong to the order Laminariales, commonly known as kelp.[149,150] Kelps consist of a holdfast (haptera) connecting them to a substrate, as well as stipes and blades (the lamina) which can be both divided and not divided.[151] *L. hyperborea* mainly grows in the northeast Atlantic Ocean, including all along the Norwegian coastline, forming extensive kelp forest at depths of around 30 meters. In Norway, the standing biomass of *L. hyperborea* is about $60x10^6$ tons, the richest fields being on the west coast.[152–154] It can grow up to 4 meters in length and has a round meristem and branched fronds, depending on factors such as age, tide, and wave-exposure (Figure 9).[155–157] *Laminaria digitata* has a semi-stiff stipe and a blade divided into long digits, while *Saccharina latissima* have a short stipe and a long, broad, and unbranched blade that can grow up to 3 m long.[158]

Ascophyllum nodosum and *Fucus vesiculosus* are bladderwrack type seaweeds of the Fucales order, found in the eulittoral zone.[143,150] They possess vesicles filled with gas that allow them to float near the surface. *F. vesiculosus* have a yellow/green to dark brown color and can grow up to 1 m in length and has a broad holdfast, a distinct midrib, and usually branched flat fronds.[159] *A. nodosum* is most commonly yellow or olive-green and can grow up to 2 m in length. It has cylindrical axes and compressed laterals and is attached to a substrate by a disc-like holdfast.[150,160]

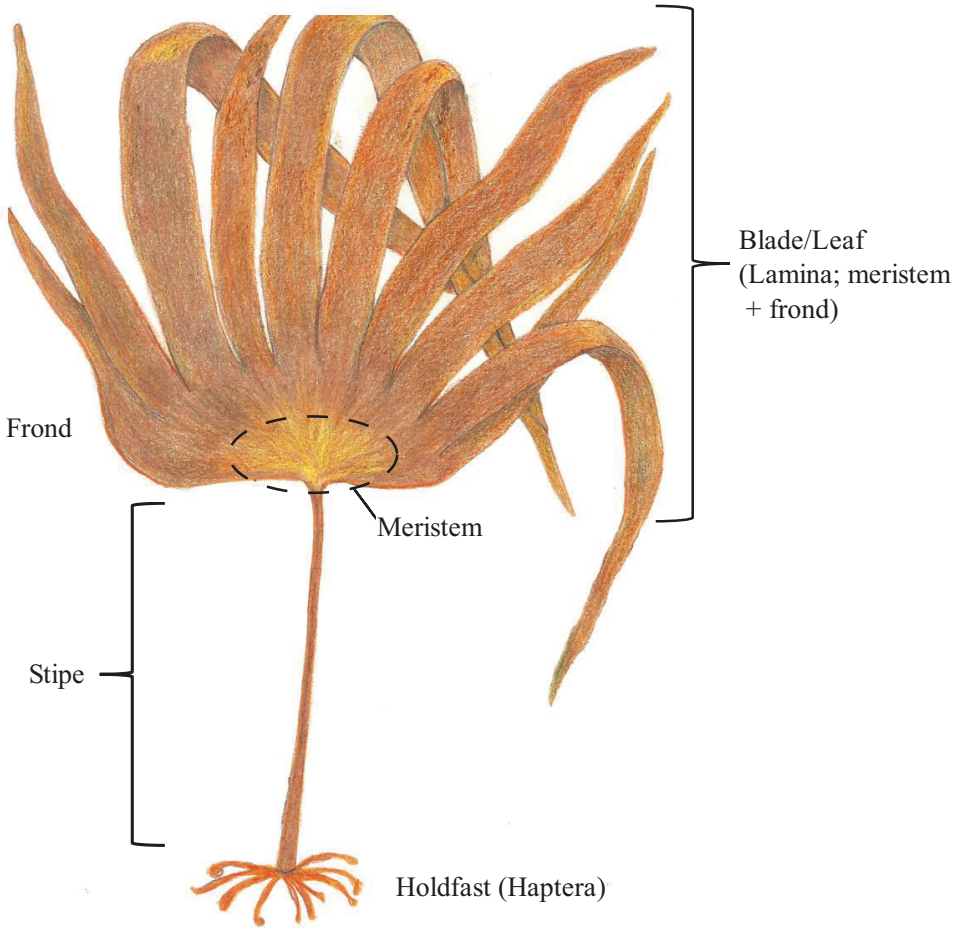


Figure 9: Illustration of *Laminaria hyperborea* showing the leaf with the meristem and frond, the stipe, and the holdfast (M. E. Wekre 2023). Adapted from Toth and Pavia (2002).[151]

1.6.1.1 Polyphenolic content

The polyphenolic content of marine macroalgae is highly variable and can be influenced by a range of intrinsic and external factors. Intrinsic factors, such as species, age, size, and harvest time, can all play a role in determining the polyphenolic composition of macroalgae. However, external factors like UV-radiation, wave exposure, temperature, wounding, salinity, nutrient enrichment, and depth can also significantly impact the synthesis of polyphenols in macroalgae.[31,45,161–166] For instance, studies have shown that

exposure to high levels of UV-radiation, can stimulate the production of polyphenols in algae.[164,167]

1.6.2 Quantitative measurements

Numerous studies investigate the quantification of total polyphenol content in macroalgae by using the Folin-Ciocalteu (FC) total polyphenol content (TPC) assay as described by Singleton and Rossi (1965).[168] Despite being nearly 100 years old, the FC method remains the most referred to method for quantification of polyphenols in not just algae, but also marine- and terrestrial plants, as well as various foods and beverages.[169–178] The TPC assay is a non-selective colorimetric method that reacts with all reducing species in the sample analyzed, rather than just polyphenols, and changes the color of the solution from yellow to blue under basic conditions (Section 3.4.2.1). The quantification is based on the measured absorbance (UV–visible spectrophotometry) and a calibration curve.[179–181] Due to this lack of selectivity, the assay is known to overestimating the polyphenolic content.[182,183] In recent years, quantitative NMR (qNMR) has emerged as a more specific method for quantifying metabolites like polyphenols in complex biological matrices.[184–190] The qNMR method is a primary ratio measurement that utilizes one-dimensional (1D) proton (^{1}H) NMR spectra and a standard (external or internal) compound significantly different from the analyte (Section 3.4.4). Peaks appearing in the aromatic region are integrated and used to quantify the sample.

Table 1 presents an overview of selected publications that analyze various macroalgae in the project, using either the TPC assay, qNMR, or both.

Table 1: Selected reported quantifications of polyphenols (PP) in seaweeds for species within the Fucaceae, Sargassaceae and Laminariaceae families as well as the green seaweeds Ulvaceae, utilizing either the TPC assay or qNMR. Adapted from paper **II**.

Seaweed	Location	Extraction solvent	Method	PP Concentration	Publication
			Fucaceae		
Fucus vesiculosus	Denmark	Ethanol	TPC	12.0 mg GAE/g *	Farvin and Jacobsen (2013)[191]
Fucus vesiculosus	France	Ethanol	qNMR	15.32% TAE	Parys *et al.* (2007)[184]
Fucus vesiculosus	France	Ethanol	TPC	15.88% PGE	Parys *et al.* (2007)[184]
Fucus vesiculosus	Ireland	60% aqueous methanol	TPC	2.5 mg GAE/g DW	O'Sullivan *et al.* (2011)[192]
Fucus vesiculosus	Canada	50% aqueous methanol	TPC	23.21% PGE	Zhang *et al.* (2006)[193]
Fucus vesiculosus	Iceland	70% aqueous acetone	TPC	242 mg PGE/g *	Wang *et al.* (2009)[194]
Fucus serratus	Ireland	80% ethanol	TPC	0.075 mg GAE/g *	Heffernan *et al.* (2014)[195]
Fucus serratus	Ireland	70% aqueous acetone	TPC	30.68 mg PGE/g	Ford *et al.* (2020)[187]
Fucus serratus	Ireland	70% aqueous acetone	qNMR	17.00 mg TAE/g	Ford *et al.* (2020)[187]
Ascophyllum nodosum	Ireland	80% ethanol	TPC	0.101 mg PGE/g *	Tierney *et al.* (2013)[196]
Ascophyllum nodosum	Spain	Water	TPC	59.2 mg PGE/g DW	Gisbert *et al.* (2021)[197]
Ascophyllum nodosum	Ireland	70% aqueous acetone	TPC	36.68 mg PGE/g	Ford *et al.* (2020)[187]
Ascophyllum nodosum	Ireland	70% aqueous acetone	qNMR	37.35 mg TAE/g	Ford *et al.* (2020)[187]
Ascophyllum nodosum	Scotland	Ethanol	TPC	0.3-1.0% PGE FW	Parys *et al.* (2009)[198]
Ascophyllum nodosum	Scotland	Ethanol	qNMR	0.6-2.2% TAE FW	Parys *et al.* (2009)[198]
Ascophyllum nodosum	France	Ethanol	TPC	13.49% PGE	Parys *et al.* (2007)[184]
Ascophyllum nodosum	France	Ethanol	qNMR	25.34% TAE	Parys *et al.* (2007)[184]
			Sargassaceae		
Sargassum muticum	France	75% ethanol	TPC	10.18% PGE	Anaëlle *et al.* (2013)[199]
Sargassum fusiforme	China	30% aqueous ethanol	TPC	63.61 mg PGE/g	Li *et al.* (2017)[200]
Cystoseira tamariscifolia	France	50% aqueous methanol	TPC	0.63% PGE	Jégou *et al.* (2015)[185]
Cystoseira tamariscifolia	France	50% aqueous methanol	qNMR	0.46% PGE	Jégou *et al.* (2015)[185]

Table 1 continued.

Seaweed	Location	Extraction solvent	Method	PP Concentration	Publication
			Laminariaceae		
Macrocystis pyrifera	Chile	70% aqueous acetone	TPC	1.47 mg GAE/g DW	Leyton *et al.* (2016)[46]
Laminaria hyperborea	Ireland	60% aqueous methanol	TPC	1.5 mg GAE/g DW	O'Sullivan *et al.* (2011)[192]
Laminaria hyperborea	Iceland	70% aqueous acetone	TPC	130 mg PGE/g *	Wang *et al.* (2009)[194]
Laminaria digitata	Iceland	70% aqueous acetone	TPC	10 mg PGE/g *	Wang *et al.* (2009)[194]
Laminaria digitata	Denmark	Ethanol	TPC	0.324 mg GAE/g *	Farvin and Jacobsen (2013)[191]
Laminaria digitata	Scotland	80% aqueous methanol	TPC	5.7% GAE	Vissers *et al.* (2017)[186]
Laminaria digitata	Scotland	80% aqueous methanol	qNMR	4.3% GAE	Vissers *et al.* (2017)[186]
Laminaria digitata	Ireland	80% ethanol	TPC	0.0022 mg GAE/g *	Heffernan *et al.* (2014)[195]
Saccharina latissima	Canada	50% aqueous methanol	TPC	2.17% PGE	Zhang *et al.* (2006)[193]
Saccharina latissima	Norway	80% aqueous acetone	TPC	5-15 mg PGE/g DW	Roleda *et al.* (2019)[201]
			Ulvaceae		
Ulva intestinalis	Ireland	80% ethanol	TPC	0.0358 mg PGE/g *	Tierney *et al.* (2013)[196]
Ulva intestinalis	India	Ethanol	TPC	59.67 mg GAE/g	Pradhan *et al.* (2021)[202]
Ulva intestinalis	Denmark	100% Ethanol	TPC	2.37 mg GAE/g *	Farvin and Jacobsen (2013)[191]
Ulva intestinalis	Australia	80% aqueous ethanol	TPC	0.0148 mg GAE/g *	Zhong *et al.* (2020)[203]

*Value recalculated to mg (GAE/PGE)/g from original publication. GAE = Gallic Acid Equivalents. PGE = Phloroglucinol equivalents. TAE = Trimesic acid equivalents.

1.6.3 Seasonal variation

The synthesis of polyphenols and other secondary metabolites in macroalgae depends on several external factors, including both biotic and abiotic, resulting in expected seasonal fluctuations. Such variations have been documented for several terrestrial- and marine plants [96,204–209], as well as for some macroalgae.[166,187,210–215] Although polyphenol content has been shown to vary with seasons, as well as intrinsic- and external factors as previously mentioned, additional factors such latitudinal gradients, geographical regions, grazing, tidal patterns, and spatial scales also influence the production of polyphenols in macroalgae.[167,211]

Mannino *et al.* (2016)[210] found that the total polyphenol content of the brown algae *Cystoseira amentacea* to be highest during winter, whereas Abdala-Díaz *et al.* (2006)[214] reported that *Cystoseira tamariscifolia* to have the highest polyphenol content during the summer months. Both algae belong to the same genus, Cytoseira, in the Sargassaceae family, and both studies utilized the TPC assay for polyphenol quantification. Gorham and Lewey (1984)[216] analyzed another alga belonging to this family, *Sargassum muticum*, and also found that the polyphenol concentration was highest in summer, using an earlier variation of the TPC assay with the Folin-Denis reagent. However, the material was harvested at different locations (Italy, Spain, and the UK) as well as during different years (2011, 1998-2000, and 1984), which will also influence the polyphenolic content. Furthermore, the seasonal variation may affect the TPC assay, as the TPC-interfering compounds in the algal biomass may also vary with seasons. Additionally, changes in the polyphenol heterogeneity may affect the TPC results due to the possible varying number of reacting groups within the polyphenolic matrix.

Several studies have investigated the seasonal variation of the polyphenolic content in the brown algae *Ascophyllum nodosum* using different analytical methods. Parys *et al.* (2009)[198] used both qNMR and the TPC assay to measure the polyphenolic content and reported the highest values during the summer months (June-July) regardless of the method. Connan *et al.* (2004)[217], Apostolidis *et al.* (2011)[218], Tabassum *et al.* (2016)[215], and Garcia-Vaquero *et al.* (2021)[219] also utilized the TPC assay and reported the polyphenolic content of *A. nodosum* to be highest during the summer months. In contrast, Ford *et al.* (2020)[187] reported some seasonal variation in the polyphenolic

content of *Ascophyllum nodosum*, with a maximum observed in February when using the TPC assay, but a maximum in May when using qNMR. These studies collectively demonstrate that the polyphenolic content in *Ascophyllum nodosum* exhibits seasonal variation, with the highest values typically observed during the summer months.

Schiener *et al*. (2014) investigated the seasonal variation in the chemical composition of four brown seaweeds from the Laminariales order, including *Laminaria hyperborea*, *Laminaria digitata*, *Saccharina latissima*, and *Alaria esculenta*, using the TPC assay. The study found that *L. hyperborea* and *S. latissima* had the highest polyphenol content in July, whereas *L. digitata* and *A. esculenta* showed the highest concentrations in May. Similarly, Garcia-Vaquero *et al.* (2021)[219] reported the highest polyphenol concentration of *L. digitata* and *L. hyperborea* in summer when using the TPC assay. However, Connan *et al.* (2004)[217] found that *L. digitata* had the highest polyphenol content in winter when analyzing various brown seaweeds from the coast of Brittany, France, also using the TPC assay. Abdulla and Fredriksen (2004)[155] also used the TPC method to analyze *Laminaria hyperborea* from the west coast of Norway and found that the maximum polyphenol content was obtained in March. However, it is clear that the choice of analytical method can also impact the results, as seen in the discrepancies between the TPC assay and qNMR results reported by Ford *et al.* (2020)[187].

2. Motivation and aim

Seaweed is a highly abundant and diverse marine bioresource with a wide range of ecological and commercial applications. Seaweed polyphenols are of particular interest due to their potential health benefits, including antioxidant and anti-inflammatory effects. The presence of more advanced polyphenols such as flavonoids in seaweed, however, is still debated.[27,95,100,220–228] The flavonoid biosynthetic pathway has long been considered exclusive to terrestrial plants or aqueous plants with a terrestrial origin, such as *Zostera marina* and other seagrasses. Some studies have reported the presence of flavonoids in seaweed, while others do not report flavonoids. Nevertheless, there are reports of flavonoids from marine algae. Although many studies have only reported total amounts of flavonoids using general assays (such as the TFC assay), a few studies have reported more thorough investigations at the molecular level using LC-MS and NMR detailing specific flavonoid structures in algae.[100,229–233] Despite the low concentration of flavonoid compounds in algae compared to those in land plants, the existence of a biosynthetic pathway to flavonoids in algae cannot be ruled out.[221]

The most common polyphenols in macroalgae are the phlorotannins, diverse oligomers of phloroglucinol, found in brown algae. These phlorotannins are produced by the condensation of malonyl-CoA by a polyketide synthase (PKS), similar to the action of chalcone synthase (CHS) in the flavonoid biosynthetic pathway. Given the large phylogenetic distance between brown algae and land plants, Davies *et al.* (2020)[221] report that this, however, may be an example of parallel evolution. The characteristics of individual polyphenols present in seaweed are not fully researched or understood. This is partly due to the polyphenolic content in seaweed often being measured with the beforementioned "total" assays, non-selective colorimetric methods which fail to reflect the chemodiversity and the composition of the polyphenolic content. The complex matrix of natural products in seaweed can interfere with the extraction and purification processes – as the high content of polysaccharides (such as alginate, and fucoidan), making it difficult to obtain pure polyphenol extracts. Additionally, the tough cell walls of seaweed can make it challenging to break down the cell structure and release the polyphenols. Furthermore, the lower concentration of polyphenols in seaweed relative to other biomasses, makes it necessary to process larger quantities of seaweed to obtain the same quantity of polyphenols, making analysis of seaweed polyphenols challenging. Extraction and purification of the phenolic content of marine macroalgae needs to be optimized to better investigate their polyphenolic content. Furthermore, gaining more knowledge about the actual polyphenol content can provide further insights into the evolution of polyphenolic production and flavonoid biosynthesis in algae, especially considering their phylogenetic distance from terrestrial plants. Therefore, research should aim to investigate the specific characteristics of individual polyphenols present in seaweed, as this will provide molecular-level insights into the composition of the polyphenolic content of seaweed. However, to be able to perform such studies, optimization of extraction and analyses must be performed.

Laminaria hyperborea, a type of brown algae common along the Norwegian coastline, has traditionally been harvested for its alginate content. Alginate is a polysaccharide widely used in industries such as food, pharmaceuticals, and cosmetics. The current harvesting and production practices are only able to utilize a small fraction of the seaweed biomass (the stipe), and most of the algae biomass (the leaves) are considered

as “waste”. This waste contains valuable compounds that could be extracted and utilized for other purposes, such as polyphenols with potential health benefits. To achieve a more sustainable and efficient use of *Laminaria hyperborea*, Alginor ASA is exploring extraction and the potential value of various other products from the liquid and solid waste streams of alginate production. The aim of utilizing the entire seaweed biomass for multiple products is referred to as a “total utilization approach” or “seaweed biorefinery”.

By researching the underexplored polyphenolic content of *Laminaria hyperborea*, we can gain new insights into the composition of the seaweed’s polyphenolic content and better be able to evaluate their potential applications. Increased information on seaweed polyphenols at a molecular level can also shed light on the evolution of polyphenolic production and flavonoids biosynthesis in algae. Ultimately, the aim of this project was to investigate the polyphenolic content of *Laminaria hyperborea* and provide new insights into its potential use as a life science ingredient. By optimizing seaweed polyphenolic quantification as well as qualitative data on molecular level, we hoped to contribute to both the development of more sustainable and comprehensive approach to seaweed biorefinery, but also further the scientific knowledge of seaweed polyphenols.

3. Methods

Experimental- and analytical methods used in this work are described in the following four sections: macroalgae sampling (3.1), extraction and purification (3.2), separation and isolation (3.3), and analytical methods (3.4). Additional and specified experimental details can be found in the individual papers (**I-III**).

3.1 Macroalga sampling

Several different macroalgae samples were collected and studied throughout the project. Paper **I** analyzed samples of *Ulva intestinalis*, which were hand-harvested in Ormhilleren, Norway. In papers **II** and **III** multiple brown seaweeds were studied. All samples of *Laminaria hyperborea* leaves and *Laminaria digitata* leaves were supplied by Alginor ASA, and samples of *Saccharina latissima* were supplied by Ocean Forest (Lerøy AS). All other samples were hand-collected at locations described in table 2. The materials were dried, cut into smaller pieces, and stored at -20^{o}C when not used.

Table 2: Overview of all macroalgae batches studied in the project.

Sample	Collection date	Location
Ascophyllum nodosum	06.06.2017	Eidsvåg, Bergen (N 60°26'63' E 05°17'87')
Ulva intestinalis	12.09.2018	Ormhilleren, Rong (N 60°29.68' E 4°55.33')
Fucus vesiculosus	17.08.2019	Storåkervik, Bergen (N 60°30.1044′ E 5°15.6726′)
Laminaria digitata	15.08.2019	Melbourne, Australia
Saccharina latissima	28.04.2021	Trollsøy, Vestland (N 60°8.42′ E 5°14.88′)
Laminaria hyperborea M20	09.03.2020	Rogaland Field 55E (N 59°11′ E 005°06')
Laminaria hyperborea S20	15.09.2020	Rogaland Field 55E
Laminaria hyperborea A21	17.08.2021	Rogaland Field 55E

3.2 Extraction and purification

3.2.1 Extraction

The extraction of polyphenols from *Laminaria hyperborea* was optimized by testing three different extraction methods, including traditional maceration, ultrasound assisted extraction (UAE), and accelerated solvent extraction (ASE), with various solvent mixtures of methanol (MeOH), ethanol (EtOH), ethyl acetate (EtOAc), and water, as well as different extraction temperatures and times. HPLC with a phloroglucinol calibration curve was initially used for quantification, however it was later determined that the TPC assay and qNMR were better suited for this purpose. Nonetheless, all samples in the initial study were analyses in the same manner, allowing for the determination of the optimal extraction method based on relative results.

While 60% methanol and ASE resulted in the greatest polyphenol recovery, the use of ASE is not practical for large scale extractions. Extraction with 50% aqueous EtOH using UAE and traditional maceration with 60% MeOH also yielded high polyphenol

recovery. Comparison of the ethanolic- and methanolic extracts using ^{1}H NMR indicated similar trends, and throughout the project, maceration with 60% MeOH was primarily employed.

3.2.2 Liquid-liquid partitioning

Liquid-liquid partitioning is a commonly used method for separating and purifying compounds based on their polarity. The principle of liquid-liquid extraction relies on the fact that different compounds have different solubilities in different solvents. By choosing appropriate solvents with different polarities, it is possible to selectively partition compounds of interest into different solvent phases, while leaving unwanted impurities behind.[234,235] In papers **I** and **III**, aqueous crude extracts were partitioned against hexane and ethyl acetate to remove compounds with less polar characteristics, such as chlorophylls and stilbenes, from the samples. However, some polar polyphenols were later observed in the ethyl acetate fraction, highlighting the complexity of macroalgae samples. Nonetheless, liquid-liquid partitioning is an important tool for separation and purification of compounds in complex mixtures and has been used in numerous studies to selectively separate high-value compounds.[46,72,116,186,187,236,237]

3.2.3 Amberlite XAD-7 (adsorption chromatography)

The purification of selected samples was accomplished by employing Amberlite XAD-7 column material, which is a type of adsorption chromatography frequently utilized in natural product chemistry for the separation and purification of compounds from complex mixtures. The technique involves the use of a solid stationary phase, in this case the Amberlite XAD-7 resin, which adsorbs specific compounds from a liquid phase. The elution of the adsorbed compounds is achieved by washing the column with an appropriate solvent, the elution profile being influenced by the physical and chemical properties of the compounds being separated.[234,238]

The Amberlite XAD-7 resin is moderately polar, rendering it suitable for the separation of non-aromatic and aromatic compounds in polar solvents. The resin selectively retains aromatic compounds such as polyphenols, while non-aromatic compounds such as free

sugars and aliphatic acids are removed by washing the column with distilled water. Once the non-aromatic compounds have been removed, the aromatic compounds can be eluted with methanol, and fractions of aromatic compounds with varying polarity can be collected. Adsorption chromatography with XAD-7 was used in paper **I** for the analysis of *Ulva intestinalis*.

3.3 Separation and isolation

3.3.1 Flash chromatography

Purification of crude extracts were performed using flash chromatography. Interchim puriFlash® was used with a Biotage Sfär C18 D column. The solvents used were water (A), methanol (B), and ethyl acetate (C), with initial conditions of 95% A and 5% B. The gradient followed as; 0–6 min: 95% A + 5% B, 6–12 min: 75% A + 25% B, 12–18 min: 50% A + 50% B, 18–24 min: 25% A + 75% B, 24–36 min: 100% B, 36–42 min: 50% B + 50% C, 42–48 min: 100% C. Flowrate was 12 mL/min and 15 mL sample were added to the column. Detection was done at 280 nm and using a spectral scan between 200-800 nm.

3.3.2 Preparative High Performance Liquid Chromatography (HPLC)

Preparative HPLC was applied to further separate and purify polyphenolic fractions obtained using either XAD-7 (paper **I**) or flash chromatography (paper **III**). Preparative HPLC is commonly used to isolate and purify compounds from complex mixtures at larger quantities. Compounds may be separated based on their physiochemical properties, such as size, charge, and polarity, and can be collected in fractions as they elute from the column. Despite successful separation attempts, no individual polyphenol could be isolated from either *Ulva intestinalis* or *Laminaria hyperborea* using preparative HPLC, but purified mixtures of polyphenols were obtained. This may be due to the complexity of the samples or the low abundance of polyphenols in the samples.

It is worth noting that two different instruments and solvent systems were employed throughout the project:

1. The preparative HPLC system consisted of a Gilson 321 pump (Gilson Inc., Middleton, WI, USA), an Ultimate 3000 variable wavelength detector (Dionex, Thermo Fisher Scientific, Sunnyvale, CA, USA), and a 25 × 2.12cm (10µm) UniverSil C18 column (Fortis Technologies Ltd., Neston, UK). The solvents were (A) super distilled water (0.1% acetic acid) and (B) acetonitrile (0.1% acetic acid) with initial conditions of 90% A and 10% B followed by an isocratic elution for the first 5 minutes, and the subsequent linear gradient conditions, 5–18 min: to 16% B, 18–22 min: to 18% B, 26–31 min: to 28% B, 31–32 min: to 40% B, 32–40 min: isocratic at 40% B, 40–43 min: to 10% B. The flow rate was 15mL/min, and aliquots of 750µL were injected. The detection was at 360nm.
2. The preparative HPLC system consisted of a Dionex Ultimate 3000 pump (Thermo Fisher Scientific, Sunnyvale, CA, USA), the Dionex 3000 variable wavelength detector (Thermo Fisher Scientific, Sunnyvale, CA, USA), and a 250 × 22mm (10µm) Econosphere C18 column (Dr. Maisch, Ammerbuch, Germany). The solvents were (A) super distilled water (0.5% TFA) and (B) acetonitrile (0.5% TFA). Initial conditions were 95% A and 5% B. Gradient (0-10 min: 95% A + 5% B, 10-20 min: 85% A + 15 % B, 20-34 min: 60% A + 40% B. 34-35 min: 95% A + 5% B). The flowrate was 18mL/min and aliquots of 2mL were injected.

System 1 was used in paper **I**, whereas system 2 was used in paper **III**.

3.4 Analytical methods

3.4.1 Analytical High Performance Liquid Chromatography (HPLC)

In both paper **I** and **III** reverse phase analytical HPLC was used to measure and quantify polyphenols from macroalgae samples. Analytical HPLC is a well-established technique for the qualitative and quantitative analysis of organic compounds, including polypehnols.[239–242] Similarly to preparative HPLC, the method involves the separation of complex mixtures of compounds in a sample based on their physiochemical

properties. However, analysis is performed with smaller quantities and no sample is collected after separation.

The HPLC system employed an Agilent 1260 infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA), which included a 1260 diode array detector (DAD) and a 200 × 4.6mm, 5µm ODS Hypersil column. The solvents were (A) super distilled water (0.5% TFA) and (B) acetonitrile (0.5% TFA) in a gradient (0-10 min: 95% A + 5% B, 10-20 min: 85% A + 15% B, 20-34 min: 60% A + 40% B. 34-35 min: 95% A + 5% B). The flow rate was maintained at 1 mL/min, and aliquots of 20µL were injected for analysis. UV-VIS spectra were recorded over a range of wavelengths (200-600nm) in steps of 2nm.

For quantification of polyphenols in the samples, various calibration curves were used with the HPLC method. The calibration curves were used to determine the concentrations of polyphenols in the samples by comparing their peak areas to those of the standard compounds.

3.4.2 Ultraviolet/Visible (UV-Vis) Spectroscopy

UV-Vis spectra were obtained during online HPLC analysis (sections 3.3.2 and 3.4.1). UV-Vis spectroscopy was also used to perform several colorimetric assays; the Folin-Ciocalteu (FC) total polyphenol content (TPC) assay and the total flavonoid content (TFC) assay (paper **I**). The assays were performed using a Biochrom Libra S32 UV instrument (Biochrom, Cambridge, United Kingdom).

3.4.2.1 Total Phenolic Content (TPC) Assay

The Folin-Ciocalteu (FC) Total Phenolic Content (TPC) assay is a widely used method for simple quantification of polyphenols in natural product samples. This method involves a non-specific redox reaction occurs between the FC-reagent, which contains molybdotungstate, and a reducing species (such as polyphenols) in alkaline conditions (Figure 10).[179,180] The reaction results in a blue-colored complex, which is measured at an absorbance of 760nm.

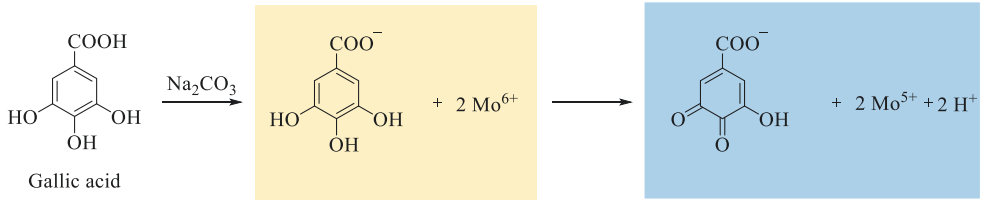


Figure 10: Reaction of molybdotungstate (FC reagent) with gallic acid (reducing species) with indications of the observed color change. Adapted from Prior *et al.* (2005) and Martono *et al.* (2019).[180,243]

To perform the TPC assay, most studies refer to the methodology described by Singleton and Rossi (1965)[168] and Singleton *et al.* (1999)[181] with slight modifications reported in some cases.[46,57,202,203,242] To optimize the TPC assay for macroalgae, an initial study was conducted, which included the optimization of reactant ratios, sample dilution, incubation time, and measure wavelength (Section 4.3.2). The results showed parameters similar to those reported by Singleton *et al.* (1999)[181], with slight modifications. Specifically, the TPC assay was performed by using 0.2mL sample, blank, or standard, 1.59mL Folin-Ciocalteu reagent, 4.0mL 20% (w/v) Na_2CO_3 and made to a total volume of 2mL with water. The mixture was incubated for 2 hours in the dark, and absorbance was measured at 760 nm. Samples were diluted to concentrations between 5000-10000ug/mL DW and always analyzed in triplicates (n = 3).

3.4.2.2 Total flavonoid content (TFC) Assay

The TFC assay is based on the ability of flavonoids to form complexes with aluminum chloride, which results in a bathochromic shift in the UV-Vis spectra.[244,245] The type of complex formed depends on the structure of the flavonoid (Figure 11). Flavonoids with hydroxyl groups at C-3 or C-5 and a carbonyl group at C-4 will form acid-stable complexes, and ortho-dihydroxyl system containing flavonoids will form acid labile complexes.

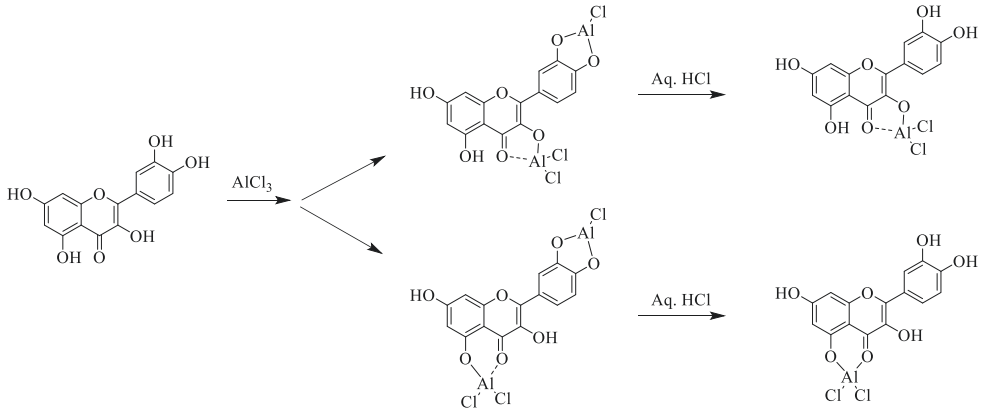


Figure 11: Reaction of quercetin with aluminum chloride forming both acid stable and acid liable complexes. Adapted from Mabry *et al.* (1970).[244]

The TFC method used was adapted within the Jordheim research group (data not published) based on procedures described by Pękal & Pyrzynska (2014)[245] and Woisky & Salatino (1998).[246] A test solution of 2mL (standard or sample) was added to four cuvettes (10 × 45mm, 3mL) and the absorbance measured at 425nm with solvent in the reference cuvette. An aliquot of $AlCl_3$ solution (0.5mL, 1%, w/v) was added to three of the four cuvettes, and the same volume of solvent was added to the fourth (blank sample). The contents of the cuvettes were stirred thoroughly, and the absorbance was measured at 1-minute intervals for 10 minutes at 22°C. For quantitative analysis apigenin was chosen as the reference compound in a concentration range of 1–500µg/mL. The TFC assay was utilized in paper **I** to determine the flavonoid content in samples of *U. intestinalis*.

3.4.3 Oxygen radical absorbent capacity (ORAC) Assay

The ORAC assay is a widely used method to measure the antioxidant capacity of a sample. The assay measures the ability of antioxidants to neutralize free radicals by monitoring the decay of fluorescent molecules in the presence of a peroxyl radical generator (in this study, AAPH).[247,248] In this study, ORAC assay was conducted by MARBIO at the University of Tromsø using 96-well microtiter plates kept in the dark. To prepare the samples, fluorescein sodium salt, AAPH (2,2'-azobis (2-methylpropionamide) dihydrochloride), and Trolox ((±)-6-Hydroxy-2,5,7,8-

tetramethylchromane-2-carboxylic acid) were dissolved in 60mM phosphate buffer (PB). Trolox was utilized as a calibration curve. Standards and samples were pipetted in duplicate with fluorescein and incubated at 37°C for 15 min. Ice cold AAPH was subsequently added, and the plate was immediately placed into the Tecan Spark multimode reader. Fluorescence was recorded (Ex 485nm ± 20 and Ex 525nm ± 20) 25 times in intervals of 90 seconds. The area under the curve (AUC) was calculated, and the AUC for 0µM Trolox was subtracted to determine the area between curves (ABC). The ABC for samples were then compared to the Trolox standard curve to obtain Trolox equivalents (TE). Results are reported as µmol TE/mg and were presented in paper **III**.

3.4.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical tool that provides valuable insights into the structure and properties of molecules. NMR is traditionally the most used method for structural elucidation of compounds in natural product chemistry.[249–253] Additionally, newer quantitative methods (qNMR) have been found to be an important tool for quantification of complex natural product samples such as macroalgae.[184,188,189,254–257] NMR experiments were obtained using a Bruker 600MHz instrument. All samples were dissolved in *d6*-DMSO (0.03% TMS), and the sample temperature was kept at 298K. For quantitative analyses, $DMSO_2$ (C = 10mM) was used as the internal standard. For specified NMR conditions refer to papers **I-III**.

One-dimensional (1D) ^1H NMR. The 1D proton experiments are useful for analyzing the hydrogen atoms in a compound. These experiments can provide information about the chemical shift and coupling constants (J_{HH}), which reveal details about the chemical environment and connectivity of atoms. The 1D ^1H NMR is a highly sensitive experiment due to the high abundance of the ^1H isotope. This technique can also be used for quantitative analysis, known as qNMR. The method is based on the fact that the area under an NMR signal is directly proportional to the number of protons producing the signal, which in turn is proportional to the amount of substance in the sample. Therefore, the quantity of a substance can be accurately determined by comparison of its NMR

signal to a known standard. To calculate the molar concentration of a sample using qNMR, equation 1 can be used:

$$\mathrm{C_{sample}}\ [\mathrm{M}] = \frac{I_{\mathrm{sample}} \times n_{\mathrm{DMSO2}} \times C_{\mathrm{DMSO2}}}{I_{\mathrm{DMSO2}} \times n_{\mathrm{sample}}} \qquad \textbf{(1)}$$

Here, C is the molar concentration, I is the signal integral, and n represents the number of protons that yield the signal. The variables n_{DMSO2} and n_{sample} represent the number of protons in the reference compound ($DMSO_2$) and the sample, respectively. By using this equation, the quantity of a substance in a sample can be accurately determined, making qNMR a valuable tool in quantitative analysis.

One-dimensional (1D) ^{13}C NMR. 1D carbon spectra provide direct information about the carbon skeleton of a compound, and can also be used to determine the type of carbon atoms.[258] The experiment is less sensitive than proton NMR due to the low abundance of the ^{13}C isotope (1.1%). The *udeft* (uniform driven equilibrium Fourier transform) pulse sequence was utilized for 1D ^{13}C NMR spectra. DEFT NMR experiments were proposed for nuclei with long relaxation times (T_1), such as carbon. The experiment recycles the carbon magnetization along the Z axis of the rotating frame after each transient.[259] UDEFT allows recording of ^{13}C spectra of slow relaxing nuclei using a short relaxation delay and thus decreases the experiment time. The experiment was chosen due to its maximized signal-to-noise ratio and increased sensitivity.[260]

HSQC. Two-dimensional Heteronuclear Single Quantum Coherence (HSQC) experiments yield spectra displaying the $^1J_{XH}$ couplings between heteronuclei (X = $^{13}C/^{15}N$) and directly coupled protons (H). In the HSQC spectrum, each peak corresponds to a particular carbon or nitrogen that is directly bonded to a proton. The position of each peak depends on the chemical shift of the heteronucleus and the J-coupling constant ($^1J_{XH}$). This experiment is useful for identifying the functional groups present in a molecule and determining their connectivity.

HMBC. The 2D ^1H-^{13}C Heteronuclear Multiple Bond Correlation (HMBC) experiment provides information about the coupling between carbon and protons that are separated by two or three bonds ($^2J_{CH}$ and $^3J_{CH}$) through cross-peaks. These cross-peaks thus

provide information about the connectivity of the molecule and can be used to identify individual spin-systems. The HMBC experiment is particularly useful for studying long-range couplings in complex molecules.

COSY. The Correlation spectroscopy (COSY) experiment is a 2D homonuclear ^{1}H-^{1}H experiment showing *J*-couplings of neighboring protons (J_{HH}) up to four bonds apart. The spectra consist of identical axes and diagonal- and off-diagonal peaks (cross-peaks).[258] Diagonal peaks indicate the protons chemical shift, and the cross peaks indicate the coupling between two protons. The COSY experiment is very useful to study small molecules and determining stereochemistry, additionally it is commonly used in natural product chemistry when several multiplets overlap and/or the 1D ^{1}H spectra are complicated.

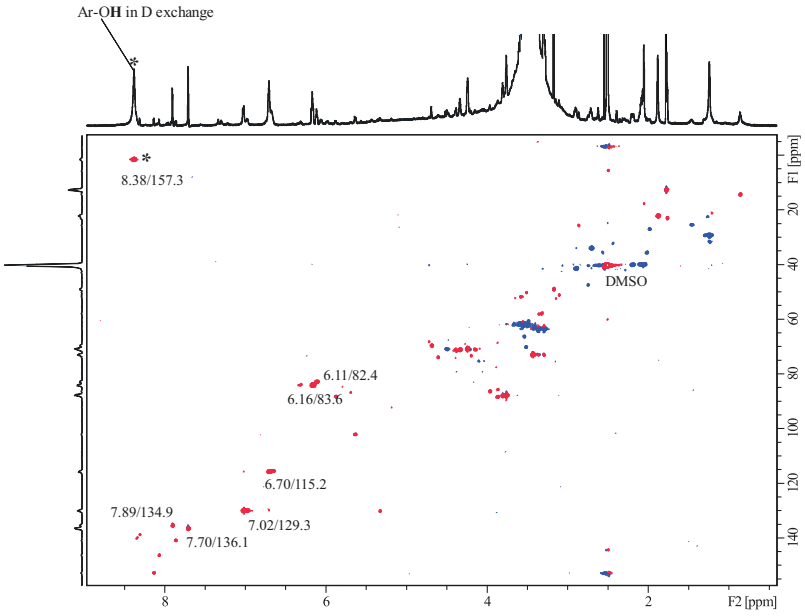


Figure 12: Example of a ^{1}H-^{13}C HSQC spectrum of a purified *Ulva intestinalis* sample, displaying chemical shift values for selected protons and carbons indicating various spin systems in the aromatic region.

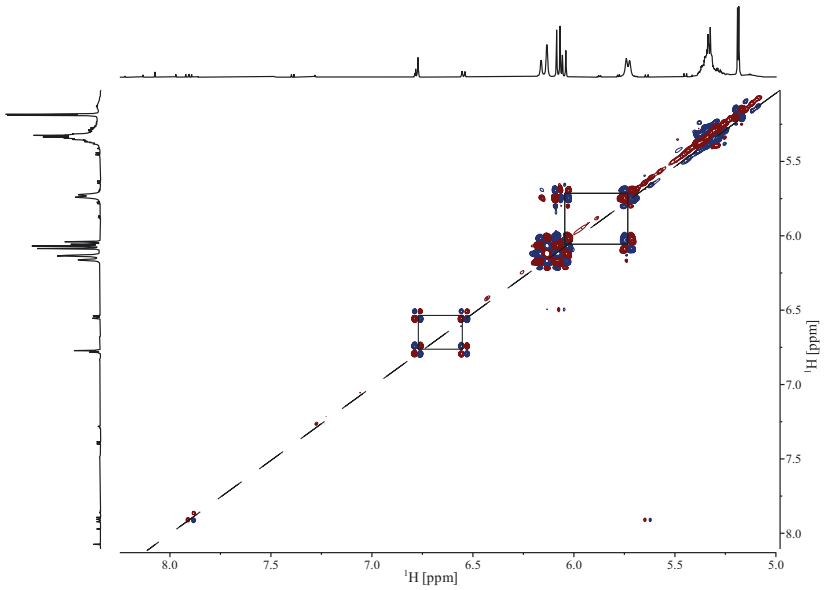


Figure 13: Example of a ^{1}H-^{1}H COSY spectrum of a purified *Ulva intestinalis* sample displaying the aromatic region showing the diagonal (chemical shifts) with cross-peaks (J_{HH}).

3.4.5 Mass Spectrometry (MS)

Mass spectrometry is a highly versatile and widely employed analytical technique for identifying and quantifying compounds in a sample. It operates by ionizing molecules and subsequently measuring the mass-to-charge ratio of the resulting ions.[258] This data can then be utilized to identify the chemical composition of a sample, as well as to determine the molecular structure of complex molecules. In this project, mass spectrometry was utilized to analyze purified samples. Low-resolution (LR) LC-MS was used for initial Total Ion Chromatogram (TIC) scans, as well as for both Selected Ion Monitoring (SIM) and Product Ion (PI) scans (MS/MS). TIC scans returned several masses detected in the samples, and SIM and PI scans further searched for these masses and their fractionation patterns. High-resolution (HR) LC-MS was also utilized to determine exact masses of compounds and as well as to obtain fragmentation spectra.

Two different gradient systems were used when performing LC-MS:

1. Agilent Technologies 1260 Infinity Series system and an Agilent Technologies 6420A triple quadrupole mass spectrometry detector. The following conditions were applied: ionization mode: positive/negative, capillary voltage = 3000V, gas temperature = 300°C, gas flow rate = 3.0L/min, acquisition range = 100–800m/z. The elution profile for HPLC consisted of the following gradient: 0–3 min: 10 % B, 3–11 min: 14% B, 11–15.5 min: 40% B, 15.5–17 min: 10% B, at a flowrate = 0.3mL/min, where solvent A was super distilled water (0.5% formic acid), and solvent B was acetonitrile (0.5% formic acid). A 50 × 2.1mm internal diameter, 1.8µm Agilent Zorbax SB-C18 column was used for separation.
2. Agilent Technologies 1260 Infinity Series system and an Agilent Technologies 6420A triple quadrupole mass spectrometry detector. The following conditions were applied: ionization mode: negative, capillary voltage = 3000V, gas temperature = 300°C, gas flow rate = 3.0L/min, acquisition range = 100–2000m/z. The elution profile for HPLC consisted of the following gradient: 0-4 min: 1% B, 4-5 min: 5-10% B, 5-7 min: 60% B, 7-9 min: 70% B, 9-11 min: 75% B, 11-13 min: 75-1% B, 13-15 min: 1% B. The flowrate was 0.3mL/min, solvent A was super distilled water (0.5% formic acid), and solvent B was acetonitrile (0.5% formic acid). A 50 × 2.1mm internal diameter, 1.8µm Agilent Zorbax SB-C18 column was used for separation.

Gradient system 1 was used in paper **I**, and gradient system 2 was used in paper **III**.

3.4.6 Raman Micro-spectroscopy

Raman spectroscopy is a robust bioanalytical technique that enables the identification and characterization of a sample's chemical composition through the inelastic scattering properties of its molecular bonds.[261] In this project, raman micro-spectroscopy was conducted at room temperature using a Bruker Senterra II Spectrometer equipped with a 785nm laser, scanning from 100–4000cm^{-1} with an integration time of 30 seconds and 3 accumulations. Due to the microscope array, a sample size less than 0.5mg was

sufficient to achieve the necessary resolution. No additional sample preparation was required. Raman was used to help identify sulfated polyphenols in paper **III**.

3.4.7 Infrared (IR) Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy is a widely used analytical technique that is based on the absorption of infrared radiation by molecular bonds.[258] In this project, FTIR spectroscopy was performed using a Bruker Alpha II instrument equipped with a Platinum ATR module, a Rocksolid^{TM} interferometer, and a deuterated triglycine sulphate (DTGS) detector. Samples were analyzed in both solid and liquid form. Liquid samples were dissolved in water or methanol and all samples were scanned from 400–4000cm^{-1} with an integration time of 360 seconds. The data was processed using the OPUS software. IR was used to help identify sulfated polyphenols in paper **III**.

4. RESULTS AND DISCUSSION

This chapter focuses on the main findings of papers **I**-**III**, which aimed to optimize the extraction, quantification, and characterization of polyphenolic compounds in macroalgae, with a focus on *Laminaria hyperborea*, for potential use as life science ingredients in the seaweed biorefinery. The published papers follow a natural evolution of knowledge, starting with a case study of the green alga *Ulva intestinalis* (paper **I**). Paper **II** focused on quantification methods for the quantification of the total polyphenolic content in *L. hyperborea* and other brown algae. The purification and characterization of the polyphenolic content of *L. hyperborea* were the focus of paper **III**.

4.1 Polyphenols in macroalgae: *Ulva Intestinalis* – a case study (paper I)

The extraction of polyphenols from the brown alga *L. hyperborea* using traditional methods is challenging. This is due to the presence of various interfering species, including alginate, mannitol, and other sugar polymers in the algal matrix, which impede the extractability of polyphenols.[161,199,200,235,236,242,262] To simplify the analysis of polyphenols and optimize the workflow, a case study was conducted on the green alga *U. intestinalis*, which has a less complex morphology and lower levels of polysaccharides and phlorotannins than brown algae. This study served as a preliminary step towards establishing a baseline for the analysis of polyphenols in algae. By using *U. intestinalis* as a reference point, the results obtained for *L. hyperborea* and other brown algae were more accurate, reliable, and comparable. Furthermore, the analysis of *U. intestinalis* provided novel insights into its polyphenolic content.

4.2 Extraction optimization of *Laminaria hyperborea* leaves

The optimal extraction conditions for polyphenol recovery from brown algae were initially tested using *L. hyperborea* harvested in March of 2020 (M20). Three different extraction methods were examined, namely accelerated solvent extraction (ASE), ultrasound assisted extraction (UAE), and maceration. Additionally, various solvents (MeOH, EtOH, EtOAc, and H_2O), temperatures, and solvent ratios were tested (Table A1, appendix A). The quantification was performed using DAD-HPLC with a phloroglucinol calibration curve, as the optimization of the TPC method was under development. Based on the DAD-HPLC analyses, the highest polyphenol recovery/extraction yield was observed for the ASE extraction with 60% aqueous methanol. However, the method gave practical challenges due to the alginate clogging of the extraction cells, resulting in major limitations on sample size loadings. ASE extraction will therefore meet challenges within industrial applications, due to the poor up-scaling potential.[263–266] Both UAE with 50% aqueous ethanol and maceration using 60% aqueous methanol yield similar polyphenol recovery. Ummat *et al*. (2020)[266] also found similar UAE conditions for optimal recovery of polyphenols from brown algae. To compare the general polyphenolic trend in extracts, ^{1}H NMR was performed. The ^{1}H

NMR spectra of *L. hyperborea* extracted with both 60% methanol (maceration) and 50% ethanol (UAE) were compared and displayed similar trends, thereby indicating that using either UAE with 50% ethanol or maceration with 60% methanol are good methods for the recovery of polyphenols from brown algae.

4.3 Quantification of Total Polyphenolic Content

Accurately quantifying the total polyphenolic content of organic biomass is generally challenging due to the lack of a single suitable method for this task.[267] Consequently, inaccurate measurements and assumptions may be made based on these data. In the case of brown seaweeds, quantifying total polyphenolic content is particularly difficult due to their challenging morphology and high content of polar polysaccharides, combined with the lower amounts of polyphenols that are less well documented. Moreover, the use of various extraction methods and analytical techniques can affect the obtained results. Therefore, it is crucial to develop more reliable and optimized methods for polyphenol quantification of marine algae for scientific purposes and the evaluation of industrial applications of marine polyphenols.
This chapter aims to present the experienced challenges of polyphenol quantification in the case study of *Ulva intestinalis*, describe the optimization of the TPC and qNMR methods in response to these challenges, and finally present how these methods were applied in the study of *Laminaria hyperborea*.

4.3.1 Quantification of polyphenols in *Ulva intestinalis* (paper I)

Polyphenol quantification of *U. intestinalis* was carried out using four methods: Folin-Ciocalteu (FC) total phenolic content assay (TPC), total flavonoid content assay (TFC), DAD-HPLC, and qNMR. Although DAD-HPLC has traditionally been the preferred method for natural product quantification due to the separation of individual compounds and thus possibility of individual quantification. However, its reliance on molar absorptivity means that using only one standard for the simultaneous quantification of different polyphenols may not yield accurate results as illustrated in paper **I**.[268] Tsao and Yang (2003)[269] proposed a time-consuming HPLC method that separates polyphenols

into five main categories, and quantification performed at different wavelengths using various reference standards. However, this method would require a large library of reference standards as well as some preliminary knowledge of the sample's polyphenolic content, which is not ideal. Optimization of the DAD-HPLC quantification method was achieved by utilizing two wavelength windows as the basis for the calculations. Peaks eluting between 1-15 minutes were quantified with a gallic acid (GA) calibration curve in the 280nm window, whereas peaks eluting between 15- 35 minutes were quantified with a GA calibration curve in the 330nm window. This was chosen due to the observed molar absorbance differences and the intensity of the eluting peaks at the two wavelengths. The polyphenolic content of the crude was found to be 11.3 ± 1.4mg GAE/g using this method.

The most used total polyphenol content quantification method is the Folin-Ciocalteu total phenolic content (TPC) assay which was also employed in this study. A protocol by Ainsworth and Gillespie (2007)[270] was used, resulting in a phenolic content of 5.2 ± 1.1mg GAE/g for the crude. However, this method was not optimized or standardized, and the results may be influenced by this (Section 4.3.2). Comparing the TPC results to other studies of *U. intestinalis* reveals significant fluctuations in polyphenolic content due to external factors such as geographical origin and physiological- and chemical environment.[271] Srikong *et al.* (2017)[272] reported a polyphenolic yield of 54.4 ± 0.3mg GAE/g for *U. intestinalis* harvested in Thailand. Akkös *et al.* (2011)[273] collected *U. intestinalis* in the Lake Acigöl (Turkey) and reported TPC results of 0.032 ± 0.003mg GAE/g sample. Pradhan *et al*. (2021)[202] analyzed samples from India and found a polyphenol recovery of 23.00 ± 0.05mg GAE/g in their methanolic extract. Pirian *et al.* (2016)[274] and Ak and Turker (2018)[275] analyzed *U. intestinalis* from the Persian Gulf and Turkey, respectively, and reported phenolic contents of 1.02mg GAE/g and 2.56mg GAE/g, respectively. These studies provide insight into the variability in the literature regarding the polyphenolic content in *U. intestinalis* harvested from different location and analyzed using various protocols of the TPC method.

qNMR is a powerful analytical technique that allows for the determination of the concentration of compounds in a sample independent of colorimetric changes, molar

absorptivity, or calibration curves. However, it requires some prior knowledge of the polyphenolic nature of the sample and selective identification of suitable NMR peaks for quantification (selective qNMR). In this study, aromatic signals in the 8.5-6.0ppm region of the ^{1}H NMR spectra were selected based on spin-system observations in two-dimensional spectra (COSY, HSQC, HMBC. Refer to section 3.4.4). These signals were individually integrated and quantified before being added together. An external reference ($DMSO_2$) and the ERETIC function in Topspin (Bruker) was used for quantification calculations. However, qNMR calculations rely on two unknown parameters: the molecular weight of the compound (MW) and the number of protons in the sample (n_{sample}, Equation 1). Three different values for the number of protons in the sample were tested (n_{sample} = 2H/4H/6H) to determine their influence on the results. The findings indicated a 33% difference between the results using n_{sample} = 2H and those using n_{sample} = 6H. Additionally, qualitative results were used to determine that the average number of protons should be 4 (n_{sample} = 4H). This was based on the fact that some of the identified compounds contained more than 4 explicitly different protons (such as flavonoids), and some contained only one or two (like phenolic acids). Consequently, assuming an average of 4 protons was considered a reasonable approximation for calculations of the total polyphenolic content of *U. intestinalis*.

Molecular weight variation was also assessed by comparing the average molecular weight of the tentatively identified polyphenols from LC-MS with that of gallic acid, a more commonly used standard. The average molecular weight of the tentatively identified polyphenols was found to be 330g/mol, which is more than double the molecular weight of gallic acid (170g/mol). However, the percent increase in results was directly proportional to the percent difference between the two molecular weights tested (64%), demonstrating that the results are directly related to the chosen molecular weight. Ultimately, gallic acid's molecular weight was used for calculations for comparison reasons, resulting in a quantification of 27.3 ± 2.7mg GAE/g.

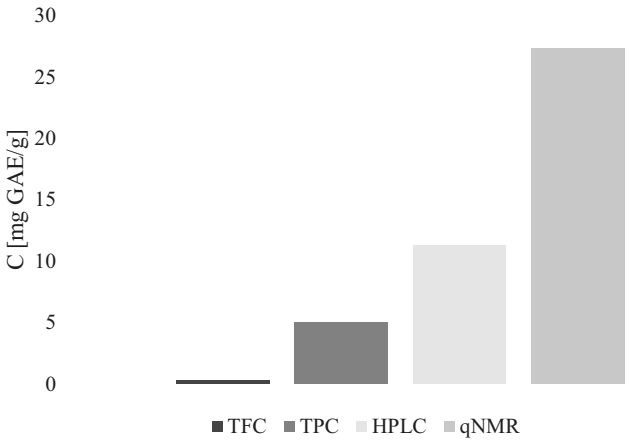


Figure 14: Quantification of total phenolic content in a *U. intestinalis* crude using a non-optimized TPC assay from literature, qNMR, and DAD-HPLC, reported in mg GAE/g. TFC values reported in apigenin equivalents [mg APE/g].

Figure 14 illustrates the quantification of polyphenols in *U. intestinalis* using various methods, with the qNMR method resulting in the highest polyphenolic yield. A total flavonoid content (TFC) assay was also performed; however, the method lacks standardization and specificity in the reaction mechanism.[245,276] The assay, which used an apigenin calibration curve, gave a yield of 0.3 ± 0.4mg APE/g for the crude sample. Aluminum chloride only forms acid stable complexes with flavones and flavonols, and acid labile complexes with ortho-dihydroxyl system, which may also include interfering species (Section 3.4.2.2).[244,276] TFC and DAD-HPLC analyses conducted on extracts from *Zostera marina* (eng.: eelgrass) within the Jordheim research group (data not published), also indicates that the TFC method underestimates the flavonoid content of this well examined seagrass[96,120,277,278], further emphasizing the uncertainty of the method.

Overall, the use of multiple quantification methods provided a comprehensive analysis of the total phenolic content of *U. intestinalis*. The results highlight the need for standardization and optimization of the TPC assay, and further development of the qNMR method, to ensure accuracy and reproducibility in the analysis of polyphenols from algal sources.

4.3.2 Folin-Ciocalteu total phenolic content assay optimization

The FC total phenolic assay (TPC) is a colorimetric assay that relies on the reaction of the FC reagent with hydroxyl groups of polyphenols in a sample. Upon initial use of the TPC assay for analyzing of *Ulva intestinalis*, it became apparent that the method needed to be optimized for marine samples. The widely referenced TPC method developed by Singleton and Rossi (1965)[168] was originally designed for wine samples, and while it has been adapted for use with terrestrial plant samples and, more recently, marine samples, the expansion of the assay's use has resulted in a variety of modifications to the experimental parameters, making accurate comparisons difficult.

To optimize the TPC assay for seaweeds, initial tests were conducted using samples of *L. hyperborea* and *U. intestinalis*, as well as *p*-coumaric acid. The ratios between all reactants (FC-reagent, salt reagent (Na_2CO_3), and sample), incubation time and wavelength were investigated individually and in relation to each other. These initial tests indicated a polynomial relationship between the amount of FC-reagent and the total polyphenol content quantified for all samples, with the maximum point identified as 7.958 ± 0.430mL/100 mL FC-reagent (Figure 15).

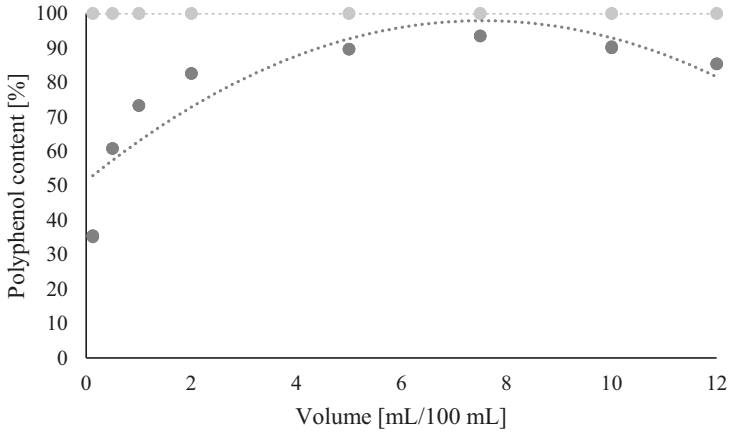


Figure 15: FC-reagent volume versus quantified polyphenols for the *p*-coumaric standard with known concentration, showing the polynomial relationship. Calculated polyphenol concentration (dark) from the TPC assay and known polyphenol concentration of *p*-coumaric acid (light) is shown.

Sodium carbonate is added to the reaction to make the reaction conditions more basic, as an acidic pH will slow down the reaction.[168,180] Thus, the amount of sodium carbonate (salt) added also influences the TPC reaction. Therefore, different volumes were tested (1-40mL/100 mL) in the same manner as the FC-reagent previously described. As the optimal FC-reagent volume was found to be 7.958mL/100 mL, this relative volume was used in all tests of the salt volume and while all other factors were kept constant. The salt reagent (20% Na_2CO_3) showed no significant increase in quantified phenolic content when extended beyond 20mL/100 mL for all samples, which was also reported by Singleton *et al*. (1999)[181] (Figure 16).

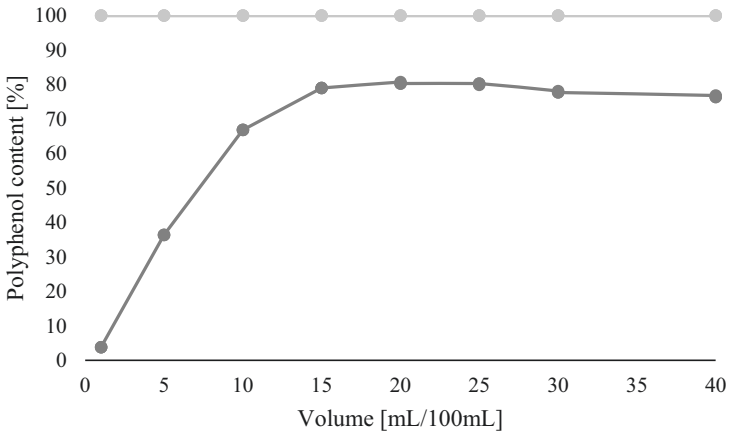


Figure 16: Relative volume of sodium carbonate (Na_2CO_3) versus quantified polyphenols for the *p*-coumaric standard with known concentration. Calculated polyphenol concentration (dark) from the TPC assay and known polyphenol concentration of *p*-coumaric acid (light) is shown, indicating no significant increase after 20mL/100 mL.

The optimal sample dilution range for algae samples was found to be between 10000 – 15000µg/mL DW. Initial tests showed λ_{max}-values varying between 750 – 770nm for the algae samples at optimal reaction conditions. Measuring the reaction at 760nm, which is the most reported, is therefore satisfactory and provides a more accurate basis of comparison. The absorbance at 760nm indicated a biological growth curve relationship with the incubation time, reaching a maximum at 30 min. Absorbance remained stable between 30 and 120 minutes, allowing incubation time to be anywhere

between 30 – 120 min as long as all samples are measured in the same manner. Most publications report a 2-hour incubation time and measurement at 760nm; thus, this was chosen to ensure more accurate comparisons.

The optimal TPC reaction conditions were found to be 0.2mL of sample, standard, or blank with 1.59mL FC-reagent and 4.0mL 20% Na_2CO_3. The mixture should be diluted with water to make the total volume 20mL and incubated for 2 hours in the dark. The absorbance should be measured at 760nm and the total phenolic content can be calculated. This optimized method was used in papers **II** and **III**. However, it is important to acknowledge that this method generally overestimates the total phenolic content due to factors such as metal contaminators and interfering compounds (i.e. sugars, amino acids, ascorbic acid, etc.).[179,180,182,183,245,279,280] Additionally, the TPC assay is dependent on the number of reactive groups in a compound. Reacting groups are hydroxyl groups (-OH) in which the FC reagent reacts with. Therefore, the polyphenolic diversity within the sample(s) will also influence the TPC results, due to the different reacting groups of the various compounds reacting differently in the FC redox reaction. By standardizing the assay using gallic acid, which Singleton *et al.* (1999)[181] report to have only two reacting group (two hydroxyl groups are in the exact same chemical environment), and then applying this to compounds possibly having several reacting groups, an overestimation will be the result. Therefore, the TPC assay should be performed with caution, as the results may only provide an estimation with low accuracy of the total phenolic content of samples with unknown compound matrices, such as algal extracts.

4.3.3 qNMR method optimization (paper II)

Quantitative ^{1}H NMR (qNMR) is an emerging quantification technique that has gained increasing attention for analysis of complex biological samples.[188,189] NMR provides rich structural information and have been proven successful for samples where traditional chromatographic methods have been ineffective.[189,281] The qNMR method is a primary ratio method in which the analyte can be correlated to a reference standard, and both internal and external reference standards may be used. In paper **I**, an external

standard was used in combination with the electronic reference method ERETIC2 (Electronic Reference To access In vivo Concentration, Bruker). The ERETIC2 method is based on PULCON, which is an internal standard method which correlates the absolute intensities of two different spectra.[282,283] However, since the external standard used ($DMSO_2$) was significantly different from the analyte, and produces spectral peaks in a different part of the 1D ^1H NMR spectra, it was decided to start using it as an internal standard in papers **II** and **III**.

The quantification of polyphenols with qNMR can be performed by integration of the -OH spectral region (14-8ppm) as described by Nerantzaki *et al.* (2011)[190], but broad and low intensity peaks are often observed in this region due to possible H-D exchange with aromatic hydroxy (-OH) groups. Therefore, a more applied method uses the aromatic region (8-6ppm) to quantify polyphenols.[184–187,189] Studies by Vissers *et al.* (2012)[186] and Ford *et al.* (2020)[187] have shown that using a narrower region of the aromatic region (7.0-5.5ppm) is sufficient for analyzing polyphenols in brown algae. This region was also chosen for studies of *L. hyperborea* due to the alga potentially having a similar polyphenolic matrix to the aforementioned studies. Additionally, the narrower region was chosen to not integrate signals potentially belonging to the same spin system. Two-dimensional NMR spectra were also utilized to eliminate additive quantification of signals belonging to the same spin system and/or eliminate quantification of compounds not originating from the polyphenolic biosynthesis (Section 3.4.4). Selective peak picking in the quantified region was performed prior to integration to yield results that better reflect the true polyphenolic content. This was named selective qNMR (paper **II**).

qNMR calculations are performed using equation 1 (Section 3.4.4) to quantify the total phenolic content. However, two calculation parameters, namely the molecular weight of the analyte and the number of protons (n_{sample}), are unknown and based on assumptions. Since equation 1 provides the molar concentration, the molecular weight is required to convert the concentration to the commonly reported mg/g unit. However, the molecular weight of phenolic compounds can vary widely, which makes it challenging to determine an accurate value. To address this issue, a reference standard molecular weight is often used. For instance, gallic acid is frequently used to quantify

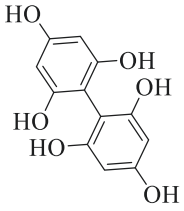
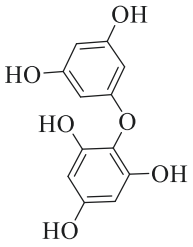
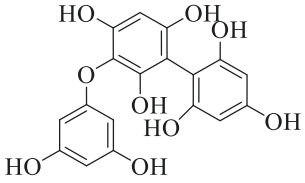
total phenolic content, and the results are expressed as gallic acid equivalents (GAE) (Table 1, Section 1.5.2). Alternatively, phloroglucinol can be used to analyze brown algae samples, and the results are reported as phloroglucinol equivalents (PGE) since brown algae are believed to contain mostly phlorotannins, which are polymers of phloroglucinol.[186,187] The molecular weight is directly proportional to the quantification results and will influence the results significantly. To facilitate comparable total phenolic content results, the TPC method and qNMR should utilize the same reference standard. Throughout this project, both gallic acid and phloroglucinol were applied. Additionally, the average number of protons per polyphenol in the sample (n_{sample}) affects the results, with a higher number leading to a lower quantification result. To obtain a more accurate value for this parameter, the phlorotannin linkages in brown algae samples were examined using ^{13}C NMR, with consideration given to the qualitative data. (Section 4.3.3.1).

4.3.3.1 Proton estimation for qNMR calculations

The identification of phlorotannin linkages in brown algae samples can be achieved using 1D ^{13}C NMR spectra, as proposed by Vissers *et al.* (2017)[186]. This method allows for the determination of the number of protons present, as the number of protons present per aromatic ring provides an estimate of the number of protons available per polyphenol in the sample.[186]

Fucols generally consist of terminal units with two protons, which yield signals in the characteristic polyphenol region of ^{1}H spectra, while the internal unit(s) contain only one aromatic proton. For phlorethols, one of the terminal units typically contains three aromatic protons, while the remaining phloroglucinol units contain two. As the phlorotannin size and branching increase, the average number of protons per aromatic ring decreases.[186] By identifying the ratio of phenyl and ether linkages in brown algae samples, an estimation of the average number of protons per aromatic ring can be made. However, this value may vary between species, so ^{13}C NMR spectra should be recorded prior to qNMR analyses of any new species and samples as the polyphenolic content may also vary with seasons.

Table 3: Overview of the phlorotannin linkage types; phenyl and ether linkages with examples.

Linkage	Type	Example	Structure
Phenyl; C-C	Fucol	Difucol	
Ether; C-O-C	Phlorethol	Diphlorethol	
Phenyl and ether; C-C / C-O-C	Fucophlorethol	Fucophlorethol	

Qualitative data was also used to estimate the average number of protons per polyphenol in the samples. Smaller phenolic acids and phlorotannins dominated the phenolic pool (see section 4.4.2). Since these phenolic acids usually contain only one or two distinctly different protons, their number of protons is low. *L. hyperborea* phlorotannins were found to be primarily composed of ether linkages with a fucol-to-phlorethol ratio of 1:3, while remaining polyphenolic matrix was dominated by phenolic acids. Based on these observations, an estimate of the average number of protons per polyphenol in the sample was made, with n_{sample} = 2H (Equation 1).

4.3.4 Quantification of polyphenols in *Laminaria hyperborea* (paper II and III)

Papers **II** and **III** utilize the optimized quantification methods described in sections 4.3.2 and 4.3.3. The quantification of crude *L. hyperborea* samples indicated relatively low polyphenolic content, with slight seasonal differences. Both paper **II** and **III** analyzed

the sample harvested in September 2020 (S20), yielding similar results (qNMR: 5.51 ± 0.00mg GAE/g, TPC: 5.72 ± 0.07mg GAE/g). Additionally, paper **II** reported that the sample harvested in March 2020 (M20) had a higher polyphenolic recovery than S20, while the sample harvested in August 2021 (A21) showed a lower polyphenolic recovery than both S20 and M20 (Table 4). Additionally, a seasonal study of the polyphenolic content in *L. hyperborea* has been started (data not shown), indicating further seasonal variation.

Table 4: Overview of the total phenolic content found for *Laminaria hyperborea* (crude- and selected purified samples), *Laminaria digitata*, *Saccharina latissima*, *Ascophyllum nodosum*, and *Fucus vesiculosus* using both the TPC assay and selective qNMR.

Sample	TPC [mg GAE/g]	qNMR [mg GAE/g]
L. hyperborea M20	6.23 ± 0.11	8.32 ± 0.00
L. hyperborea S20	5.72 ± 0.07	5.51 ± 0.00
L. hyperborea A21	5.35 ± 0.04	6.57 ± 0.00
L. hyperborea PuriFlash	19.69 ± 0.04	17.65 ± 0.00
L. hyperborea Prep. HPLC	103.0 ± 0.1	37.34 ± 0.00
L. digitata	6.94 ± 0.09	6.86 ± 0.00
S. latissima	13.1 ± 0.04	16.8 ± 0.0
A. Nodosum	17.6 ± 0.04	11.57 ± 0.00
F. vesiculosus	37.0 ± 1.0	14.8 ± 0.0

The *L. hyperborea* S20 crude sample was further purified using flash chromatography (PuriFlash) and preparative (prep) HPLC (Sections 3.3.1 and 3.3.2). Each fraction was quantified with TPC and qNMR and tested for antioxidant activity using the ORAC assay (Section 3.4.3). No antioxidant activity was detected for the crude sample, whereas the purified fractions showed increasing antioxidant activity with increasing purity. These data were also supported by the observed increasing total polyphenolic content of the purified fractions, seen from both the TPC and qNMR method.

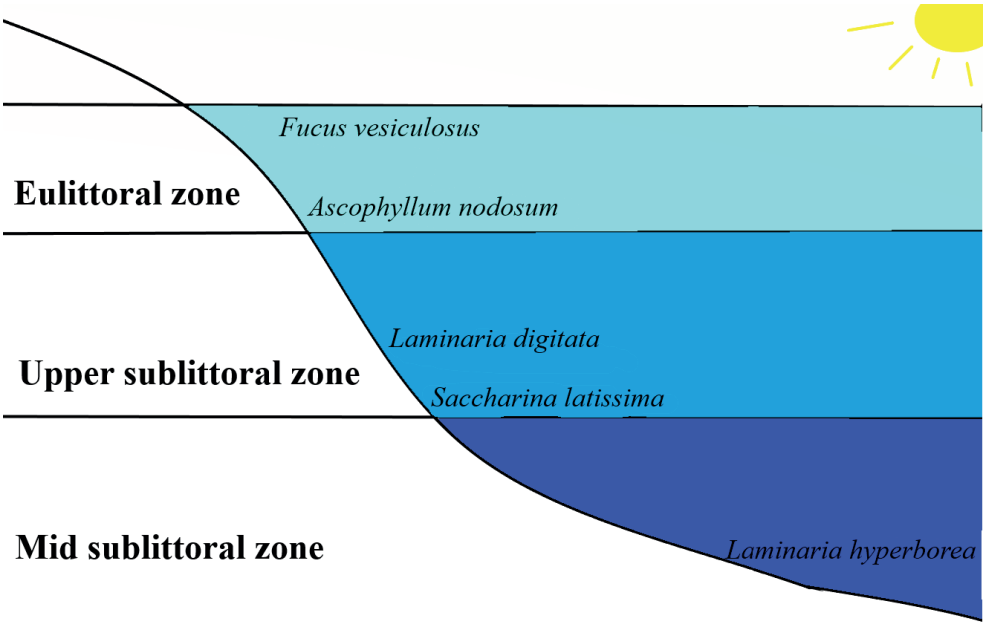


Figure 17: Indication of approximate locations within the intertidal zone of the brown algae studied in paper **II**. Adapted from Dodson *et al.* (2013)[284] and Kon *et al.* (2020)[285].

According to the findings in paper **II**, there was a small difference observed between the quantitative polyphenolic data obtained from the TPC and selective qNMR methods. Based on this observation, it is assumed that the leave biomass of *Laminaria hyperborea* contains lower levels of relatively simple, low molecular weight polyphenols. This assumption is rooted in the understanding that low molecular weight polyphenols possess fewer hydroxyl groups per molecule, which reduces the ability of the FC reagent to overestimate due to the similarity in reacting groups between the standard and the analyte. Additionally, the similarity of the TPC and qNMR results suggests that *L. hyperborea* leaves may contain less TPC-interfering compounds.

In paper **II** five brown algae species were analyzed, including *L. hyperborea*, *Laminaria digitata*, *Saccharina latissima*, *Ascophyllum nodosum*, and *Fucus vesiculosus*. Compared to the other brown algae, *L. hyperborea*, *L. digitata*, and *S. latissima* exhibited a smaller discrepancy between the TPC and selective qNMR results. On the other hand, the two Fucaceae algae, *A. nodosum* and *F. vesiculosus*, both had high polyphenolic recovery, but the observed difference between the TPC and selective qNMR results was large. This larger difference may indicate an increased heterogeneity

of the polyphenolic matrix for algae growing closer to the ocean surface, suggesting presence of polyphenols with an increased number of reacting groups – thus resulting in increased TPC values.

As both Fucaceae algae are eulittoral growing, they are more exposed to external factors such as UV-radiation and temperature fluctuations, which can lead to more heterogenous polyphenolic matrices and/or increase the polyphenolic content (Figure 17). Furthermore, these factors can vary within different sites, habitats, and seasons, affecting not only the polyphenolic production but also the production of other metabolites in algae. Since the TPC assay is non-selective, interfering species (i.e., other algal metabolites) will also affect the TPC results, possibly leading to higher results. In contrast, the selective qNMR can reduce the impact of such interfering species by selectively picking peaks in the aromatic region, accounting for the larger difference observed in polyphenolic recovery for eulittoral growing algae. Therefore, given that *L. hyperborea* is the deepest growing algae analyzed (Figure 17) and shows minimal difference between the TPC and selective qNMR result, it is likely to have a low, homogenous polyphenolic content.

4.4 Characterization

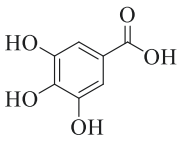
4.4.1 Characterization of polyphenols in *Ulva intestinalis* (Paper I)

Polyphenols were characterized in *U. intestinalis* using both high-resolution (HR) and low resolution (LR) LC-MS. Characterization was based on a personal database with over 600 polyphenols and other natural products reported for algae in the literature and identified within the research group. Purified fraction of *U. intestinalis* were obtained using gradient system 1 in preparative HPLC, as described in section 3.3.2., were used for characterization. LC-MS experiments were performed using gradient system 1, as described in section 3.4.5.

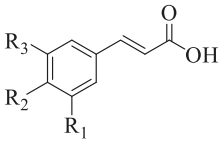
Table 5: Overview of tentatively identified polyphenols in *Ulva intestinalis* with LC-MS.

Compound	Molecular weight [g/mol]	Confirmed with standard	Compound class
Gallic acid	170.12	+	HBA
Vanillic acid*	168.15	-	HBA
Veratric acid*	182.17	-	HBA
Luteic acid*	320.21	-	HBA
Valoneic acid*	474.33	-	HBA
Caffeic acid	180.16	+	HCA
Coumaric acid	164.16	+	HCA
Chicoric acid*	474.40	-	HCA
Ferulic acid	194.18	+	HCA
Sinapic acid	224.21	+	HCA
Chrysin*	254.24	-	Flavone
Rhamnazin*^{HR}	330.29	-	Flavone
Myricetin*	318.23	-	Flavone
Luteolin*^{HR}	286.24	+	Flavone
Apigenin	270.24	+	Flavone
Diosmetin	300.26	+	Flavone
Quercetin	302.23	+	Flavonol
Isorhamnetin*	316.26	-	Flavonol
(+)-Catechin	290.27	+	Flavan-3-ol
Naringenin*	272.25	-	Flavanone
Hesperetin*	302.28	-	Flavanone
Aromadendrin*	288.25	-	Flavanonol/flavanone
Ellagic acid*	302.19	-	HT
Procyanidin B1*	578.50	-	PAC
Phloroglucinol*	126.11	-	Benzentriol

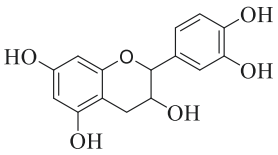
HCA = Hydroxycinnamic acid, HBA = Hydroxybenzoic acid, HT = hydrolysable tannin, PAC = proanthocyanidin, *= several possible isomers; ^{HR} = confirmed with HR LC-MS.



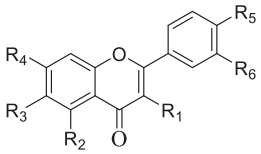
Gallic acid



Caffeic acid: R_1 = H, R_2 = OH, R_3 = OH
Coumaric acid: R_1 = H, R_2 = OH, R_3 = H
Ferulic acid: R_1 = H, R_2 = OH, R_3 = OCH_3
Sinapic acid: R_1 = OCH_3, R_2 = OH, R_3 = OCH_3



(+)-Catechin



Luteolin: R_1 = H, R_2 = OH, R_3 = H, R_4 = OH, R_5 = OH, R_6 = OH
Apigenin: R_1 = H, R_2 = OH, R_3 = H, R_4 = OH, R_5 = OH, R_6 = H
Diosmetin: R_1 = H, R_2 = OH, R_3 = H, R_4 = OH, R_5 = OCH_3, R_6 = OH
Quercetin: R_1 = OH, R_2 = OH, R_3 = H, R_4 = OH, R_5 = OH, R_6 = OH

Figure 18: Structures of the polyphenols identified with a reference standard in *U. intestinalis*.

Several low molecular weight polyphenols were tentatively identified in *U. intestinalis*, most of the compounds had observable masses below 400Da. About half of the tentatively identified compounds were flavonoids, and five were confirmed using a reference standard mix. Apigenin was partially isolated from one of the purified prep fractions, showing overlap with the reference standard in the LC-MS and DAD-HPLC analyses. The concentration of apigenin in the sample was high enough for quantification, and the apigenin content of the purified sample was found to be 2.62ng/g. Both luteolin and rhamnazin were confirmed with HR LC-MS showing a mass error below 5ppm (Δppm(luteolin) = 1.50; Δppm(rhamnazin) = 1.24). Diosmetin, quercetin, and (+)-catechin were confirmed in the extract by comparison of retention times in the gradient with the reference standard mix.

Although *U. intestinalis* samples were found to contain several flavonoids, the total flavonoid content (TFC) assay showed very low values. However, the method is somewhat debatable due to the lack of standardization, as discussed in section 3.3.1.

Results from this characterization compared to the TFC results illustrate the weaknesses and limitations of this assay.

Various phenolic acids were also identified in *U. intestinalis*, and five were confirmed with reference standards. These included both hydroxycinnamic acids (HCA) and hydroxybenzoic acids (HBA), found in a 50:50 ratio. Three other polyphenols (ellagic acid, procyanidin B1, and phloroglucinol) were tentatively identified, but without confirmation from standards or HR LC-MS. It should be noted that many of the identified compounds have several isomers with the same molecular weight, exact mass, elemental composition, and sometimes even the same fragmentation pattern.[286] For flavonoids, only aglycones were identified, which are very stable and typically do not fragment when analyzed with ESI LC-MS.[99] Therefore, fragmentation patterns of these compounds were not observed and could not be used for identification.

While there is still debate about the presence of flavonoids in algae, several flavonoids were identified in *U. intestinalis*. However, the absence of more advanced flavonoids, such as flavonoid glucosides, may suggest a limited biosynthetic capacity in this alga (see figure 8, Section 1.4.2). Still, the presence of the colored anthocyanins is recognized as adaptations within terrestrial plants. Studying flavonoids in algae can be challenging due to the diversity, structural complexity, and relatively low abundance of polyphenols. Nonetheless, understanding the diversity and distribution of these compounds could have broad implications for a range of industries, such as pharmaceutical, agricultural, and environmental industries, as well as for research of the chemical ecology of algae and for ocean monitoring. It is also worth noting that the biosynthetic pathways for secondary metabolites can vary widely between different species of plants and algae, and thus further research is needed to fully understand the diversity and distribution of flavonoids in algae.

4.4.2 Characterization of polyphenols in *Laminaria hyperborea* (paper III)

Eleven polyphenols were identified in *L. hyperborea* leaves (Table 6). The major group were phenolic acids, dominated by hydroxybenzoic acids. Four phlorotannins were identified based on fragmentation patterns, with one being a sulfated phlorotannin.

Sulfated phenolic acids were also identified. All compounds were confirmed either through HR LC-MS with mass deviations below 5ppm, or by their fragmentation patterns.

Table 6: Overview of identified polyphenols in *Laminaria hyperborea* using HR- and LR LC-MS.

Compound	Molecular ion [M-H]-	MS/MS[#]	Compound class
Salicylic acid	137.0245	**93**	HBA
Veratric acid	181.0510	-	HBA
5-Carboxyvanillic acid	211.0252	151, 107, 83, **65**, 63	HBA
5-Sulfosalicylic acid*	216.9815	137, **93**	HBA
Vanillic acid 4-sulfate*	246.9923	121, **108**, 93, 80	HBA
Sinapic acid	223.0616	208, 193, **149**, 93	HCA
Dihydrocaffeic acid 3-sulfate*	261.0082	**181**, 166, 122, 81	HCA
Trimer*	373.1196	265, 229, **126**	PT
Tetramer*	497.1867	371, 298, 254, 241, 197, **155**, 126	PT
Hexamer*	745.9562	**681**, 461, 331, 281, 249, 229	PT
Sulfated dimer*	328.9978	**249**	PT

[#]MS/MS ion marked in bold represent the base peak. HCA = Hydroxycinnamic acid, HBA = Hydroxybenzoic acid, PT = phlorotannins, * = several possible isomers.

Several phenolic compounds were tentatively identified from initial TIC scans using the personal database. The polyphenolic matrix of *L. hyperborea* was found to consist of low molecular weight compounds, with a majority of the tentatively identified compounds having masses below 800Da. While 211 polyphenols were tentatively identified as bibliographic matches, only 11 were confirmed through exact mass deviations ($\Delta ppm \leq 5$) or fragmentation patterns. This can be attributed to the lower concentration of individual polyphenols in the purified fractions.

Phenolic acids were the most dominant of the compounds identified, particularly hydroxybenzoic acids were found. In addition, three sulfated phenolic acids were identified: 5-sulfosalicylic acid, vanillic acid 4-sulfate, and dihydrocaffeic acid 3-sulfate (Figure 19). All phenolic acids were identified through a low mass deviation when

analyzed with HR LC-MS and partially through their fragmentation patterns. Commonly observed losses were of the acid groups (CO_2, $[M-44-H]^-$), as well as the loss of methyl side groups (CH_3, $[M-15-H]^-$). The sulfated phenolic acids were confirmed by the loss of a SO_3^- group ($[M-80-H]^-$) in the fragmentation spectra.

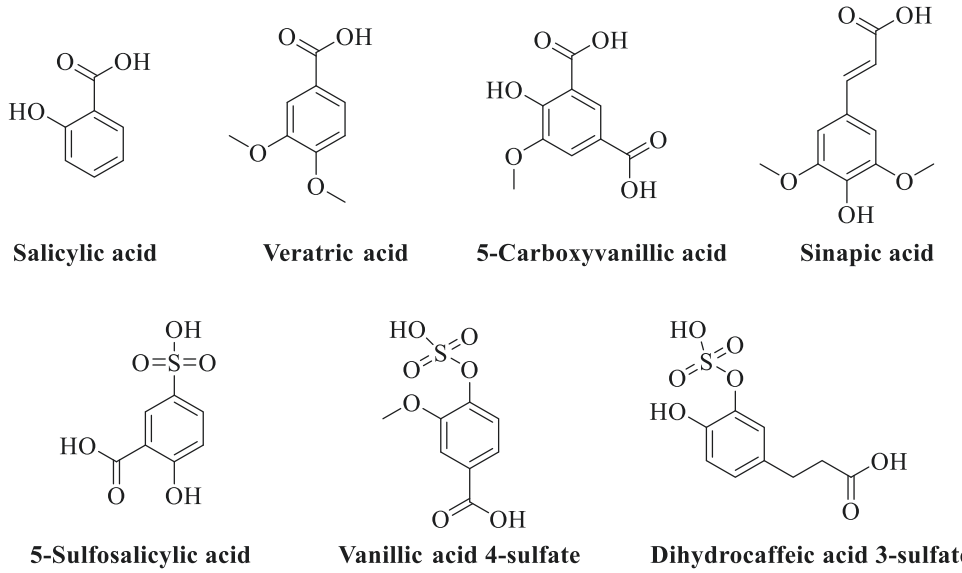


Figure 19: Structures of the seven phenolic acids identified in *L. hyperborea* with three being sulfated phenolic acids.

Phlorotannins are unique to brown algae and have been the focus of many studies analyzing the phenolic content of brown algae.[24,46,59,116,117,185,186,200] Four phlorotannins were identified in the leaf biomass of *L. hyperborea* by utilizing their fragmentation patters to determine their linkages and polymerization degrees. While similar phlorotannins have been found in other brown algae, this is the first report of such compounds in *L. hyperborea*. The phlorotannins ranged in size from dimers (two phloroglucinol units) to hexamers (six phloroglucinol units). One sulfated dimer phlorotannin was identified, which was characterized by the loss of SO_3^- ($[M-80-H]^-$) and the resulting characteristic phlorotannin fragment at *m/z* 249 (refer to figure 20). The dimer could be connected by either a phenyl linkage or an ether linkage. However, due to the observed dominance of ether linkages in the ^{13}C NMR spectra (Section 4.3.3.1), the compound is likely to be sulfated diphlorethol (see figure 21).

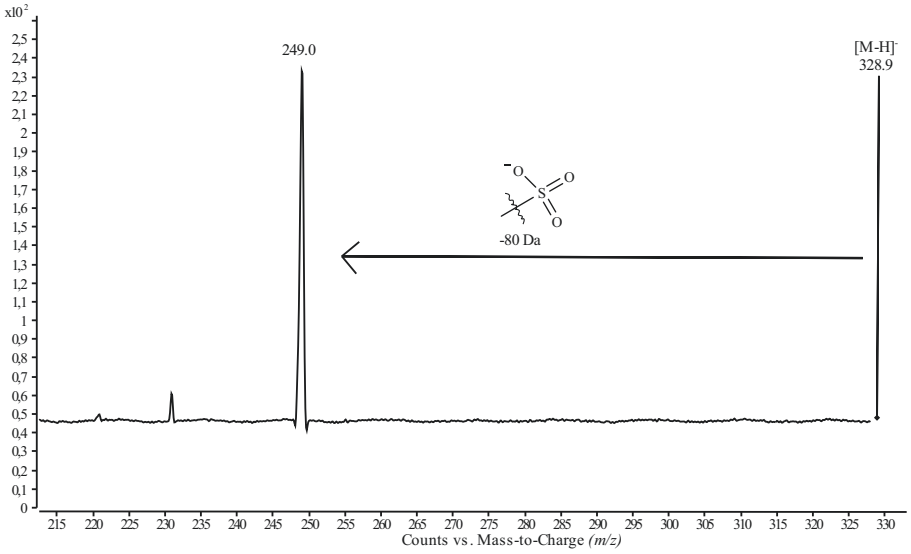
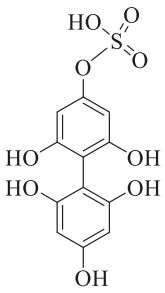
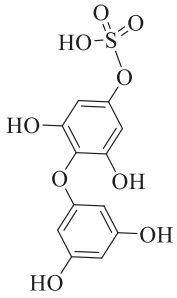


Figure 20: Excerpt from the fragmentation spectra of the sulfated dimer phlorotannin ($[M-H]^-$ = 328.9) showing the loss of the sulfate group (SO_3^-, -80Da), and the resulting phlorotannin fragment at *m/z* 249.

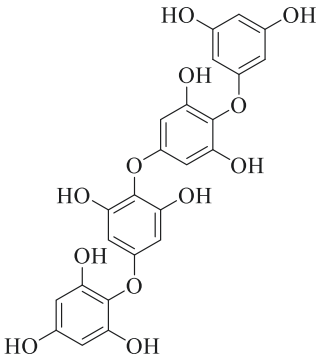
Three non-sulfated phlorotannins were also identified, a trimer, a tetramer, and a hexamer. The trimer phlorotannin was observed by its quasi-molecular peak at *m/z* 373.1196 ($[M-H]^-$) and was found to be a fuhalol-type phlorotannin, possibly Triphlorethol A, with ether linkages connecting the phloroglucinol units. The tetramer was observed at *m/z* 497.1867 ($[M-H]^-$), and although its fragmentation pattern differed from those previously reported in literature, it was identified as a phlorotannin. The hexamer was observed at *m/z* 745.9562 ($[M-H]^-$) and showed different fragmentation from those reported in literature. However, various fragmentation patterns of tetramers and hexamers are reported, showing the increased isomerism of larger and more branched phlorotannins. Fragmentation experiments were performed at varying fragmentation energies, with minor differences observed. The fragmentation pattern of the tetramer did not provide any information regarding the types of linkages connecting the phloroglucinol. However, the hexamer's fragmentation pattern showed the compound having both phenyl- and ether linkages, indicating it is a phlorethol-type phlorotannin (see figure 21 for selected possible structures).



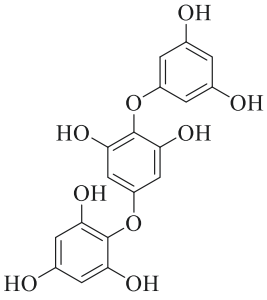
Sulfated difucol



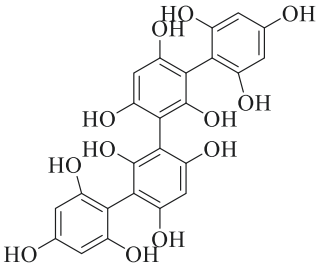
Sulfated diphlorethol



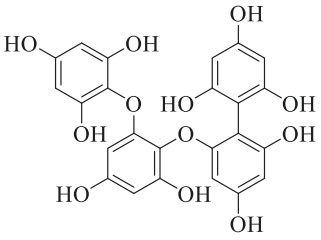
Tetramer



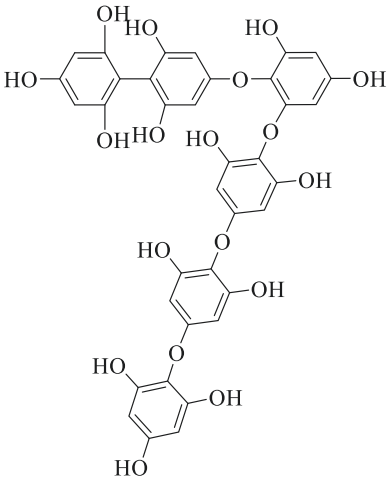
Trimer



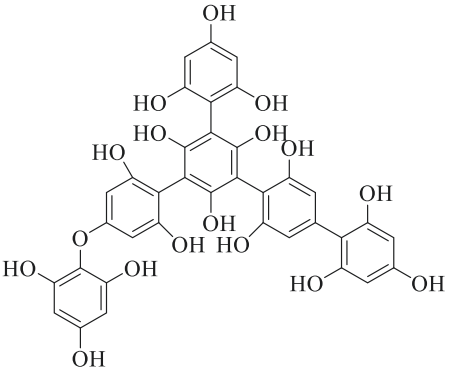
Tetramer



Tetramer



Hexamer



Hexamer

Figure 21: Possible structures of phlorotannins identified in *L. hyperborea*, showing the selected possible isomers.

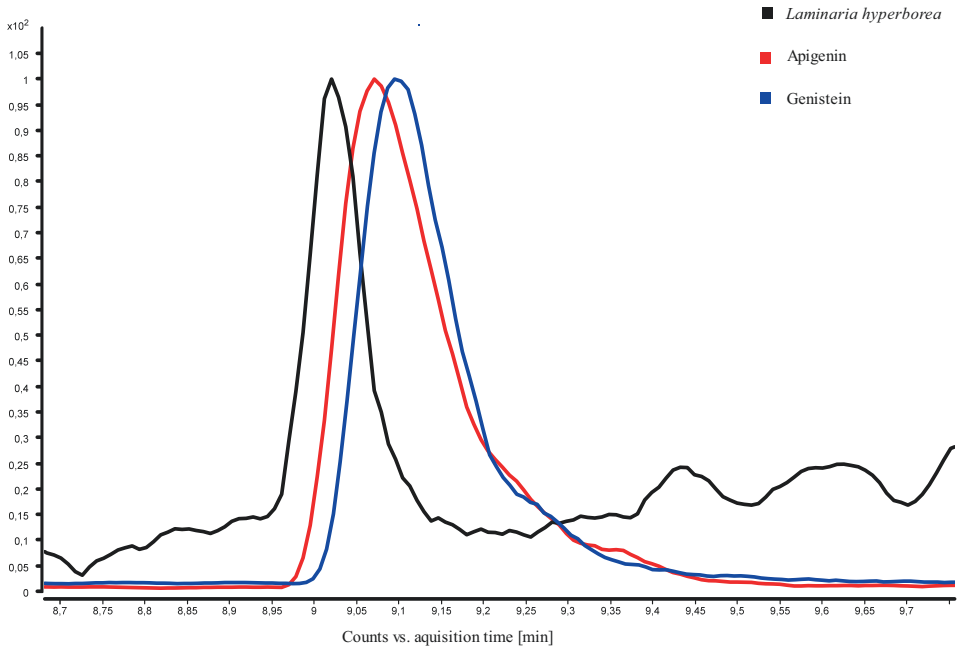


Figure 22: Overlaid LC-MS chromatogram of a possible flavonoid peak with $[M-H]^-$ = *m/z* 269 of a purified *L. hyperborea* leaf sample (black), apigenin (red), and genistein (blue).

The presence of both phlorotannins and phenolic acids in *Laminaria hyperborea* raises the question, as mentioned by Davies *et al*. (2020)[221], of whether the similarity between the CHS enzyme used for production of flavonoids and PKS enzyme needed for synthesis of phloroglucinol is an example of parallel evolution or not. Phenolic acids are synthesized early in the biosynthetic pathway of flavonoids; however, it is the CHS enzyme that is vital for production of flavonoids. Although no flavonoids were identified with HR LC-MS or fragmentation patterns from *L. hyperborea*, several of the tentatively identified compounds indicated the presence of flavonoids. Specifically, a compound matching apigenin (and its isomers) was detected in *L. hyperborea* ($[M-H]^-$ = 269). However, the mass deviation was well above 5ppm, and no fragmentation or characteristic UV-spectrum was observed. It is worth noting that flavonoid aglycones, such as apigenin, are not easily fragmented using ESI LC-MS. The reference standard of apigenin and one of its isomers, genistein, were analyzed and compared to the *L.*

hyperborea sample, yielding indications that the detected compound might be a similar flavonoid aglycone, although not a perfect match (see figure 22). However, no further information of the potential flavonoid compound could be acquired, such as a characteristic UV-Vis-spectrum, and thus it is not possible to conclude whether brown algae contain flavonoids or not. A UV-Vis-spectrum was not possible to acquire as the concentration of the compound was very low. Nevertheless, more research on the polyphenolic content of brown algae is important to further our knowledge of these compounds in marine algae and to determine if flavonoids are present in brown algae.

In summary, the natural polyphenolic composition of *L. hyperborea* leaves is predominantly composed of soluble low molecular weight polyphenols, with phenolic acids being the most abundant. In addition, a high occurrence of sulfated compounds was observed. Furthermore, non-phenolic compounds were also identified in *Laminaria hyperborea*, as listed in appendix B. This project provides the first comprehensive characterization of individual polyphenols in *L. hyperborea*. The high homogeneity and water solubility of the polyphenolic content of *L. hyperborea* enables extraction of high value products from both solid and liquid side streams of commercial alginate production.

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Appendix A & B

Appendix A

Table A1: Overview of selected extraction optimizations experiments performed with *L. hyperborea* M20 (Section 4.2). Total phenolic quantification using HPLC with a phloroglucinol calibration curve was performed.

					Solvent ration [%]				Quantification
Method	**m(biomass) [g]**	**DW [mg]**	**Temperature [°C]**	**Time [h]**	**H_2O**	**MeOH**	**EtOH**	**EtOAc**	**%PP [PGE]**
ME	5.029	57	23	3.0	100	0	0	0	0.449
ME	5.103	432	23	2.5	0	100	0	0	0.246
ME	5.201	10	23	2.5	0	0	0	100	0.0690
ME	5.920	148	23	3.0	40	60	0	0	0.822
ME	5.029	55	23	2.5	80	20	0	0	0.405
ME	5.201	69	23	3.0	80	20	0	0	0.666
ASE	6.249	401	70	0.75	40	60	0	0	1.14
ASE	3.587	138	70	0.75	60	40	0	0	0.756
ASE	3.566	119	70	0.75	80	20	0	0	0.639
UAE	5.048	61	23	2.0	80	20	0	0	0.719
UAE	5.013	223	23	0.50	50	50	0	0	0.570
UAE	5.151	324	23	0.50	50	0	50	0	0.890
UAE	5.034	175	23	0.75	60	40	0	0	0.429
UAE	5.036	271	23	0.75	40	60	0	0	0.781

ME = Maceration Extraction, ASE = Accelerated Solvent Extraction, UAE = Ultrasound Assisted Extraction, MeOH = methanol, EtOH = ethanol, EtOAc = ethyl acetate, PP = polyphenols, PGE = Phloroglucinol Equivalents, DW = dry weight of extract.

Appendix B

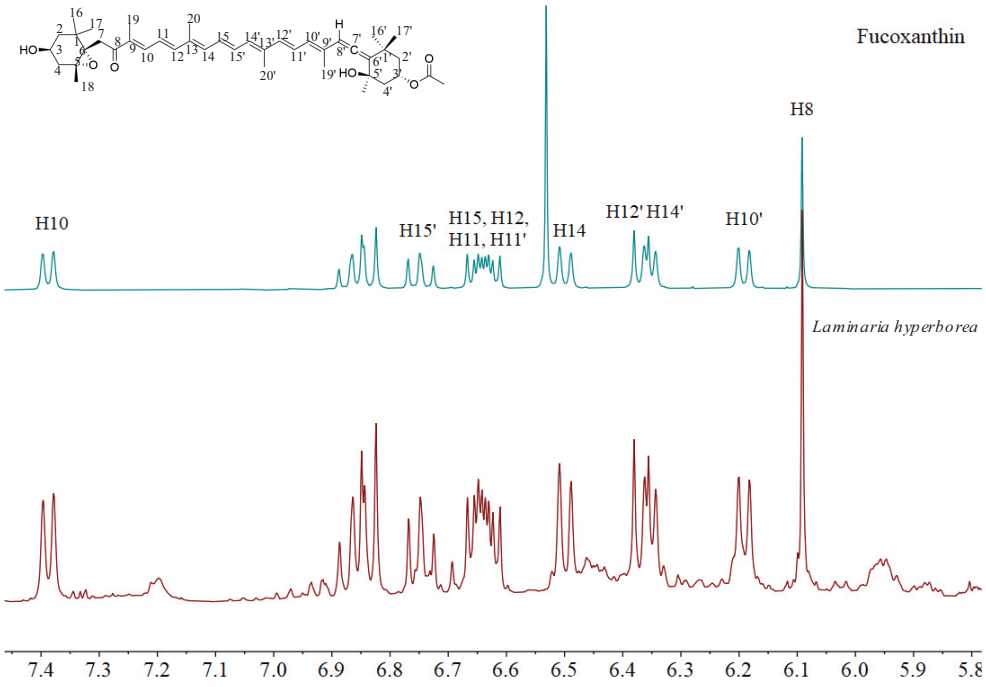

Figure B1: Identification of the carotenoid fucoxanthin using 1D ^{1}H NMR. Showing a fucoxanthin reference standard (blue) and a purified *Laminaria hyperborea* fraction from PuriFlash (red), with structural elucidation.

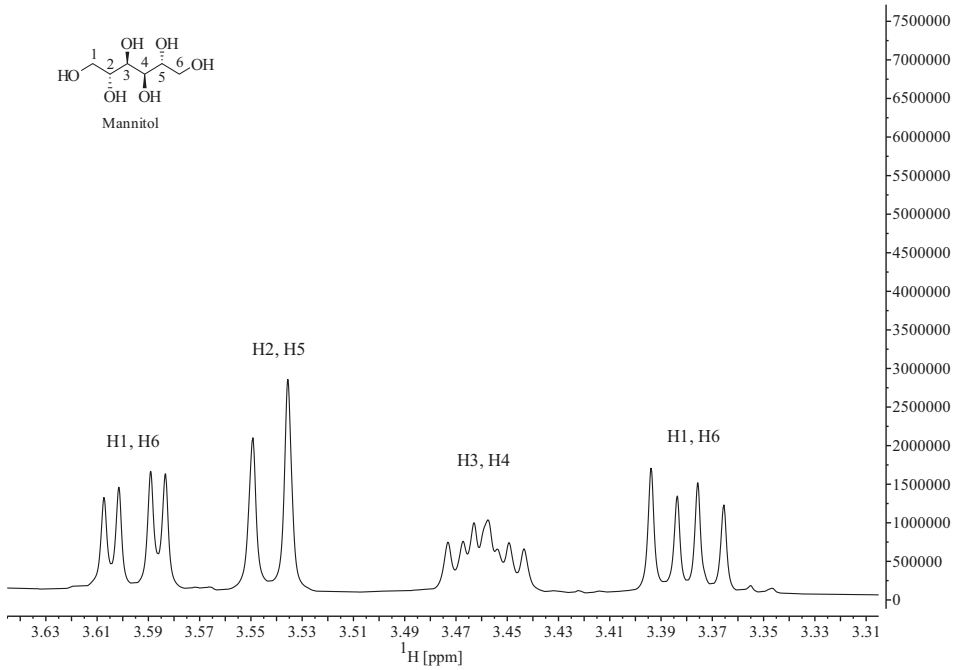

Figure B2: Structural elucidation of mannitol in *Laminaria hyperborea* using 1D ^{1}H NMR.

Table B1: Non-phenolic acids identified in *L. hyperborea* using HR LC-MS.

Compound	Molecular formula	$[M-H]^-$	Mass deviation [ppm]
Citric acid	$C_6H_8O_7$	191.0202	2.27
Ascorbic acid	$C_6H_8O_6$	175.0252	1.93

Papers I-III

PAPER I

antioxidants

MDPI

Article

Quantification of Polyphenols in Seaweeds: A Case Study of *Ulva intestinalis*

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Abstract: In this case study, we explored quantitative ^{1}H NMR (qNMR), HPLC-DAD, and the Folin-Ciocalteu assay (TPC) as methods of quantifying the total phenolic content of a green macroalga, *Ulva intestinalis*, after optimized accelerated solvent extraction. Tentative qualitative data was also acquired after multiple steps of purification. The observed polyphenolic profile was complex with low individual concentrations. The qNMR method yielded 5.5% (DW) polyphenols in the crude extract, whereas HPLC-DAD and TPC assay yielded 1.1% (DW) and 0.4% (DW) respectively, using gallic acid as the reference in all methods. Based on the LC-MS observations of extracts and fractions, an average molar mass of 330 g/mol and an average of 4 aromatic hydrogens in each spin system was chosen for optimized qNMR calculations. Compared to the parallel numbers using gallic acid as the standard (170 g/mol, 2 aromatic H), the optimized parameters resulted in a similar qNMR result (5.3%, DW). The different results for the different methods highlight the difficulties with total polyphenolic quantification. All of the methods contain assumptions and uncertainties, and for complex samples with lower concentrations, this will be of special importance. Thus, further optimization of the extraction, identification, and quantification of polyphenols in marine algae must be researched.

Keywords: seaweeds; green algae; marine algae; *Ulva intestinalis*; *Enteromorpha intestinalis*; quantification; polyphenols; flavonoids; apigenin; accelerated solvent extraction; ASE; HPLC-LRMS; HPLC-HRMS; HPLC; TPC; Folin–Ciocalteu; TFC; qNMR

1. Introduction

Marine macroalgae, or seaweed, is a large group of macroscopic organisms that are an important component in aquatic ecosystems. The wide diversity of marine organisms is being recognized as a rich source of functional materials and, in 2015, the global seaweed aquaculture production reached 30 million tons [1]. Although marine algae have gained increasing attention over the last years due to the fact of their bioactive natural substances with potential health benefits, they are still identified as an underexploited resource [2–6].

Natural antioxidants with multifunctional potential are of high interest, and numerous studies have focused on natural antioxidants, including polyphenols and flavonoids, from terrestrial plants [7–9]. However, the application potential of polyphenolic analyses of marine sources suffers from several factors, most importantly, the lack of exactness with respect to quantitative and qualitative data at a molecular level. Marine plant material with analytic matrices at very low concentrations and a high

and variable dissolved salt concentration makes polyphenol analyses challenging [4,10]. The diversity of phenolic compounds also varies from simple to highly polymerized substances which makes qualitative and quantitative procedures, involving sample preparation and extraction, difficult to standardize. Thus, this makes for a further challenge in the analyses and in furthering the research in this field.

Colorimetric assays, such as Folin-Ciocalteu, have been extensively used to quantify phlorotannins and polyphenolic content in seaweeds. However, since the assay is difficult to standardize and not selective, it has been recommended to use the assay for approximate measurements of an extract's antioxidant potential only [11–15]. Since the colorimetric assays neither separate nor give a correct quantitative measurement of the individual compounds, high-performance liquid chromatography (HPLC) has been the method of choice for separation and quantification of polyphenols in plants. The HPLC with multiple diode array UV-Visible detection (DAD) quantifies according to Lambert-Beer's law ($A = \varepsilon cl$). A compound's ability to absorb UV-Visible light (A) is related to the compound's molar absorptivity value (ε) and molar concentration (c). The diversity of molar absorptivity values of polyphenols is almost as large as the number of polyphenols existing; even within the same polyphenol class, there will be differences [16]. In the lack of commercially available standards, one standard is often chosen when total amounts of polyphenols or phlorotannins are quantified. Gallic acid (GA) seems to be the most used standard for total polyphenolic quantification and phloroglucinol (PG) for the phlorotannin quantification in brow algae [17–20]. In addition to the limitations with commercially available standards, HPLC will also suffer from a lack of separation of complex extract matrices and loss of compound amounts due to the irreversible retention on the HPLC column during elution.

In recent years, quantitative ^{1}H NMR (qNMR) have gained increasing attention as a method for quantitative determination of metabolites in complex biological matrices [21–23]. According to the review by Pauli et al. (2012) [22] and references therein, qNMR methods have proven successful when standard chromatographic methods have been ineffective [22]. In general, qNMR can be considered a primary ratio method of measurement in which the analytes can be correlated directly to a calibration standard, and since the reference compound differs from the analytes, generating a calibration curve becomes unnecessary. However, the quantification needs to be validated with reference compounds. Some work on quantification of phlorotannins in brown algae (*Ascophyllum nodosum*, *Fucus vesiculosus*, and *Cystoseira tamariscifolia*) with qNMR has been done using internal standards [14,23].

In this case study, we examined the polyphenolic content of the green algae *Ulva intestinalis* (syn. *Enteromorpha intestinalis*) collected on the west coast of Norway. An optimized extraction of the polyphenolic content was performed. The extract and semi-purified fractions were further analysed utilizing qNMR with an external reference for quantification of the total phenolic content. For comparison, HPLC-DAD and TPC assay analyses were also performed. To further explore the diverse group of polyphenols in *Ulva intestinalis*, qualitative analyses were performed with HPLC-DAD, HPLC-LR, and HR-MS. We entered this case study with the overarching goal of examining which analytical methods could lead to a more reliable value of polyphenolic content in seaweed and, thus, obtain a better view of the grand potential of seaweed phenolics.

2. Materials and Methods

2.1. Plant Materials

Samples of *Ulva intestinalis* (syn. *Enteromorpha intestinalis*) were collected in June from the western coast of Norway; Rogn, Ormhilleren (60°29′38.8″ N 4°55′11.9″ E). The voucher specimen of *Ulva intestinalis* was deposited in the Herbarium BG (Voucher no. BG-A-75) at the University Museum of Bergen, Bergen.

2.2. Chemicals

All chemicals used were of analytical grade. Methanol (≥99.9%), acetonitrile (≥99.8%), trifluoroacetic acid (TFA) and Folin-Ciocalteu reagent were all acquired form Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Formic (98–100%) and acetic (99.8%) acids were both acquired from Riedel-de Haën (Honeywell Inc., Charlotte, NC, USA). Luteolin, apigenin, myrcetin, diosmetin, quercetin, caffeic acid, coumaric acid, ferulic acid, sinapic acid, and gallic acid reference standards were all purchased from Sigma–Aldrich (Sigma-Aldrich, St. Louis, MO, USA). The analytical standard of tricin was purchased from PhytoLab (PhytoLab BmbH & Co. KG, Vestenbergsgreuth, Germany), (+)-catechin was purchased from USP (USP, Rockville, MD, USA), and DPPH free radical was purchased from Merck (Merck, Kenilworth, NJ, USA). Deionized water was deionized at the University of Bergen (Bergen, Norway).

2.3. Extraction and Purification

The collected plant material was washed thoroughly in fresh water and air dried. Dried plant material was stored at −20 °C when not used. Dried material was extracted using ASE (Accelerated Solvent Extraction) (Dionex™ ASE™ 350, Thermo Fisher Scientific, Waltham, MA, USA). A dried sample of *Ulva intestinalis* (55.9 g) was mixed with Dionex ASE prep DE sand and added to 66 mL stainless-steel cells with two glass fiber filters placed at the bottom end of the cell, before being extracted using a Dionex ASE 350 Accelerated Solvent Extractor. The extraction procedure consisted of two different methods, one being a pre-soak method, and the other being the primary extraction method. Pre-soaking consisted of extraction at 23 °C under 1500 psi. The static extraction period was 1 min with a flush volume of 50% of cell volume, purged with N_2 for 70 s, and 100% deionized water was used as the solvent in the pre-soak method. The primary extraction method consisted of preheating for 5 min, and samples were then extracted at 70 °C under 1500 psi. Static extraction time was 5 min with a flush volume of 60% of the cell volume, purged with N_2 for 100 sec. The solvent used for the primary extraction was a mixture of deionized water and methanol (40:60, *v/v*). Primary extraction was repeated two times. The volume of the combined extract was reduced using a rotavapor, and the concentrated aqueous extract was partitioned against ethyl acetate (EtOAc) four times. The contents of both the EtOAc phase and the water phase were examined using HPLC-DAD, HPLC-LRMS, HPLC-HRMS, and colorimetric assays including Total Phenolic Content Assay (TPC) and Total Flavonoid Content Assay (TFC). Before analysis, all phases were carefully reduced to dryness using rotavapor, and, finally, the samples were dried under N_2 gas.

The aqueous extract was applied to an Amberlite XAD-7 column and washed with distilled water. Methanol was applied for elution. The pre-eluted washing water was analyzed for polyphenols with HPLC. Collected methanolic fractions (XAD7-A, XAD7-B, XAD7-C) were reduced using a rotavapor and analyzed on analytical HPLC. The XAD-7 fraction A contained the highest number of polyphenols and was chosen to be submitted to preparative HPLC to obtain three purified fractions; prepLC-A1, -A2, and -A3 (Figure 1).

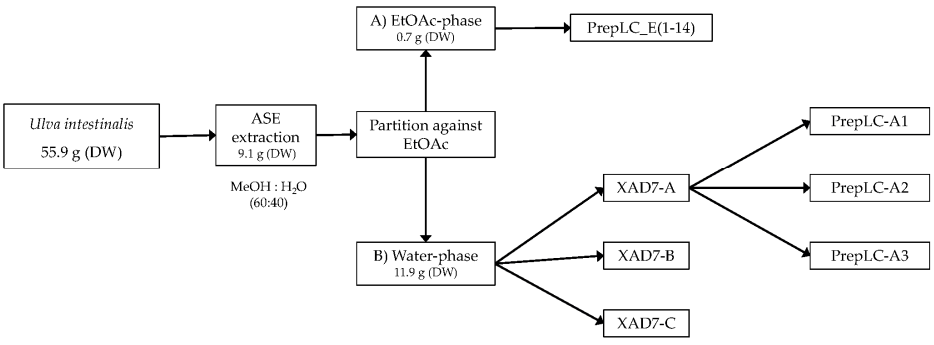

Figure 1. Overview of the extraction and purification steps in the *Ulva intestinalis* analysis.

2.4. General Instrumentation

2.4.1. Preparative HPLC

The preparative HPLC system consisted of a Gilson 321 pump (Gilson Inc., Middleton, WI, USA), an Ultimate 3000 variable wavelength detector (Dionex, Thermo Fisher Scientific, Sunnyvale, CA, USA), and a 25 × 2.12 cm (10 μm) UniverSil C18 column (Fortis Technologies Ltd., Neston, UK). Two solvents were used: (A) super distilled water (0.1% acetic acid) and (B) acetonitrile (0.1% acetic acid) with initial conditions of 90% A and 10% B followed by an isocratic elution for the first 5 minutes, and the subsequent linear gradient conditions, 5–18 min: to 16% B, 18–22 min: to 18% B, 26–31 min: to 28% B, 31–32 min: to 40% B, 32–40 min: isocratic at 40% B, 40–43 min: to 10% B. The flow rate was 15 mL/min, and the aliquots of 750 μL were injected.

2.4.2. Analytical HPLC-DAD

All HPLC-DAD analyses were performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 1260 diode array detector (DAD) and a 200× C analysis was performed using two solvents, (A) super distilled water (0.5% TFA) and (B) acetonitrile (0.5% TFA), in a gradient (0–10 min: 95% A + 5% B, 10–20 min: 85% A + 15% B, 20–34 min: 60% A + 40% B. 34–35 min: 95% A + 5% B). The flow rate was 1.0 mL/min, and aliquots of 20 μL were injected with an Agilent 1260 vial sampler. UV-Vis absorption spectra were recorded during the HPLC analysis over the wavelength range of 200–600 nm in steps of 2 nm.

The established HPLC method was validated for linearity, sensitivity, precision, and accuracy. Table 1 presents data for calibration curves, test ranges, limit of detection (LOD), and limit of quantification (LOQ) for gallic acid. The LOD and LOQ were calculated based on the standard deviation of *y*-intercepts of the regression line (S_y) and the slope (S), using the equations LOD = 3.3 × S_y/S and LOQ = 10 × S_y/S.

Table 1. Calibration curve, limit of detection (LOD), and limit of quantification (LOQ) for gallic acid (GA) (Sigma-Aldrich) at 280 nm and 330 nm.

Standard	Calibration Curve (μg/mL)	R^2	Test Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid (280 nm)	$y = 65.536x - 366.51$	0.9988	10–500	14.1	42.8
Gallic acid (330 nm)	$y = 0.2603x - 0.8339$	0.9993	10–500	18.5	56.0

2.4.3. HPLC-LRMS and HPLC-HRMS

Liquid chromatography low-resolution mass spectrometry (HPLC-LRMS) (ESI+/ESI−) was performed using an Agilent Technologies 1260 Infinity Series system and an Agilent Technologies 6420A triple quadrupole mass spectrometry detector. The following conditions were applied: ionization mode: positive/negative, capillary voltage = 3000 V, gas temperature = 300 °C, gas flow rate = 3.0 L/min, acquisition range = 100–800 *m/z*. The elution profile for HPLC consisted of the following gradient: 0–3 min: 90%A + 10%B, 3–11 min: 86%A + 14%B, 11–15.5 min: 60%A + 40%B, 15.5–17 min: 90%A + 10%B, at a flowrate = 0.3 mL/min, where solvent A was super distilled water (0.5% formic acid), and solvent B was acetonitrile (0.5% formic acid). A 50 × 2.1 mm internal diameter, 1.8 μm Agilent Zorbax SB-C18 column was used for separation. Calibration curve of Apigenin ran on HPLC-LRMS and used for quantification is listed in Table 2.

Table 2. Calibration curve, limit of detection (LOD), and limit of quantification (LOQ) for apigenin (Sigma-Aldrich) acquired using HPLC-LRMS.

Standard	Calibration Curve (mM)	R^2	Test Range (mM)	LOD (mM)	LOQ (mM)
Apigenin	$y = (2.0 \times 10^{-6})x - 2054.6$	0.995	0.00156–0.0125	0.0014	0.0041

Liquid chromatography high-resolution mass spectrometry (HPLC-HRMS) (ESI+/TOF) was performed using an AccuTOF JMS-T100LC (JEOL, Peabody, USA) mass spectrometer in combination with an Agilent Technologies 1200 Series HPLC system. The following instrumental settings/conditions were used: ionization mode: positive, ion source temperature = 220 °C, needle voltage = 2500 V, desolvation gas flow = 4 L/min, nebulizing gas flow = 3 L/min, orifice1 temperature = 125 °C, orifice2 voltage = 10 V, ring lens voltage = 20 V, ion guide RF voltage = 1600 V, detector voltage = 2350 V, acquisition range = 15–1000 *m/z*, spectral recording interval = 0.50 sec, wait time = 0.033 nsec, and data sampling interval = 2 nsec. The elution profile for HPLC consisted of the same gradient and column as described for HPLC-LRMS, but the flowrate was increased to 0.35 mL/min.

2.4.4. NMR Spectroscopy

Quantification of the extracts of *Ulva intestinalis* was performed using ^{1}H NMR analyses on a Bruker 600 MHz instrument (Bruker BioSpin, Zürich, Switzerland). All spectra were recorded in DMSO-d_6 at 25 °C. The pulse sequence applied was *zg30* with the following acquisition parameters: sweep width of 19.8 ppm, 64 k data points, 16 scans, and 2 dummy scans. The relaxation delay, d1, was set to 40 sec (equal to $5 \times T_{1,\max}$) to ensure complete relaxation between scans. The spectra were processed using a line broadening of 0.3 Hz. The crude extract was used for T_1 measurements, utilizing the *t1ir* pulse sequence with a sweep width of 19.8 ppm, 16 k data points, 8 scans, 2 dummy scans, and 9 different inversion recovery delays between 1 ms and 5 s. Measured T_1 values ranged from 1.0–8.1 s.

Quantification using the ^{1}H NMR spectra was performed using the ERETIC2 function in TopSpin with $DMSO_2$ (10 mM) as an external reference. The $DMSO_2$ signal (~3.0 ppm) was integrated and defined as the ERETIC reference (No. H = 6, Mm = 94.13 g/mol, V(sample) = 0.75 mL, C = 10 mM).

Reference compounds for validation were gallic acid (GA), *p*-coumaric acid, ferulic acid, (+)-catechin, and luteolin (10 mM, DMSO-d_6). An average standard deviation of < 10% was observed. The integrations were repeated three times.

Two-dimensional heteronuclear single quantum coherence (^{1}H-^{13}C HSQC), heteronuclear multiple bond correlation (^{1}H-^{13}C HMBC), and double quantum filtered correlation (^{1}H-^{1}H DQF COSY) spectra were also recorded on the Bruker 600 MHz instrument.

2.5. Total Phenolic Content Assay

For the determination of total phenolic content, the Folin-Ciocalteu total phenolic content assay (TPC) was used. The method used was adapted from Ainsworth and Gillespie (2007) [24]. 200 µL of the sample or standard was added to the cuvettes (10 × 45 mm, 3 mL), followed by 400 µL 10% (*v/v*) Folin–Ciocalteu reagent in super distilled water. Further, 1600 µL 700 mM Na_2CO_3 in super distilled water was added to the cuvettes. The mixture was incubated for 30 minutes, and the absorbance was measured at 765 nm using a Shimadzu UV-1800 UV spectrophotometer and a Shimadzu CPS-100 cell positioner (Shimadzu, Kyoto, Japan). Data was expressed as gallic acid equivalents (GAE). An incubation time of 2 h was also tested.

2.6. Total Flavonoid Content Assay

For the determination of the total flavonoid content, 2 mL test solution (standard or sample) was added to four cuvettes (10 × 45 mm, 3 mL) and the absorbance measured at 425 nm with solvent in the reference cuvette. An aliquot of AlCl3 solution (0.5 mL, 1%, *w/v*) was added to three of the four

cuvettes, and the same volume of solvent was added to the fourth (blank sample). The content of the cuvettes was stirred thoroughly, and the absorbance measured at 1 minute intervals at 425 nm for 10 minutes at 22 °C. For quantitative analysis apigenin was chosen as the reference compound (concentration range of 1–500 μg/mL). Procedure modified from Pękal and Pyrzynska (2014) [25].

3. Results and Discussion

3.1. Quantification of Polyphenols in Ulva Intestinalis

In this work, extraction of polyphenols was performed after optimization of extraction parameters utilizing a Dionex ASE 350 extraction instrument (see Section 2.3). Aliquots (10 mL) of the different phases, ASE (Accelerated Solvent Extractor) Crude, (A) EtOAc and (B) water (see Figure 1) were sampled and dried for weight determination and further quantification with HPLC-DAD, qNMR, TPC, and TFC. The results of the different quantification methods are shown in Tables 3–5.

Table 3. Quantification of polyphenols in the crude extract and liquid–liquid extraction phases of crude with HPLC.

Sample	g DW	%PP GAE	mg (GAE)/g DW
ASE crude	9.1	1.1 ± 0.14	11.3 ± 1.4
(A) EtOAc	0.7	0.7 ± 0.2	6.7 ± 0.2
(B) Water	11.9	0.6 ± 0.1	5.5 ± 0.9
A + B	12.6	1.2 ± 0.1	12.1 ± 0.5

PP = polyphenol; (A) EtOAc = ethyl acetate phase; (B) water phase; GAE = gallic acid equivalents; DW = Dry Weight.

Table 4. Quantification of polyphenols in the crude extract and liquid–liquid extraction phases of crude with qNMR.

Sample	DW	GAE			330 Mw eq.			mg (GAE)/g DW	mg (330 Mw eq.) g DW
	g	%PP			%PP				
		2H	4H	6H	2H	4H	6H	4H	4H
ASE Crude	9.1	5.5 ± 0.5	2.7 ± 0.3	1.8 ± 0.2	10.6 ± 1	5.3 ± 0.5	3.5 ± 0.4	27.3 ± 2.7	52.9 ± 5.2
(A) EtOAc	0.7	0.502 ± 0.002	0.251 ± 0.001	0.167 ± 0.001	1.01 ± 0.07	0.50 ± 0.04	0.30 ± 0.03	2.51 ± 0.01	5.0 ± 0.4
(B) Water	11.9	4.9 ± 0.3	2.5 ± 0.2	1.7 ± 0.1	9.7 ± 0.7	4.8 ± 0.3	3.2 ± 0.1	24.9 ± 1.5	48.5 ± 3.3
A + B	12.6	5.5 ± 0.2	2.7 ± 0.3	1.9 ± 0.1	10.7 ± 0.4	5.3 ± 0.2	3.6 ± 0.1	27.4 ± 1.1	53.5 ± 2.1

PP = polyphenol; (A) EtOAc = ethyl acetate phase; (B) water phase; GAE = gallic acid equivalents; 330 Mw eq. = equivalents of average mass found from MS; 2H, 4H, and 6H = assumptions made related to the number of aromatic ^{1}H in each polyphenolic spin system; DW = Dry Weight.

Table 5. Quantification of polyphenols in the crude extract and liquid–liquid extraction phases of crude with total phenolic content (TPC).

Sample	g DW	GAE %PP	mg (GAE)/g DW
ASE crude	9.1	0.4 ± 0.1	5 ± 1
(A) EtOAc	0.7	0.035 ± 0.001	0.3 ± 0.2
(B) Water	11.9	0.4 ± 0.1	3.6 ± 1.5
A + B	12.6	0.5 ± 0.1	4 ± 1

PP = polyphenol; (A) EtOAc = ethyl acetate phase; (B) water phase; GAE = gallic acid equivalents; DW = Dry Weight.

3.2. Quantification Utilizing High-Performance Liquid Chromatography (HPLC) with Wavelength Detector (DAD)

Quantification of polyphenols in plants and foods has been a topic of discussion and research for years, and among the different methods HPLC-DAD it has been the method of choice due to the possibility of separation of compounds before individual quantification. However, with the use of retention times, absorption spectra, and molar absorptivity, the technique is often limited when

it comes to simultaneous determination of polyphenols of different groups [9]. Table 6 illustrates the different area responses observed in HPLC for different standards with the same concentration, reflecting the molar absorptivity differences.

Table 6. Illustration of molar absorptivity differences expressed with HPLC integrated peak areas (280 nm and 330 nm) of selected standards (5 mM) used in polyphenolic quantification.

Standard	Compound Class	$\lambda_{max\ (nm)}$	280 nm	330 nm
p-Coumaric acid	HCA	(230), 310	2754 ± 43	4743 ± 4
Gallic acid (GA)	HBA	272	2884 ± 2	8.7 ± 0.3
(+)-Catechin	Flavan-3-ol	279	5687 ± 6	2.1 ± 0.4
Apigenin	Flavone	(267), 340	801,120 ± 2361	131,812 ± 1525

HCA = hydroxycinnamic acid, HBA = hydroxybenzoic acid

When dealing with complex polyphenolic mixtures with unknown identities, which is the case for seaweeds, one standard is often selected for quantification. Traditionally, gallic acid is chosen for total polyphenolic quantification and phloroglucinol (PGE) for total phlorotannin quantification as seen for brown algae [17–20]. In this work, gallic acid (GA) was chosen as the reference standard, since the nature of the polyphenols in the green algae *U. intestinalis* was unknown, and since we wanted to compare different quantification methods. However, there is no doubt that the estimation of the total polyphenol content will suffer from this.

The HPLC peaks with maximum intensity in the 280 nm (R_t: 1–15 min) were quantified according to the 280 nm GA standard curve (Table 1), while peaks with maximum intensity in the 330 nm (R_t: 15–35 min) window were quantified according to the 330 nm GA standard curve. This resulted in an HPLC-DAD quantification of 1.1% polyphenols in the algae, based on quantification on the ASE crude extract (11.3 ± 1.4 mg GAE/g DW) (Table 3). The recovery of the polyphenols after the liquid-liquid ethyl acetate partition was quantified to be 1.2% (12.1 ± 0.5 mg GAE/g DW), almost evenly distributed into the (A) EtOAc phase (0.7%) and the (B) water phase (0.6%). Thus, the total recovery for A + B was relatively close to the initial amounts found in the crude.

3.3. Quantitative NMR (qNMR)

In order to get closer to a "true" estimation of polyphenol content in seaweeds, quantifications using ^{1}H NMR (qNMR) were performed (Table 4). One of the advantages of qNMR is that there is no need to consider the large variation observed regarding the molar absorptivity of different phenolic compounds (Table 6) nor the loss of sample during chromatography as with HPLC analyses. When quantifying polyphenols from NMR, one can consider two regions for quantification: the –OH spectral region, as shown by Nerantzakie et al. [23], or the aromatic 1H region [14,26]. Nerantzaki et al. presented a method for total phenolic content determination of crude plant extracts based on phenol type –OH resonances in the region between 14–8 ppm. Signals were selected after observation of elimination, or reduction, of the signal intensities after irradiation of the residual water resonance. In our marine *U. intestinalis* samples, the phenol –OH type resonances were observed at low intensities and were too broad to perform reliable integration. The broad signals may be attributed to the nature of the marine extract, containing many different types of phenol –OH resonances. Additionally, the ASE crude and the water phase contained some water, even after careful drying, which increases the phenol –OH exchange with the water peak. The 10–8.5 ppm region of the EtOAc phase (Figure 2) showed several sharp signals; however, these signals were found to not represent phenol –OH resonances due to the fact of their observed $^1J_{CH}$ correlations in the HSQC spectrum.

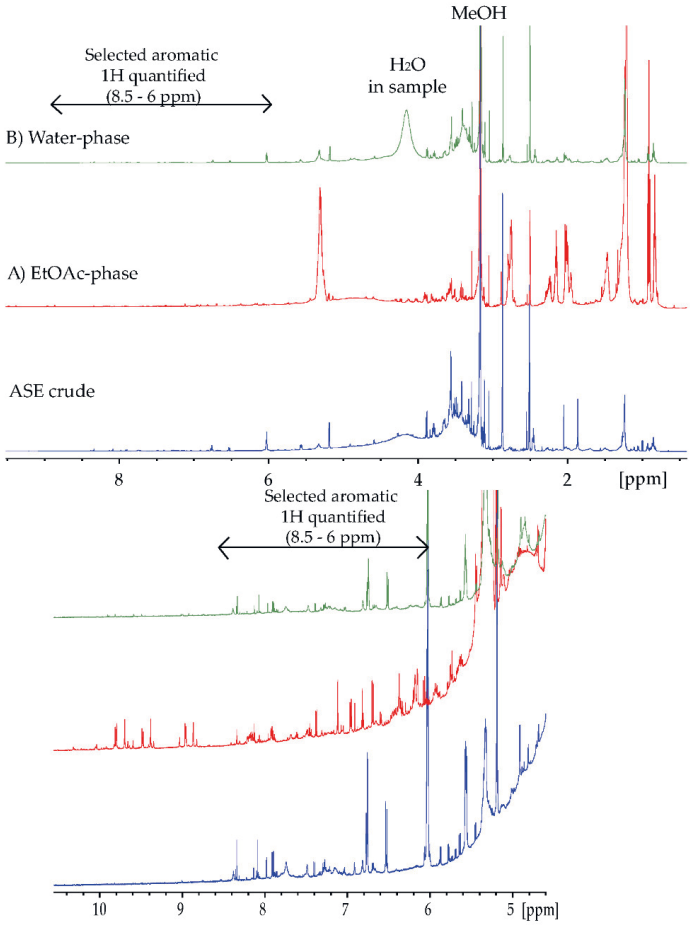

Figure 2. ^{1}H-NMR spectrum (600 MHz) for ASE crude (blue), (**A**) EtOAc phase (red), and (**B**) water phase (green) recorded in DMSO-d_6 at 25 °C. 2D spectra were used to deselect peaks in the 8.5–6 ppm region belonging to the same spin system, avoiding multiple quantification.

For qNMR calculations, characteristic aromatic signals in the 8.5–6 ppm region of the ^{1}H NMR spectra were integrated individually, and quantifications were added together to yield the total phenolic content (Section 2.4.4, Figure 2) [21,25]. Additionally, two-dimensional NMR spectra, such as COSY, HSQC and HMBC, were recorded to deselect signals belonging to the same molecule as far as possible in order to avoid multiple quantifications. The qNMR calculations were validated with quantification of standards (Section 2.4.4). Quantifications were calculated using the ERETIC2 function in TopSpin (Bruker) with $DMSO_2$ as an external reference (C = 10 mM). However, to quantify the signals, a molar mass is needed. The molar mass of gallic acid was chosen in order to obtain comparable results. Quantifications were also calculated using an average molar mass of 330 g/mol based on observed masses from the MS analyses (Table 4). Additionally, an average value of aromatic protons found in each polyphenolic spin system must be chosen. This assumption will also introduce uncertainty. Nerantzakie et al. [23] made their quantification on phenol –OH and used an average of 2 OH for each spin system related to their standard, caffeic acid. In Table 4, the polyphenolic content calculation utilizing different average aromatic protons are shown, resulting in a 33% difference between the maximum (2 aromatic H) and minimum (6 aromatic H) values calculated. Based on our tentatively

identified compounds in Table 7 it seemed like 4 aromatic protons (H) was a reasonable assumption. The qNMR method thus yielded a polyphenolic content of 5.3% in the crude (52.9 ± 5.2 mg 330 Mw eq./g DW). Due to the parallel numbers, using gallic acid (170 g/mol) and 2 aromatic protons yielded similar results (Table 4).

Table 7. Overview of tentatively identified low-mass polyphenols/simple phenolics at different stages of purification with HPLC-LRMS.

Observed R_t (min)	$(M+H)^+$	Tentative identification	LC-MS R_t Confirmed with Standard	Compound Class	Phase
1.56	171	Gallic acid	+	HBA	XAD7-A
4.74	127	Phloroglucinol *	–	benzentriol	EtOAc
6.93	291	Catechin	+	flavan-3-ol	EtOAc
8.11	181	Caffeic acid	+	HCA	EtOAc, XAD7-C
8.67	169	Vanilic acid *	–	HBA	EtOAc
9.02	165	Coumaric acid	+	HCA	EtOAc
10.10	475	Chicoric acid *	–	HCA	XAD7-B
10.27	195	Ferulic acid	+	HCA	EtOAc
10.27	183	Veratric acid *	–	HBA	EtOAc
10.51	225	Sinapic acid	+	HCA	XAD7-B
10.65	321	Luteic acid *	–	HBA	XAD7-A
12.31	475	Valoneic acid *	–	HBA	Crude, H_2O, XAD7-A
12.50	319	Myricetin *	–	flavone	XAD7-A, prepLC-A3
12.90	287	Luteolin $^{*, HR}$	+	flavone	EtOAc, prepLC-A3
12.98	303	Quercetin	+	flavonol	EtOAc, PrepLC-A3
13.16	273	Naringenin *	–	flavanone	PrepLC-A3
13.69	271	Apigenin (2.62 ng/g)	+	flavone	PrepLC-A3
14.43	303	Hesperetin *	–	flavanone	PrepLC-A3
14.76	289	Aromadendrin/eriodictyol *	–	flavanonol/flavanone	EtOAc
14.93	301	Diosmetin	+	flavone	XAD7-A, PrepLC-A2
14.95	303	Ellagic acid *	–	HT	XAD7-A
15.61	331	Rhamnazin $^{*, HR}$	–	flavone	EtOAc, prepLC-A3
16.12	579	Procyanidin B1 *	–	PAC	PrepLC-A2, EtOAc
16.76	256	Chrysin *	–	flavone	Crude
16.80	317	Isorhamnetin *	–	flavonol	PreLC-A3

HCA = hydroxycinnamic acid, HBA = hydroxybenzoic acid, HT = hydrolysable tannins, PAC = proanthocyanidin. * Several possible isomers; ^{HR} HR-LC-MS mass; + = identity confirmed with standard on LR-LC-MS, - = identity not confirmed with standard on LR-LC-MS.

3.4. Colorimetric Assays: Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The Folin–Ciocalteu assay is the most common assay used to quantify phenolic content (TPC) in both terrestrial plants and seaweeds. However, the assay is debatable due to the lack of standardization and lack of specificity in the reaction mechanism resulting in the colorimetric quantification [11–15,27]. This is of importance for all colorimetric assays, including the total flavonoid content (TFC) assay [25,28]. With increasing purity of the samples, direct quantitative measurements seem to be more reliable. However, the difficulty of standardizing this assay does not seem to be without importance.

The TPC assay (Table 5) resulted in a total of 0.4% in the ASE crude (5 ± 1 mg GAE/g DW), with a recovery of 0.04% in the (A) EtOAc phase (0.035 ± 0.001 mg GAE/g DW) and 0.4% in the (B) water phase (0.4 ± 0.1 mg GAE/g DW). Relatively high standard deviations were observed for the aqueous phases, potentially reflecting the lack of reliability of the method and difficulties with standardization.

The relative partition of polyphenols found between the two phases (A:B) in the TPC assay seem to follow the pattern observed from the qNMR quantification (10:90) (Table 4), rather than the partition ratio found in the HPLC-DAD analyses (50:50) (Table 3). The different ratio observed from the HPLC analyses is most likely due to the impact of molar absorptivity difference between the standard used and the compounds present.

The occurrence of flavonoids in algae is a central topic [29–32], and we chose to run a TFC assay in parallel with our attempts to identify flavonoids in our extracts (Table 8). The TFC assay gave a total of 0.03% flavonoids in the ASE Crude (0.2 ± 0.4 mg apigenin eq./g DW) and 0.13% in the (A) EtOAc phase (0.2 ± 0.4 mg apigenin eq./g DW). No flavonoids were detected in the (B) water phase with the TFC method.

Table 8. Quantification of flavonoids in the crude extract and liquid–liquid extraction phases of crude with total flavonoid content (TFC).

Sample	g DW	mg Apigenin Equivalents	mg (Apigenin eq.)/g DW
ASE crude extract	9.1	0.03 ± 0.04 [a]	0.3 ± 0.4 [a]
(A) EtOAc phase	0.7	0.13 ± 0.01	1.3 ± 0.1
(B) Water phase	11.9	n.d.	n.d.
A + B	12.6	0.13 ± 0.01	1.3 ± 0.1

[a] Three parallels measured from (0–34 mg); n.d.= not detected; PP = polyphenol; FL = flavonoid; (A) EtOAc = ethyl acetate phase; (B) water phase; TFC = total flavonoid content; DW = Dry Weight.

3.5. Qualitative Analysis of Polyphenols in Ulva intestinalis

After ASE extraction of the polyphenols (Figure 3; HPLC profile and selected UV-Vis spectra) and partition of the aqueous crude extract against ethyl acetate, the concentrated water phase (B) was applied to a XAD-7 column, washed with distilled water, and then eluted with methanol (Figure 1). The pre-eluted washing water was analyzed for polyphenols with HPLC-DAD. Collected methanolic fractions (XAD7 A–C) were reduced using a rotavapor and analyzed using analytical HPLC. The XAD-7 fraction A showed the highest polyphenol content and was chosen to be submitted to preparative HPLC to obtain three major fractions (prepLC A1–A3, Figure 1). The EtOAc phase was also submitted to preparative HPLC. The liquid–liquid partition with ethyl acetate gave some selectivity with respect to separation of compounds as seen in Figure 4. The compounds found in the EtOAc phase were most likely less polar and seemed to have a shorter chromophore compared to compounds observed in the water phase. The compounds in the water phase also showed an additional absorption band around 412–414 nm.

The preparative HPLC gave some separation of compounds; however, the samples were still complex. All the phases and fractions underwent extensive analyses with HPLC-DAD, HPLC-LRMS, HPLC-HRMS, and NMR. The results of the HPLC-LRMS analyses are shown in Table 7, giving an overview of the tentatively identified compounds.

Fragmentation patterns were difficult to obtain due to low concentrations. The ESI-MS spectra were recorded in both positive and negative modes. The masses of a luteolin-isomer ($(M+H)^+$, calculated: 287.05556, exact: 287.05599, $C_{15}H_{10}O_6$, Δppm 1.5) and a rhamnazin-isomer ($(M+H)^+$, calculated: 331.08178, exact: 331.08178, $C_{17}H_{14}O_7$, Δppm 1.24) were confirmed with HPLC-HRMS. The rhamnazin-isomer (*m/z* 331.08178) did not overlap with the commercial standard tricin (330 Mw) in the HPLC-LRMS SIM scan.

The most conclusive evidence of the presence of flavonoids in the green algae *U. intestinalis* was found in the late preparative fraction: prepLC-A3 (Figure 5). This fraction contained many of the peaks observed between 15 and 35 min in the HPLC profile of the crude (330 nm) (Figure 3). Several of the flavonoid masses found were tentatively identified from this fraction (Table 7) which has its origin from the water phase (B). The TFC assay did not detect any flavonoids in the water phase (Table 8) which illustrates the problem with relaying on these colorimetric assays. One flavonoid in the prepLC-A3 fraction was identified to be apigenin, using overlaid an HPLC-LRMS SIM scan at *m/z* 271 $(M+H)^+$ with an apigenin standard (Figure 5). The amount of the apigenin in the algae was found to be 2.617 ng/g (DW) using an apigenin calibration curve (Table 2).

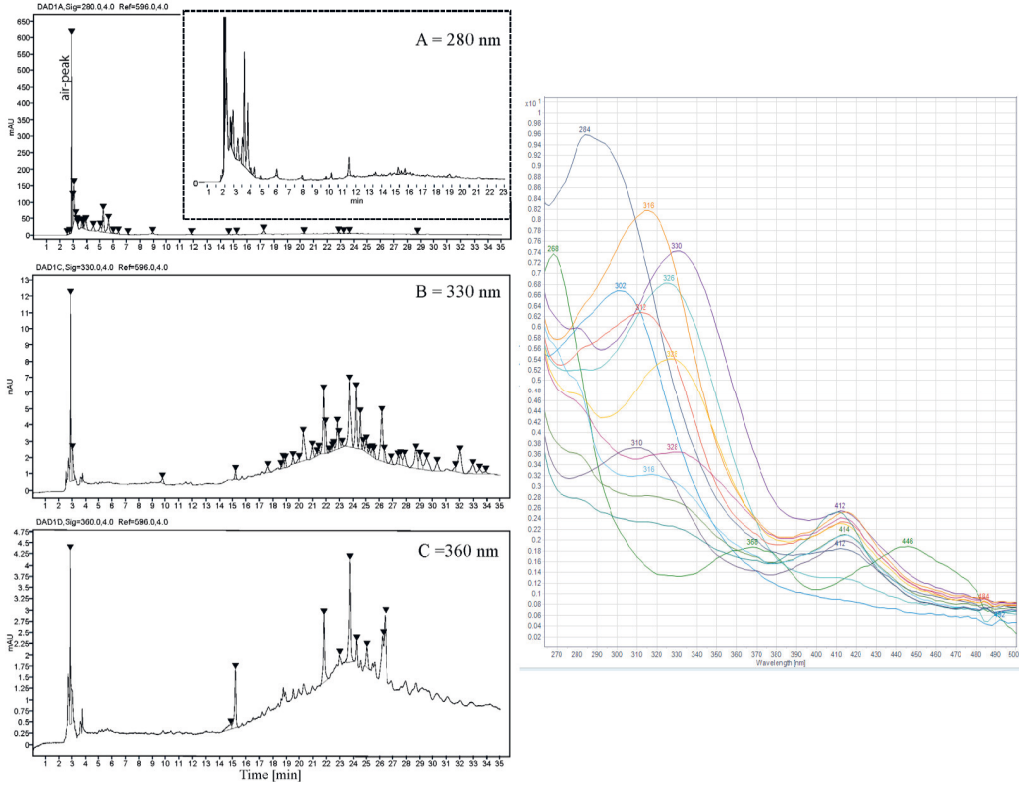

Figure 3. (**Left**) HPLC profile of ASE crude extract of *U. intestinalis* shown at three different wavelengths (A: 280 nm, B: 330 nm, and C: 360 nm). (**Right**) UV-Visible spectrum of selected HPLC-peaks from 15 to 35 min in the (B) 330 nm window.

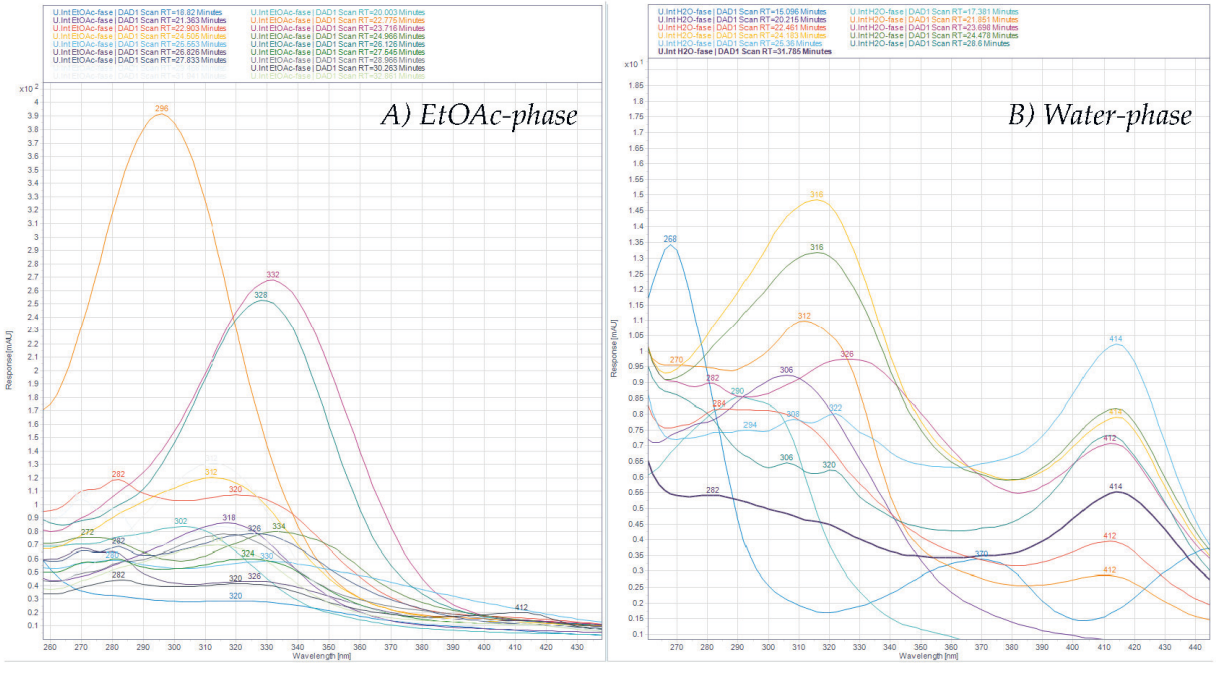

Figure 4. UV-Visible spectra of HPLC peaks found in the (A) EtOAc phase (**left**) and the (B) water phase (**right**) recorded at 330 nm from 15 to 35 min in the chromatograms.

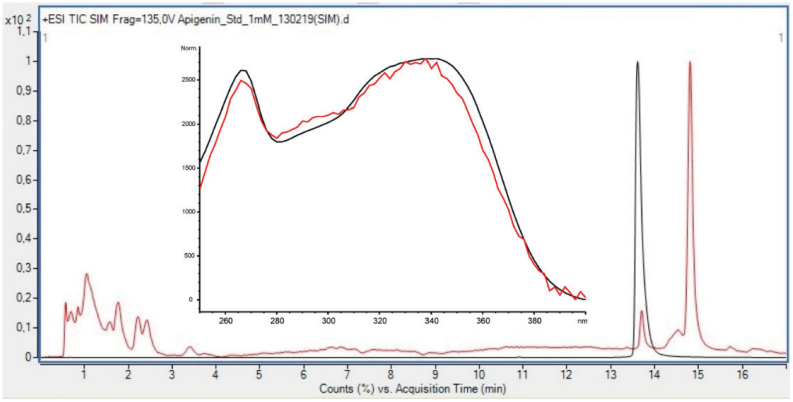

Figure 5. Overlaid HPLC-LRMS (+ESI) SIM Scan at *m/z* 271 of prepLC-A3 fraction (red line, C (Api, HPLC-LRMS) = 2.62 ng/g DW) and apigenin standard (C = 1.00 mM) (black line).

4. Conclusion

This case study provides an optimized extraction process for polyphenolic extraction of algae. The total polyphenolic content was quantified with qNMR (5.3%), HPLC-DAD (1.1%), and TPC (0.4%). Flavonoids and polyphenolic acids were tentatively identified in *Ulva intestinalis* samples. Apigenin was confirmed in one of the semi-purified fractions.

The same samples yielded different total phenolic contents when utilizing the different analytical methods, highlighting the difficulties related to polyphenolic quantification in extracts. All methods utilized in this study depend on assumptions and, thus, also uncertainty. This will be of special importance when analyzing complex samples at low concentrations as is the case for the polyphenolic content in marine algae. Further standardization and optimization of total phenolic quantifications of marine algae samples should be researched.

Author Contributions: M.J. and M.E.W. conceived and designed the experiments; M.J. collected the algae materials; J.U. contributed with support and discussions concerning the qNMR experiments; B.H. contributed with support and discussions concerning the HPLC-LRMS methods and instrumentation; B.H. recorded the HPLC-HRMS data; M.E.W. performed all the laboratorial work, the HPLC-DAD, TPC, qNMR, and HPLC-LR and HRMS experiments; K.K. developed the ASE-350 extraction method and the HPLC-LRMS method, and modified and performed the TFC assay. M.E.W. and M.J. analyzed the data. M.J. and M.E.W. wrote the paper. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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PAPER II

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RESEARCH ARTICLE

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Advancing quantification methods for polyphenols in brown seaweeds—applying a selective qNMR method compared with the TPC assay

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Abstract

Introduction: Brown seaweeds are a sustainable biomass with a potential for various industrial applications. Polyphenols are an important contributor to this potential.

Objective: The aim was total quantification of polyphenols in brown seaweeds from different tidal zones, using a selective ^{1}H quantitative NMR (qNMR) method, comparing the results with the colorimetric Folin–Ciocalteu total phenolic content (TPC) assay.

Method: qNMR was performed with integration of selected peaks in the aromatic region (7–5.5 ppm). Deselection of non-polyphenolic ^{1}H signals was based on information from 2D (^{1}H-^{13}C, ^{1}H-^{15}N) NMR spectra. ^{13}C NMR phlorotannin characterisation facilitated the average number of protons expected to be found per aromatic ring used for the ^{1}H quantification.

Results: Selective qNMR and the TPC assay showed similar results for the three sublittoral growing species from the Laminariaceae; lower amounts for *Laminaria hyperborea* and *Laminaria digitata* (qNMR: 0.4%–0.6%; TPC: 0.6%–0.8%, phloroglucinol equivalents (PGE), dry weight (DW)) and higher amounts for *Saccharina latissima* (qNMR: 1.2%; TPC: 1.5%, PGE, DW). For the eulittoral Fucaceae, *Fucus vesiculosus* (qNMR: 1.1%; TPC: 4.1%; PGE, DW) and *Ascophyllum nodosum* (qNMR: 0.9%; TPC: 2.0%; PGE, DW), the TPC results were found to be up to three times higher than the qNMR results. The ^{13}C NMR characterisation showed the highest phlorotannin polymerisation degree for *F. vesiculosus*.

Conclusion: The TPC assay provided similar polyphenolic amounts to the selective qNMR method for sublittoral species. For eulittoral growing species, the TPC method showed amounts up to three times higher than the qNMR method—most likely illustrating the lack of selectivity in the TPC assay.

KEYWORDS
phlorotannins, polyphenols, qNMR, quantification, seaweeds

1 | INTRODUCTION

The world's population is projected to reach 9 billion by 2050, and utilisation of bioresources will be increasingly important for food, feed, and health applications. Seaweeds, or macroalgae, are a sustainable biomass and play an increasing role in aquaculture and marine bioresource development.[1–4] Macroalgae grow in abundance in their natural habitat and can be both harvested and farmed. Seaweed farming is a sustainable industry with minimal environmental impact, as it does not require fertilisers or irrigation and does not compete for agricultural land.[1,5–9] Several products, for various applications, can be extracted from macroalgae including alginate, fucoidan, mannitol, cellulose, proteins, carotenoids, and polyphenols.[10,11]

Polyphenols are bioactive compounds synthesised by macroalgae during plant growth and as a response to external stressors such as UV radiation, wounding, and climate.[12–21] Bioactivities of polyphenols include antioxidant, antiviral, anticancer, antibacterial, antidiabetic, and neuroprotective activities as well as antiallergic effects.[22–35] Various polyphenols have been identified in macroalgae, with phlorotannins being the predominant polyphenol group in the class of Phaeophyta (brown algae).[12,36–42]

Phlorotannins, which are exclusive to brown algae, are oligomers of phloroglucinol and are separated into different subgroups depending on the linkage of the phloroglucinol units. These linkages can be either phenyl linkages (C-C), ether linkages (C-O-C), or both (Figure 1).[9,12,18,43–45]

Extensive analysis and identification of polyphenols in algae is important to uncover and understand algae's potential in industrial applications. However, existing methods for polyphenolic analysis have significant shortcomings, such as lack of exactness at the molecular level. This is due to the low concentrations of polyphenols in the large compound matrix of the algae.[36,46,47] Furthermore, the diversity of seaweed polyphenols complicates data collection and standardisation of procedures.[9] Being able to quantify the polyphenolic content in crude seaweed materials with higher accuracy is important in order to fully explore seaweeds' potential applications.[48–51] Table 1 displays selected literature with variation in methods, standards, and polyphenolic amounts found for different species representing three different brown seaweed families; Fucaceae, Sargassaeae, and Laminariaceae.

The Folin–Ciocalteu (FC) total phenolic content (TPC) colorimetric assay was introduced nearly 100 years ago and is still the most used method for polyphenol quantification.[66] However, the method depends on a non-selective redox reaction and has been evaluated to yield only estimates of polyphenol content.[36,67–70] High performance liquid chromatography (HPLC) with UV-visible diode array detection (DAD) is also used for polyphenol quantification but rarely for quantification of the total polyphenolic content. This is due to the quantification method being based on molar absorptivity (ε; Beer–Lambert's law, $A = \varepsilon cl$). Molar absorptivity (ε) values vary greatly, even within polyphenol classes, making a precise "one standard" total polyphenolic quantification with HPLC-DAD impossible.[71] Quantitative NMR is a quantification method independent of colorimetric changes, molar absorptivity, and calibration curves.[36,44,45,53,72–74] The method quantifies polyphenols based on correlations between signals of polyphenols and an internal or external standard significantly different from the analyte. However, the method should not be used without some knowledge of the polyphenolic nature of the extract and reasonable selection of NMR peaks for quantification.[36]

In this study, we continue our examination of total phenolic quantification methods for seaweeds by optimising the quantitative ^{1}H NMR method and compare the results with the FC TPC assay.[36] Three brown seaweeds from the Laminariaceae, *Laminaria hyperborea*, *Laminaria digitata*, *Saccharina latissima* (syn. *Laminaria saccharina*), and two Fucaceae species, *Ascophyllum nodosum* and *Fucus vesiculosus*, were selected for the examination. The selected Laminariaceae species are distributed in the sublittoral zone, while the Fucaceae species have their natural habitat from the middle littoral to lower intertidal zone: the eulittoral zone. Thus, the Fucaceae species are more exposed to greater variation in environmental conditions such as solar radiation (UV) and temperature fluctuation than the Laminariaceae species, possibly reflected in their polyphenol content.[75] This study's main objective was to advance toward optimised quantification tools for analysis of polyphenols in seaweeds to increase the accuracy of these assessments. ^{13}C NMR was used to assess the different linkage profiles of the phlorotannins in the examined brown seaweed species.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All chemicals used were of analytical grade. The FC reagent, gallic acid, phloroglucinol, methanol (≥ 99.9%), ethanol (absolute), ethyl acetate (≥ 99.5%), $DMSO_2$ (TraceCERT®), and DMSO-d_6 (0.03% TMS)

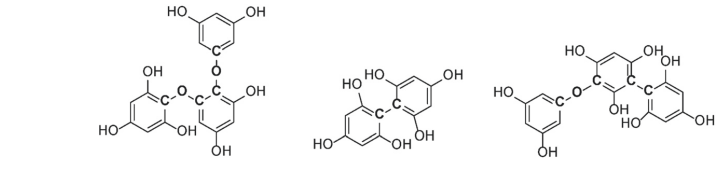

FIGURE 1 Examples of phlorotannins containing the different phloroglucinol linkage types triphlorethol A (ether linkage, **C-O-C**, phlorethol type), difucol (phenyl linkage, **C-C**, fucol type), and fucophlorethol (ether and phenyl linkage, fucophlorethol type)

TABLE 1 Selected reported quantifications of polyphenols (PP) from brown seaweeds found for species within the Fucaceae, Sargassaceae, and Laminariaceae families, utilising either the TPC assay or qNMR, indicating variation in reference standards used

Seaweed	Location	Extraction solvent	Quantification method	PP concentration	Publication
Fucaceae					
Fucus vesiculosus	Denmark	Ethanol	TPC	12.0 mg GAE/g *	Farvin and Jacobsen (2013)[52]
F. vesiculosus	France	Ethanol	qNMR	15.32% TAE	Parys et al. (2007)[53]
F. vesiculosus	France	Ethanol	TPC	15.88% PGE	Parys et al. (2007)[53]
F. vesiculosus	Ireland	60% aqueous methanol	TPC	2.5 mg GAE/g DW	O'Sullivan et al. (2011)[54]
F. vesiculosus	Canada	50% aqueous methanol	TPC	23.21% PGE	Zhang et al (2006)[55]
F. vesiculosus	Iceland	70% aqueous acetone	TPC	242 mg PGE/g *	Wang et al. (2009)[56]
Fucus serratus	Ireland	80% ethanol	TPC	0.075 mg GAE/g *	Heffernan et al. (2014)[57]
F. serratus	Ireland	70% aqueous acetone	TPC	30.68 mg PGE/g	Ford et al. (2020)[45]
F. serratus	Ireland	70% aqueous acetone	qNMR	17.00 mg TAE/g	Ford et al. (2020)[45]
Ascophyllum nodosum	Ireland	80% ethanol	TPC	0.101 mg PGE/g *	Tierney et al. (2013)[58]
A. nodosum	Spain	Water	TPC	59.2 mg PGE/g DW	Gisbert et al. (2021)[59]
A. nodosum	Ireland	70% aqueous acetone	TPC	36.68 mg PGE/g	Ford et al. (2020)[45]
A. nodosum	Ireland	70% aqueous acetone	qNMR	37.35 mg TAE/g	Ford et al. (2020)[45]
A. nodosum	Scotland	Ethanol	TPC	0.3%–1.0% PGE FW	Parys et al. (2009)[60]
A. nodosum	Scotland	Ethanol	qNMR	0.6%–2.2% TAE FW	Parys et al. (2009)[60]
A. nodosum	France	Ethanol	TPC	13.49% PGE	Parys et al. (2007)[53]
A. nodosum	France	Ethanol	qNMR	25.34% TAE	Parys et al. (2007)[53]
Sargassaceae					
Sargassum muticum	France	75% ethanol	TPC	10.18% PGE	Anaëlle et al. (2013)[61]
Sargassum fusiforme	China	30% aqueous ethanol	TPC	63.61 mg PGE/g	Li et al. (2017)[62]
Cystoseira tamariscifolia	France	50% aqueous methanol	TPC	0.63% PGE	Jégou et al. (2015)[63]
C. tamariscifolia	France	50% aqueous methanol	qNMR	0.46% PGE	Jégou et al. (2015)[63]
Laminariaceae					
Macrocystis pyrifera	Chile	70% aqueous acetone	TPC	1.47 mg GAE/g DW	Leyton et al. (2016)[64]
Laminaria hyperborea	Ireland	60% aqueous methanol	TPC	1.5 mg GAE/g DW	O'Sullivan et al. (2011)[54]
L. hyperborea	Iceland	70% aqueous acetone	TPC	130 mg PGE/g *	Wang et al. (2009)[56]
Laminaria digitata	Iceland	70% aqueous acetone	TPC	10 mg PGE/g *	Wang et al. (2009)[56]
L. digitata	Denmark	Ethanol	TPC	0.324 mg GAE/g *	Farvin and Jacobsen (2013)[52]
L. digitata	Scotland	80% aqueous methanol	TPC	5.7% GAE	Vissers et al. (2017)[44]
L. digitata	Scotland	80% aqueous methanol	qNMR	4.3% GAE	Vissers et al. (2017)[44]
L. digitata	Ireland	80% ethanol	TPC	0.0022 mg GAE/g *	Heffernan et al. (2014)[57]
Saccharina latissima	Canada	50% aqueous methanol	TPC	2.17% PGE	Zhang et al. (2006)[55]
Saccharina latissima	Norway	80% aqueous acetone	TPC	5–15 mg PGE/g DW	Roleda et al. (2019)[65]

*Value recalculated to mg (GAE/PGE)/g from original publication.
Abbreviations: DW, dry weight; GAE, gallic acid equivalents; PGE, phloroglucinol equivalents; TAE, trimesic acid equivalents; TPC, total phenolic content.

were acquired from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Deionised water was deionised at the University of Bergen (Bergen, Norway).

2.2 | Seaweed material

Laminaria hyperborea leaves were acquired from Alginor ASA. Samples were harvested in March 2020 (M20), September 2020 (S20), and August 2021 (A21) along the coast of Haugesund, Norway (Rogaland field 55E; N 59°11′ E 005°06′). *Laminaria digitata* leaf samples were also acquired from Alginor ASA. The material was collected in August 2019 along the southern Australian coast, Melbourne, Victoria. *Fucus vesiculosus* samples were collected from Storåkervika, Bergen, Norway (N 60°30.1044′ E 5°15.6726′) in August 2019. *Saccharina latissima* (syn. *L. saccharina*) was acquired from Lerøy AS. The material was harvested outside Trollsøy, Vestland (N 60°8.42′ E 5°14.88′) in June 2021. *Ascophyllum nodosum* was collected in Eidsvåg, Bergen (N 60°26.63′ E 05°17.87′) in September 2017. All samples were rinsed thoroughly with fresh water and air dried. The plant material was stored at −20°C when not used.

2.3 | Sample preparation

Crude extracts of each macroalgae were obtained using similar extraction parameters to the ones established by Ummat et al (2020).[76] A total of 10–20 g of dried material was pre-soaked with water (500 mL) for 30 min in an ultrasound bath (35 kHz). The same material was further extracted with aqueous ethanol (50:50, v/v; 2 x 500 mL) in the ultrasound bath for 30 min. All extractions of the same material were pooled and dried for analysis. When not used, dried crude extracts were stored at −20°C.

2.4 | Folin–Ciocalteu TPC assay

Procedures described by Singleton et al. (1999) and Singleton and Rossi (1965) with slight modifications optimised for brown seaweeds were used to determine the TPC using the FC reagent.[77,78] Briefly, in the method 0.2 mL sample, blank or standard, 1.59 mL FC reagent, and 4.0 mL 20% (w/v) Na_2CO_3 were used and made to a total volume of 20 mL with water. The mixture was incubated for 2 h in the dark, and absorbance was measured at 760 nm using a Biochrom Libra S32 UV instrument (Biochrom, Cambridge, United Kingdom). Gallic acid and phloroglucinol calibration curves were used to validate the linearity, sensitivity, precision, and accuracy of the TPC method (Table 2). Three parallels ($n = 3$) of each sample or standard were analysed to ensure statistically significant results.

2.5 | NMR analyses

Dried samples were dissolved in 0.6 mL DMSO-d_6 (0.03% TMS) containing the internal standard $DMSO_2$ (C = 10mM). Quantification using ^{1}H NMR analyses was performed employing a Bruker 600 MHz instrument (Bruker BioSpin, Zürich, Switzerland). All spectra were recorded at 298 K. For accurate quantification, the T_1 value of each sample was measured to ensure complete relaxation between scans. The T_1 measurements were performed by applying the *t1ir* pulse sequence with a sweep width of 19.8 ppm, 16 k data points, 8 scans, 4 dummy scans, and 9 different inversion recovery delays between 1 ms and 5 s. To ensure complete relaxation, the d1 value was set to 5 × T_1 for all ^{1}H spectra obtained for quantitative NMR (qNMR) analysis.[36,79]

The one-dimensional (1D) ^{1}H NMR spectra used for quantifications were recorded using the *zg30* pulse sequence with a sweep width of 19.8 ppm, 65 k data points, 128 scans, 2 dummy scans, and the relaxation delay (d1) was 5 × T_1 for the selected sample. The spectra were processed using a line broadening of 0.3 Hz.

All quantifications were performed based on Equation 1 with $DMSO_2$ (10mM, No. H = 6, MW = 94.13 g/mol) as the internal standard.

$$C_{\text{sample}}\,[\text{M}] = \frac{I_{\text{sample}} \times n_{\text{DMSO2}} \times C_{\text{DMSO2}}}{I_{\text{DMSO2}} \times n_{\text{sample}}} \quad (1)$$

where C = molar concentration [M], I = signal integral, and n = number of protons yielding the signal.

Aromatic signals in the region of 7.0–5.5 ppm were individually integrated and quantified and then added together to obtain the estimated TPC. Standard samples of gallic acid and phloroglucinol were analysed, integrated, and quantified to yield the standard deviation of the qNMR method (Table 3).

Two-dimensional (2D) ^{1}H-^{13}C and ^{1}H-^{15}N NMR spectra (heteronuclear multiple-bond correlation (HMBC) and heteronuclear single-quantum coherence (HSQC)) were used to eliminate non-aromatic signals in the polyphenol region (7.0–5.5 ppm).

^{1}H-^{13}C HMBC spectra were acquired using the *hmbcetgpl3nd* pulse sequence with non-uniform sampling (50%), 352 scans, 16 dummy scans, ^{1}H sweep width of 13.02 ppm, ^{13}C sweep width of 220.0 ppm, and a relaxation time of 2.0 seconds.

^{1}H-^{13}C HSQC spectra used the *hsqcedetgpsisp2.3* pulse sequence with 128 scans, 32 dummy scans, ^{1}H sweep width of 13.02 ppm, and ^{13}C sweep width of 200.0 ppm.

Additionally, ^{1}H-^{15}N HSQC spectra were recorded utilising the *hsqcetgp* pulse sequence. Number of scans was 32 with 8 dummy scans, ^{1}H sweep width of 15.15 ppm, and ^{15}N sweep width of 200.0 ppm.

1D ^{13}C NMR spectra were used qualitatively to identify linkage differences of phlorotannins in the samples. Spectra were recorded

TABLE 2 Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for gallic acid and phloroglucinol at 760 nm using the optimal total phenolic content (TPC) conditions

Standard	Calibration curve	r^2	Range [ug/mL]	LOD [ug/mL]	LOQ [ug/mL]
Gallic acid	y = 0.00115x – 0.00101	0.999	1000–30	18.610	56.391
Phloroglucinol	y = 0.00102x – 0.00177	0.998	1000–30	44.067	133.54

TABLE 3 Standard deviations of the qNMR method meaured with two standards, gallic acid and phloroglucinol, with known concentrations (C_{known}) using $DMSO_2$ as the internal reference

Standard	C_{known} [M]	Chemical shift [ppm]	Integral	Number of protons (n)	$C_{measured}$ [M]	Standard deviation
Gallic acid	0.0355	6.91	1.18	2	0.0353	0.000115
Phloroglucinol	0.0362	5.70	2.36	3	0.0471	0.00544

using the *udeft* pulse sequence with 21 k data points, 236.65 ppm sweep width, 5,120 scans, and 8 dummy scans. The *udeft* pulse sequence was used, so more sensitive ^{13}C spectra with maximised signal-to-noise ratio could be acquired in shorter time.[80,81] Signals of 95–160 ppm were used to obtain carbon ratios distinguishing characteristic phlorotannin carbons.

3 | RESULTS AND DISCUSSION

3.1 | Phlorotannin characterisation with ^{13}C NMR

As phlorotannins are the dominating polyphenolic compounds in brown seaweeds, ^{13}C NMR was used to assess the linkage profiles of the phlorotannins in the five seaweeds examined (Table 4). ^{13}C NMR spectra of *L. hyperborea* (M20), *L. digitata*, *S. latissima*, *A. nodosum*, and *F. vesiculosus* were interpreted based on predicted chemical shifts and literature data.[44,45] Figure 2 displays the ^{13}C NMR spectra of the examined species (A-E) and indicates the characteristic signal regions for typical phlorotannin linkages. The two carbons of the phlorotannin ether linkages (C-O-C) were observed between 124 and 128 ppm and 156 and 161 ppm in the ^{13}C NMR spectrum, while signals from phenyl linkages (C-C) were found between 100 and 105 ppm. Signals representing the C-H bonds in the aromatic phlorotannin were found between 96 and 99 ppm. To make an overall characterisation of the phlorotannin content present for each species, the relative occurrence of ether linkages (C-O-C) and phenyl linkages (C-C) in the ^{13}C NMR spectrum can be compared. The measured intensities of the different linkage signals are calculated relative to the aromatic phlorotannin C-H carbon: I (C-H) = 1 (Table 4).[44,45]

TABLE 4 Measured intensity (^{13}C NMR) of characteristic phlorotannin linkages in the examined seaweed species presented relative to each species' aromatic C-H carbon

	Intensity ratio measured		
Species	C-O-C	C-C	C-H[a]
Laminaria hyperborea M20	3.08	1.00	1.00
Laminaria digitata	0.94	0.32	1.00
Saccharina latissima	2.73	0.68	1.00
Fucus vesiculosus	20.1	1.11	1.00
Ascophyllum nodosum	4.95	5.67	1.00

[a]I(C-H) = 1.0; C-O-C = ether linkage; C-C = phenyl linkage.

Ford et al. (2020) and Vissers et al. (2017) report on characterisation of phlorotannins in brown seaweeds using ^{13}C NMR data. *A. nodosum* and *Fucus serratus* in the study by Ford et al. (2020) were both found to be dominated by phlorethol-like (ether linkage) phlorotannins.[45] Vissers et al. (2017) report a higher abundance of ether linkages compared with phenyl linkages in *L. digitata*.[44] In the same study, they also present a molar fucol-to-phlorethol ratio of 1:26, which means that for each phenyl linkage there are 26 ether linkages within the phlorotannins in the extract, indicating an abundance of phlorethol-like phlorotannins. The intensity data from our analysis (Table 4) indicate that phlorotannins with ether linkages are more abundant compared with those with phenyl linkages in four of the five seaweeds in this study (*L. hyperborea*, *L. digitata*, *S. latissima*, and *F. vesiculosus*). Of these, *F. vesiculosus* shows, by far, the highest fucol-to-phlorethol ratio (1:18). The calculated linkage ratio of these species indicates a larger presence of phlorethol-type phlorotannins compared with fucol-type phlorotannins (Figure 1, Table 4). The fifth brown alga, *A. nodosum*, showed a distinct ratio of an approximate equal occurrence of phenyl linkages compared with ether linkages (1:0.8), in accordance with fucophlorethol-type phlorotannins. However, an even distribution of the two linkages, resulting in a similar ratio, is also

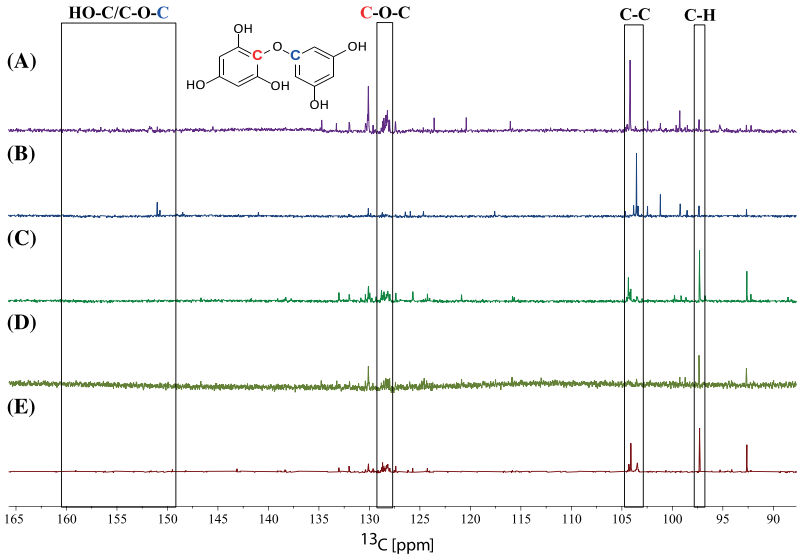

FIGURE 2 ^{13}C NMR spectra of *Fucus vesiculosus* (A), *Ascophyllum nodosum* (B), *Saccharina latissima* (C), *Laminaria digitata* (D), and *Laminaria hyperborea* M20 (E) demonstrating the structural linkage differences. The boxes indicate the peaks representing ether linkages (C-O-C), phenyl linkages (C-C), and the C-H bonds. A hypothetical structure is drawn to indicate the difference in chemical shifts of the two carbons in the ether linkages (C-O-C). Figure 1 illustrates the different phlorotannin linkages.

a possibility. Although Ford et al. (2020) did not report a fucol-to-phlorethol ratio, calculations based on the data provided in their study yield the fucol-to-phlorethol ratio is 1:7 for *A. nodosum* and 1:2 for *F. serratus*. The linkage ratios are, to some extent, sample specific because the intensities of each characteristic carbon signal are measured relative to the aromatic C-H carbon of that selected sample. This will cause results to vary, but the differences could also reflect both seasonal and geographical variation in the seaweeds' phlorotannin content. However, the dissimilar results might also reflect the natural variation of the polyphenolic content.[82–84] Overall, the presented data indicate that brown algae contain more phlorethol-like phlorotannins (Table 4), supporting the reports by Visser et al and Ford et al and reinforced by mass spectrometry analyses previously reported.[41,85,86]

Furthermore, the phlorotannin ^{13}C NMR characterisation can facilitate an estimate of the number of protons per aromatic phlorotannin ring in the seaweed samples. This estimate is made to improve the accuracy of the total polyphenolic content quantification using ^{1}H qNMR.[44,53] phlorethols (Figure 1) generally consist of one terminal aromatic ring containing three aromatic hydrogens, yielding signals in the characteristic polyphenol region of the ^{1}H spectrum, whereas the remaining phloroglucinol units only have two aromatic hydrogens. In fucols, the terminal units contain two aromatic hydrogens and the internal unit(s) only one. Additionally, when a phloroglucinol unit is connected to ≥ 3 subunits (polymerisation degree ≥ 4), the average number of aromatic hydrogens decreases.[44] The species' polymerisation degree is investigated to some extent, as the ratios of ethyl (C-O-C) and phenyl (C-C) linkages are reported relative to the aromatic carbon (C-H). Table 4 indicates that the Laminariaceae have the lowest polymerisation degree, with *L. digitata* having the lowest. Both *A. nodosum* and *F. vesiculosus* show high degree of polymerisation with either I(C-O-C) or I(C-C) well above 3. Additionally, other studies have found indications that seaweeds belonging to the Fucaceae species contain phlorotannins consisting of 2–16 phloroglucinol units.[41,85,87,88] Montero et al. (2016) found that for the brown algae *Sargassum muticum*, the degree of polymerisation of the phlorotannins ranged from 2 to 10 for samples collected in Norway.[89] Taking these studies and the indications of the relative intensity ratios measured into account, it can be assumed that phlorotannins with polymerisation degrees ≥ 4 make up a large part of the phlorotannin matrix of the alga in this study. Considering the ^{13}C ratios and the knowledge of the expected structures, the average number of protons per aromatic ring was set to be 2H ($n_{sample} = 2$, Equation 1). The number of hydrogens present per aromatic ring estimates the number of protons available per polyphenol in the sample, and thus this educated assumption of the number of protons in the samples was used in the qNMR calculations (Table 5).

3.2 | Total quantification of polyphenols

^{1}H qNMR can be performed by integrating the -OH spectral region (14–8 ppm), as proposed by Nerantzaki et al. (2011).[74] More conventional methods, however, integrate the aromatic region (8–6 ppm).[36,44,53,60,63,72,73] Due to possible H-D exchange with aromatic–OH groups, leading to loss of intensity and broad peaks, the aromatic ^{1}H-region was selected for polyphenolic quantification. Based on knowledge of chemical ^{1}H-shifts of polyphenolic aromatic signals, a narrower region (7.0–5.5 ppm) was selected in order not to integrate signals from the same aromatic system twice.[44,45,53] Two-dimensional NMR spectra (HMBC and HSQC) of the seaweed extracts were analysed to explore the nature of the proton signals in the defined region followed by a selective peak-picking process prior to integration. For example, all samples revealed a similar peak around 6 ppm in the proton spectra (Figure 4). ^{1}H-^{15}N HSQC spectra indicated that this ^{1}H peak was coupled to a nitrogen δ 5.97/81.8 ($^{1}H/^{15}N$), meaning this signal is unlikely to originate from the polyphenolic biosynthesis (Figure 3). Therefore, this peak was not quantified. Similar peak picking was performed based on recorded ^{1}H-^{13}C HMBC spectra for each alga. Figure 4 displays the quantified region of the ^{1}H NMR spectrum for the five algae with eliminated signals indicated. Signals were individually integrated and quantified (Equation 1), then summed to yield the total polyphenolic content. All signals belonging to the same aromatic ring structure in the 2D spectra were averaged, rather than summed, prior to the quantification calculation so as to not yield overestimations. Quantification using Equation 1 is dependent on an unknown factor, namely the number of protons per aromatic ring in the samples (n_{sample}). Increasing this value will decrease the molar concentration calculated; however, using ^{13}C NMR to estimate this value provides a more accurate quantification.[44] Furthermore, a standard molecular weight is required to report the quantification in mass units (mg/g). This value is directly proportional to the quantification result and has a significant impact on the quantification; a high molecular weight standard will yield a higher quantification result.

Sample	Family	Zone	C [mg GAE/g DW]	C [mg PGE/g DW]
L. hyperborea M20	Laminariaceae	Sublittoral	8.32 ± 0.00	6.17 ± 0.01
L. hyperborea S20	Laminariaceae	Sublittoral	5.51 ± 0.00	4.08 ± 0.01
L. hyperborea A21	Laminariaceae	Sublittoral	6.57 ± 0.00	4.87 ± 0.01
L. digitata	Laminariaceae	Sublittoral	6.86 ± 0.00	5.09 ± 0.01
S. latissima	Laminariaceae	Sublittoral	16.8 ± 0.0	12.4 ± 0.0
A. nodosum	Fucaceae	Eulittoral	11.57 ± 0.0	8.57 ± 0.0
F. vesiculosus	Fucaceae	Eulittoral	14.8 ± 0.0	11.0 ± 0.0

TABLE 5 Total polyphenol content obtained for *Laminaria hyperborea*, *Laminaria digitata*, *Saccharina latissima*, *Ascophyllum nodosum*, and *Fucus vesiculosus* extracts using the selective qNMR method. Results are expressed as both gallic acid equivalents (GAE) and phloroglucinol equivalents (PGE) per dry weight (DW).

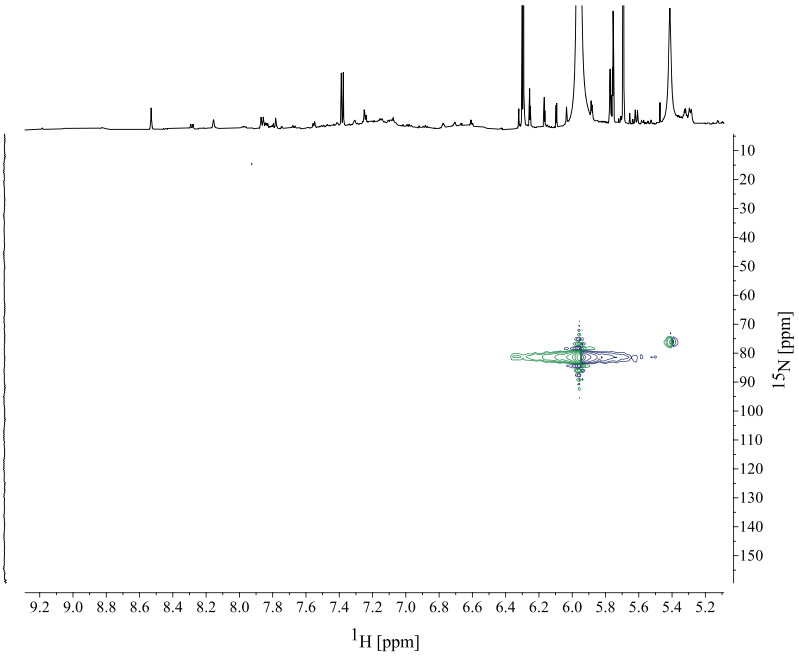

FIGURE 3 ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectrum of *Laminaria hyperborea* M20 indicating a large peak at 5.97 ppm coupling to a nitrogen at 81.8 ppm, indicating this signal does not represent a polyphenol. Similar peaks observed at 6 ppm in other algae analysed were also eliminated prior to quantification.

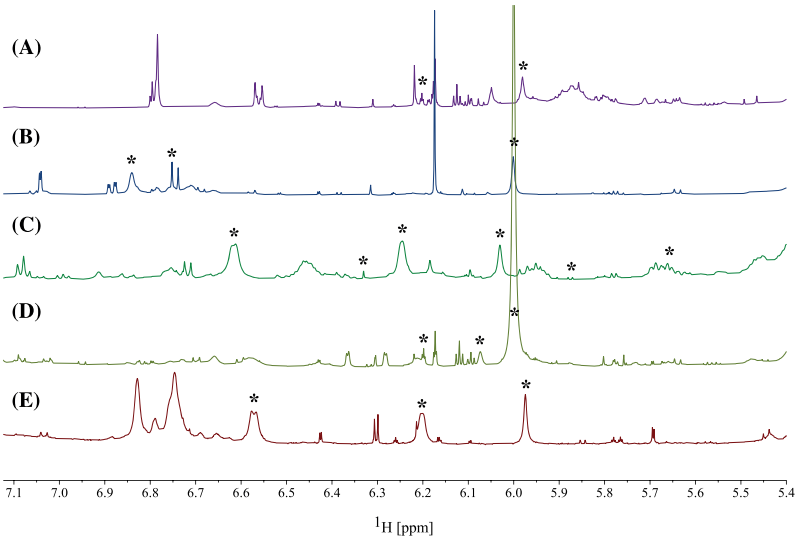

FIGURE 4 ^{1}H NMR spectra displaying the polyphenolic region (7.0–5.5 ppm) used for quantification of *Fucus vesiculosus* (A), *Ascophyllum nodosum* (B), *Saccharina latissima* (C), *Laminaria digitata* (D), and *Laminaria hyperborea* M20 (E). Signals labelled with asterisk (*) were deselected based on 2D NMR prior to quantification.

The FC TPC assay was also used for quantification. This is a colorimetric assay dependent on the redox reaction of the FC reagent with hydroxyl groups of polyphenols in a sample. Singleton and Rossi's TPC method from 1965, optimised for wine samples, is one of the most cited.[78] Slight modifications of this method were made prior to the analysis to optimise the assay for seaweed samples.

In Table 1, selected reported quantifications of polyphenols from brown seaweeds utilising the TPC assay and/or qNMR are shown.[36,44,45,53] The majority of the works quantify using only the TPC assay; however, some studies use both methods such as Parys et al. (2007), Parys et al. (2009), Vissers et al. (2012), and Ford et al. (2020).[44,45,53,60] Comparison and evaluation of analytical methods for

total quantification of polyphenols in seaweeds is relevant to gain new knowledge of the polyphenolic content in seaweeds and to search for the optimal method to assess the total content of this highly diverse group of compounds.[9,36,53,60] However, some studies report results from the TPC and qNMR as different standard equivalents or without any explanation of the standard used for quantification. This makes both interpretation and comparison difficult and highlights the limitation of not having standardised methods.

The quantifications presented using a selective qNMR method resulted in the highest polyphenol content found for *S. latissima* (1.2% phloroglucinol equivalents (PGE), dry weight (DW)) (Table 5), while the two other Laminariales were found to contain the lowest observed polyphenolic content in the study with 0.41– 0.62% (PGE, DW). *Fucus vesiculosus* and *A. nodosum* showed similar values to *S. latissima*, with polyphenol contents of 1.1% and 0.9% (PGE, DW), respectively. The qNMR results calculated using phloroglucinol (MW = 126.11 g/mol) were slightly lower than once calculated using gallic acid (MW = 170.12 g/mol) due to the lower molecular weight of phloroglucinol, as previously discussed.

Parys et al. (2007) and Ford et al. (2020) both analysed *A. nodosum* (Fucaceae) and found the total polyphenol content, using qNMR, to be 25.34% trimesic acid equivalents (TAE) and 37.35 mg TAE/g, respectively (Table 1).[45,53] These results are considerably higher than the amounts found for the same species in our investigation (Table 5). Whether the reported data by Parys et al and Ford et al are calculated based on fresh or dry weight is not clear, although dry weight concentrations are most frequently used. Additionally, both studies perform NMR quantification using a larger molecular weight for standardisation (MW (trimesic acid) = 210.14 g/mol) and a smaller number of protons (n_{sample} = 1.7).[45,53] These two parameters will make a significant impact on the quantification as mentioned previously and therefore contribute to the higher quantification reported by Ford et al More comparable to our results are studies performed by Jégou et al. (2015) reporting 0.46% polyphenol content in *Cystoseira tamariscifolia* (Fucaceae) using qNMR, and Roleda et al. (2019) reporting TPC amounts of 5–15 mg PGE/g DW in *S. latissima*, with the latter being season dependent (Table 1).[63,65]

Applying the TPC assay to the three Laminariales resulted in polyphenolic contents of 0.61%–1.5% (PGE, DW) and 0.54%–1.3% (GAE, DW), with the greatest amounts observed for sugar kelp (*S. latissima*) (Table 6). *Fucus vesiculosus* and *A. nodosum* showed TPC results of approximately 4% and 2% PGE, respectively. Based on the TPC results only, the eulittoral *F. vesiculosus* and *A. nodosum*, growing in more shallow waters, show a higher polyphenolic concentration compared with all three Laminariaceae species growing in the sublittoral zone. Parys et al. (2007) and Zhang et al. (2006) present even higher TPC values for species belonging to the Fucaceae family (15.9% PGE and 23.2% PGE, respectively), while Farvin and Jackobsen (2013), O'Sullivan et al. (2011), and Heffernan et al. (2014) all have found lower TPC values for *Fucus* species (Table 1).[52–55,57] A study of Icelandic seaweeds by Wang et al. (2009) reports higher total polyphenol contents for both *F. vesiculosus* (~ 24%) and *L. hyperborea* (~ 13%). However, their results for *L. digitata* are comparable to our investigation (10 mg PGE/g, ~ 1%).[56]

3.3 | Comparing the selective qNMR method with the TPC assay

In Figure 5 the quantified polyphenolic content of *L. hyperborea*, harvested in various seasons, *L. digitata*, *S. latissima*, *A. nodosum*, and *F. vesiculosus* using the selective qNMR method and the TPC assay are shown—including a trendline for the two methods compared with a non-selective qNMR method. The general trend shows higher TPC values compared with the corresponding qNMR quantifications. Minor differences were observed between the two methods for the sublittoral growing Laminariaceae species (*L. hyperborea*, *L. digitata*, and *S. latissima*) (Figure 5). Indications of possible seasonal differences were observed for the selected samples, although a complete seasonal study was not performed. However, variations of the polyphenol content in regard to harvest season have previously been reported in literature.[9,45,60,65,90] A significant difference between the TPC and qNMR results was observed for both eulittoral growing Fucaceae species *A. nodosum* and *F. vesiculosus*, where the TPC assay yields up to three time the amount found with the qNMR method for the *Fucus* species. Ford et al. (2020) also reports higher TPC values compared with their qNMR results for a Fucaceae species *F. serratus*.[45] However, they use a non-selective qNMR method, and their qNMR quantification of *A. nodosum* is approximately 20% higher compared with their TPC assay. Parys et al. (2009) also compare the FC TPC assay with a (non-selective) qNMR method in a seasonal investigation of the polyphenol concentration in *A. nodosum*.[60] Their FC TPC assay yields 1.5–4 times higher polyphenolic amounts than their qNMR method, and Parys et al. conclude that the results from the two methods cannot be compared due to their principal differences. Both Ford et al. and Parys et al. apply a non-selective qNMR method, and

Seaweed	Family	Zone	C [mg GAE/g DW]	C [mg PGE/g DW]
L. hyperborea M20	Laminariaceae	Sublittoral	6.23 ± 0.11	7.15 ± 0.15
L. hyperborea S20	Laminariaceae	Sublittoral	5.72 ± 0.07	6.56 ± 0.08
L. hyperborea A21	Laminariaceae	Sublittoral	5.35 ± 0.04	6.14 ± 0.05
L. digitata	Laminariaceae	Sublittoral	6.94 ± 0.09	7.93 ± 0.09
S. latissima	Laminariaceae	Sublittoral	13.1 ± 0.04	15.0 ± 0.05
A. nodosum	Fucaceae	Eulittoral	17.6 ± 0.04	20.1 ± 0.05
F. vesiculosus	Fucaceae	Eulittoral	37.0 ± 1.0	42.0 ± 1.1

TABLE 6 Overview of the total polyphenol content obtained for *Laminaria hyperborea*, *Laminaria digitata*, *Saccharina latissimia*, *Ascophyllum nodosum*, and *Fucus vesiculosus* extracts using the optimised total phenolic content (TPC) reaction conditions. Results are expressed as gallic acid and phloroglucinol equivalents (GAE/PGE) per dry weight (DW) (mean ± SD, n = 3).

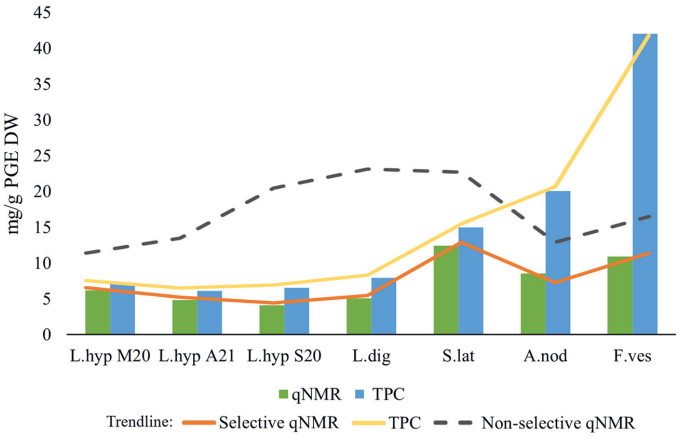

FIGURE 5 *Polyphenolic content of Laminaria hyperborea (L.hyp)* (harvested in various seasons M20 = March 2020, A21 = August 2021, S20 = September 2020), *Laminaria digitata (L.dig)*, *Saccharina latissima*, *Ascophyllum nodosum*, and *Fucus vesiculosus* quantified using qNMR (green) and total phenolic content (TPC) assay (blue), reported as milligram phloroglucinol equivalents (PGE) per gram dry weight (DW). The orange and yellow lines represent the trend lines of the displayed bar chart, and the grey line indicates non-selective qNMR values.

these results are higher than the ones presented in our study. A significant difference can be observed between selective and non-selective qNMR quantification, as illustrated in Figure 5.

In general, the colorimetric TPC assay using the FC reagent has been assumed to overestimate the polyphenolic content.[69,70,91,92] This is due to several factors, such as the presence of metal contaminants or high levels of reducing sugars or other compounds, for instance ascorbic acid or amino acids, which interfere with the FC reaction.[68,91,93] However, increased polyphenol diversity within the extract, such as hydroxybenzoic acids, hydroxycinnamic acids, hydrolysable tannins, proanthocyanins, and flavonoids—seen in seaweed species in rather shallow waters as well as in aquatic and terrestrial plants—may also result in higher TPC quantifications due to the possibly larger number of reacting groups within a molecule not accounted for in the TPC standardisation.[9,36,75,77,78,90,94–96] Quantitative NMR as a method is in general not as sensitive as the colorimetric TPC assay to interfering species such as metal contaminants, high levels of reducing sugars, or pigments. The reported selective qNMR method is also less influenced by the diversity of the polyphenols, as the ^{13}C NMR partial characterisation prior to the quantification facilitates the estimate of number of protons (H) per aromatic ring of the dominating polyphenol group (phlorotannins) in the extract—increasing the accuracy of the method.

The polyphenolic content will always reflect the variety of biosynthesis' found within different species and external factors such as temperature, UV exposure, pathogens, etc. that will always vary within habitats, sites, and seasons, influencing both the polyphenolic production and the production of other metabolites—the latter particularly affecting the non-selective colorimetric TPC quantification. Results reported herein reveal that the TPC method can possibly be safely applied to sublittoral growing Laminariales species, which most likely possess a less diverse polyphenolic content and fewer interfering species. However, for the shallower-growing seaweed species, such as the eulittoral *F. vesiculosus* and *A. nodosum*, the TPC assay and the qNMR method show significant differences, most likely reflecting the shortcomings of the colorimetric assay. By applying a selective qNMR method for total polyphenolic quantification, the results will be less influenced by the diversity of the polyphenols in the sample and the presence of interfering compounds than when using the TPC assay. Hence, this approach will provide a polyphenolic quantification assumed to be closer to the "true" polyphenol concentration of brown seaweeds.

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DATA AVAILABILITY STATEMENT

The supplementary information is deposited in UoB Open Research Data (https://doi.org/10.18710/VZHSWT). The deposited data contain information on the TPC assay and qNMR results.

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PAPER III

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Characterization of high value products in the side-stream of *Laminaria hyperborea* alginate production - Targeting the phenolic content

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ABSTRACT

Sustainable production based on renewable biomass and efficient bioprocesses are important elements in the growing blue bioeconomy. The traditional *Laminaria hyperborea* alginate production disposes approximately 80 % of the raw material, ignoring large amounts of potential high-value products from the alga. Particularly, the leaf fraction of the seaweed is often disregarded. This study aimed to characterize high value products from the alginate production side-stream – focusing on the leaf biomass and particularly targeting the phenolic content. After extraction and solvent optimization, 60 % methanol was used for the extraction. The extract was further purified with PuriFlash and semi-preparative chromatography and increasing phenolic selectivity and purity was observed with TPC and qNMR, as well as antioxidant activity (ORAC). In the purified fractions, the LR LC-MS analyses displayed several masses, where 96 % (n = 1376) were of lower molecular weights (< 800 Da). Fifteen high value compounds were further identified using HR LC-MS (MS/MS) and/or NMR. This also included non-phenolics such as fucoxanthin, aliphatic acids and mannitol. Nonetheless, most compounds were identified as the targeted phenolics, consisting of lower molecular weight phenolic acids (salicylic acid, veratric acid, 5-caboxyvanillic acid, sinapic acid, 5-sulfosalicylic acid, vanillic acid 4-sulfate, and dihydrocaffeic acid 3-sulfate) and phlorotannins (trimer, tetramer, hexamer, and a sulfated dimer). None of the identified phenolics have previously been reported in *L. hyperborea*. In general, a high occurrence of sulfated phenolic compounds was observed and a sulfated diphlorethol/difucol was characterized for the first time. The isolation and characterization of high value components in the leaf biomass of L. *hyperborea* strongly supports the development of a total utilization of commercial alginate production. The characterization also adds information on the phenolic content of seaweeds at a molecular level, valuable to research on seaweed biosynthesis and development, chemical ecology, and ocean monitoring.

1. Introduction

Seaweeds are a natural source of a diverse chemical matrix with potential uses in food-, feed-, cosmetical-, and pharmaceutical industries [1,2]. This includes alginates, fucoidan, laminarin, cellulose, mannitol, polyphenols, and other pigments. Brown algae are one of the most promising marine biomasses as a renewable source contributing to the growing blue bioeconomy [3]. Human consumption of seaweed includes direct consumption as food, as food supplements, or as thickening agents such as alginate [4]. Additionally, natural antioxidants with multi-functional potential from seaweeds are of increasing interest. The brown alga *Laminaria hyperborea* is predominantly found in the subtidal zone of the Northern Atlantic Ocean. Due to its large content of structural and functional polysaccharides, specifically its unique alginate composition, *L. hyperborea* has long been exploited for alginate production. However, the traditional alginate production disposes approximately 80 % of the raw material either as liquid or solid waste streams, ignoring large amounts of potential high-value products from the alga [3,5]. Phenolics have been found to have several bioactivities including antioxidant, anti-cancer, antidiabetic, and antimicrobial activity, making them sought after by several industries [6–11]. Phenolics are a large and heterogenous group with structures ranging from simple monomer units to complex polymerized structures. They are synthesized in both marine- and terrestrial plants via either the acetate-malonate- or the

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shikimic pathway [12–16]. Terrestrial plant phenolics have been largely studied, whereas studies on seaweed phenolics are fewer. The occurrence of sulfated polyphenols seen in several marine plants and some algae species, is suggested to be an ecological adaptation to the marine environment [17–26]. However, the phenomenon is also observed for phenolics in some terrestrial species [27,28]. Sulfation increases the hydrophilicity and solubility of a molecule which increases the bioavailability of the compound [22,29,30]. It is also believed to influence the inactivation of toxic products and play a role in plant growth regulation [22,31–33]. The increased water solubility of sulfated polyphenols may also contribute to the colour of the seaweed biomass, as it can form stable complexes with other pigments [34]. However, the sulfate binding is susceptible to hydrolyzation, and this may create challenges with respect to degradation during extraction and isolation [27,31]. Characterization of polyphenols in seaweed is challenging, as they often occur at lower concentrations and are similar in structure. Particularly phlorotannins have been reported for several brown algae species. They are polymers of phloroglucinol and include several structural and conformational isomers [35]. Phlorotannins can be divided into groups based on the linkage of phloroglucinol units. Compounds with only ether linkages (C-O-C) are called fuhalols, compounds with only phenyl linkages (C-C) are called fucols, and compounds having both ether- and phenyl linkages are called fucophlorethols [36–40]. Due to the challenge of separation and isolation, phlorotannins are primarily characterized using LC-MS and classified according to their linkage type (C-C vs. C-O-C bond) and polymerization degree [18,39,41–44].

The extraction of phenolics, pigments and other high-value products from L. *hyperborea* as a side-stream of alginate production would provide access to several previously discarded bioactive compounds. Particularly the leaves are regarded as lower value compared to the stipe fraction, due to their alginate composition being lower in guluronic acid content [45]. By evaluating the by-products of the commercial alginate production and characterizing potential high-value products, the raw material utilization as well as the sustainability of the full seaweed biorefinery is increased [3,4,46]. This study aimed to evaluate the chemical composition of high-value products in *Laminaria hyperborea* to explore the possibility of producing high-value products from side streams of the alginate production and seaweed biorefinery.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade. The Folin-Ciocalteu (FC) reagent, gallic acid, phloroglucinol, sinapic acid (≥ 98 %), salicylic acid (≥ 99 %), fucoxanthin (≥ 95 %), methanol (≥ 99.9 %), ethanol (absolute), ethyl acetate (≥ 99.5 %), acetone (≥ 99.5 %), formic acid (≥ 98 %), $DMSO_2$ (TraceCERT®), and DMSO-d_6 (0.03 % TMS) were acquired from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Hexane (mixture of isomers) was acquired from Honeywell (Honeywell Inc., Charlotte, NC, USA). Deionized water was deionized at the University of Bergen (Bergen, Norway). Fluorescein sodium salt, AAPH, and Trolox used for the ORAC assay was acquired from Merck (Oslo, Norway). All reference standards were acquired from Merck.

2.2. Seaweed material

Laminaria hyperborea (Laminariaceae, Phaeophyceae) leaves (lamina) were acquired from Alginor ASA. The biomass was harvested in September 2020 along the coast of Haugesund, Norway (Rogaland field 55E; N 59°11′ E 005°06′). All samples were rinsed thoroughly with fresh water and air-dried. The dried plant material was stored at -20 °C when not used.

2.3. Sample preparation

Crude extracts were obtained using traditional maceration. 20 g dried material was dissolved in 500 mL 60 % methanol and left stirring for 24 h. Two parallels were extracted simultaneously, and all extractions of the same material were pooled and dried for analysis. When not used, samples were dried and stored at -20 °C.

2.4. Flash chromatography

The crude extract was fractioned with flash chromatography using an Interchim puriFlash® with a Biotage Sfär C18 D column. The solvents used were water (A), methanol (B), and ethyl acetate (C), with initial conditions of 95 % A and 5 % B. The gradient followed as; 0–6 min: 95 % A + 5 % B, 6–12 min: 75 % A + 25 % B, 12–18 min: 50 % A + 50 % B, 18–24 min: 25 % A + 75 % B, 24–36 min: 100 % B, 36–42 min: 50 % B + 50 % C, 42–48 min: 100 % C. Flowrate was 12 mL/min and 15 mL sample were added to the column. Detection was done at 280 nm and using a spectral scan between 200 and 800 nm. Eight fractions were obtained using flash chromatography.

2.5. Preparative HPLC

Further, the first eluting fraction from the flash chromatography was further purified using preparative HPLC. The preparative HPLC system consisted of a Dionex Ultimate 3000 pump (Thermo Fisher Scientific, Sunnyvale, CA, USA), the Dionex 3000 variable wavelength detector (Thermo Fisher Scientific, Sunnyvale, CA, USA), and a 250 × 22 mm (10 μm) Econosphere C18 column (Dr. Maisch, Ammerbuch, Germany). The solvents were (A) super distilled water (0.5 % TFA) and (B) acetonitrile (0.5 % TFA). Initial conditions were 95 % A and 5 % B. Gradient followed as; 0–10 min: 95 % A + 5 % B, 10–20 min: 85 % A + 15 % B, 20–34 min: 60 % A + 40 % B. 34–35 min: 95 % A + 5 % B. The flowrate was 18 mL/min and aliquots of 2 mL were injected. Twelve fractions were obtained using preparative HPLC.

2.6. Total polyphenolic content (TPC) assay

Folin-Ciocalteu TPC assay was performed using a method optimized for seaweeds, as described in Wekre et al. (2022) [47]. Briefly, the method used 0.2 mL sample, blank, or standard, 1.59 mL Folin-Ciocalteu-reagent, 4.0 mL 20 % (*w/v*) Na_2CO_3 and made to a total volume of 20 mL with water. The mixture was incubated for 2 h in the dark, and absorbance was measured at 760 nm using a Biochrom Libra S32 UV instrument (Biochrom, Cambridge, United Kingdom). Gallic acid and phloroglucinol calibration curves were used to validate the linearity, sensitivity, precision, and accuracy of the TPC method (Table 1). Three parallels ($n = 3$) of each sample or standard were analyzed to ensure statistically significant results.

2.7. NMR analyses

Dried samples were dissolved in 0.6 mL DMSO-d_6 (0.03 % TMS) containing the internal standard $DMSO_2$ (C = 10 mM). Quantification using ^{1}H NMR analyses were performed employing a Bruker 600 MHz AVANCE NEO instrument with a QCI cryoprobe (Bruker BioSpin, Zürich, Switzerland). All spectra were recorded at 298 K. For accurate

Table 1
Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for gallic acid at 760 nm using the optimal TPC conditions.

Standard	Calibration curve	r^2	Range [ug/mL]	LOD [ug/mL]	LOQ [ug/mL]
Gallic acid	y = 0.00115× − 0.00101	0.999	1000–30	18.610	56.391

quantification, the T_1 value of each sample was measured to ensure complete relaxation between scans. The T_1 measurements were performed by applying the *t1ir* pulse sequence with a sweep width of 19.8 ppm, 16 k data points, 8 scans, 4 dummy scans, and 9 different inversion recovery delays between 1 ms and 5 s. To ensure complete relaxation, the d1-value was set to 5 x T_1 for all ^{1}H spectra obtained for qNMR analysis [48,49].

The one-dimensional (1D) ^{1}H NMR spectra used for quantifications were recorded using the *zg30* pulse sequence with a sweep width of 19.8 ppm, 64 k data points, 128 scans, aq = 2.75 s, fidres = 0.36 Hz, and the relaxation delay (d1) was 5 x T_1 for the selected sample. The spectra were processed using a line broadening of 0.3 Hz.

Selective qNMR was performed as described in previous publications [47]. All quantifications were performed based on Eq. (1) with $DMSO_2$ (10 mM, No. H = 6) as the internal standard.

$$C_{sample}\ [M] = \frac{I_{sample} \times n_{DMSO2} \times C_{DMSO2}}{I_{DMSO2} \times n_{sample}} \quad (1)$$

where C = molar concentration [M], I = signal integral, and n = number of protons yielding the signal.

2.8. LC-electrospray mass spectrometry (ESI-/TOF)

Low-resolution liquid chromatography mass spectrometry (LC-ESI MS(Q); LR LC-MS) was performed using an Agilent Technologies 1260 Infinity Series system and an Agilent Technologies 6420A triple quadrupole mass spectrometry detector. The following conditions were applied: ionization mode: negative, capillary voltage = 3000 *V*, gas temperature = 300 °C, gas flow rate = 3.0 L/min, acquisition range = 100–2000 *m/z*. The elution profile for HPLC consisted of the following gradient: 0–4 min: 1 % B, 4–5 min: 5–10 % B, 5–7 min: 60 % B, 7–9 min: 70 % B, 9–11 min: 75 % B, 11–13 min: 75–1 % B, 13–15 min: 1 % B, at a flowrate = 0.3 mL/min, where solvent A was super distilled water (0.5 % formic acid), and solvent B was acetonitrile (0.5 % formic acid). A 50 × 2.1 mm internal diameter, 1.8 μm Agilent Zorbax SB-C18 column was used for separation. UV–Vis spectral data for all peaks were accumulated in the range of 200–800 nm and the chromatographic profiles were recorded at 280 nm, 330 nm, and 360 nm.

High-resolution mass spectrometry (LC-ESI MS(QqTOF); HR LC-MS) was performed using the same gradient and solvent system as for LR-MS, using a similar column (Agilent Zorbax Eclipse HD, 2.1 × 50 mm, 1.8 μm) and flowrate = 0.250 mL/min, capillary voltage = 4000 *V*, gas temperature = 280 °C, gas flow rate = 8.0 L/min. MS/MS experiments (LC-ESI MS(QqQ)) were acquired for precursor ions using helium as collision gas with a collision energy of 15–35 eV.

2.9. High performance liquid chromatography (HPLC-DAD)

HPLC-DAD analyses were performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 1260 diode array detector (DAD) and a 200 × 4.6 mm, 5 μm ODS Hypersil column (Agilent Technologies, Santa Clara, CA, USA). HPLC analysis was performed using two solvents, (A) super distilled water (0.5 % TFA) and (B) acetonitrile (0.5 % TFA), in a gradient (0–10 min: 95 % A + 5 % B, 10–20 min: 85 % A + 15 % B, 20–34 min: 60 % A + 40 % B. 34–35 min: 95 % A + 5 % B). The flow rate was 1.0 mL/min, and aliquots of 20 μL were injected with an Agilent 1260 vial sampler. UV–Vis absorption spectra were recorded during the HPLC analysis over the wavelength range of 200–600 nm in steps of 2 nm.

2.10. Free radical scavenging by oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed in black 96-well microtiter plates. Fluorescein sodium salt AAPH (2,2′-azobis(2-methylpropionamide) dihydrochloride) and Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were dissolved in 60 mM phosphate buffer (PB). Trolox was used as a calibration curve (Table 2). Standards and samples were pipetted into a microtiter plate in duplicate with fluorescein and incubated at 37 °C for 15 min. Ice cold AAPH was added, and the plate was immediately moved to the Tecan Spark multimode reader. Fluorescence (Ex 485 nm ± 20 and Ex 525 nm ± 20) was recorded 25 times in intervals of 90 s. The area under the curve (AUC) was calculated and the AUC for 0 μM Trolox was subtracted to find the area between curves (ABC). ABC for samples were compared to the Trolox standard curve to find Trolox equivalents (TE). Results are reported as μmol TE/mg DW. All ORAC tests were performed at MARBIO (Tromsø, Norway).

2.11. Vibrational spectroscopy - infrared and Raman

Selected purified L. *hyperborea* samples (PuriFlash and preparative HPLC) were scanned with FTIR and Raman micro spectroscopy. Infrared spectroscopy was performed using a Bruker Alpha II, equipped with a Platinum ATR module, a Rocksolid™ interferometer and a deuterated triglycine sulphate (DTGS) detector. Samples were analyzed both as solids and in liquid form. Liquid samples were dissolved in water or methanol and scanned from 400 to 4000 cm^{-1} (integration time of 360 s). The OPUS software was used to process the data.

Raman micro spectroscopy was performed at room temperature using a Bruker Senterra II Spectrometer equipped with a 785 nm laser and scanned from 100 to 4000 cm^{-1} (integration time 30 s, 3 accumulations). A sample size of <0.5 mg gave sufficient resolution due to the microscope array. No further sample preparation was necessary.

3. Results and discussion

3.1. Extraction and quantification of polyphenols

Several methods were tested on dried L. *hyperborea* biomass for extraction of potential high-value side stream products of alginate production, primary targeting polyphenols. The extraction methods included accelerated solvent extraction (ASE), ultrasound assisted extraction (UAE), and maceration. Different ratios of aqueous methanol and aqueous ethanol were tested as extraction solvents [50–53]. The ASE method yielded slightly higher phenolic contents; however, the method has limitations with respect to low sample size, application, and clogging of extraction cells due to the polysaccharides in the biomass. The UAE and maceration extractions showed similar phenolic extraction yields – making the traditional maceration preferable. Maximum and similar extraction yields were observed when using 60 % aqueous methanol or ethanol.

Additional purification of the maceration crude extract was performed to improve characterization accuracy of the phenolics [35]. Flash chromatography and preparative HPLC were used for pre-fractionation and purification, the target being high polyphenol recovery (Sections 2.3-2.5). The effect of increased purification was observed with increased quantitative phenolic content (TPC and qNMR), as well as with increased antioxidant values (ORAC) (Fig. 1).

3.1.1. Selective extraction of phlorotannins

The use of liquid-liquid extraction for increased selectivity is particularly seen for phlorotannins, often succeeding maceration, either

Table 2
Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for Trolox used in ORAC assay.

Standard	Calibration curve	r^2	Range [μM]	LOD [μM]	LOQ [μM]
Trolox	y = 10,454,393.2× + 12,880,433	0.952	12.5–0.78	3.899	11.82

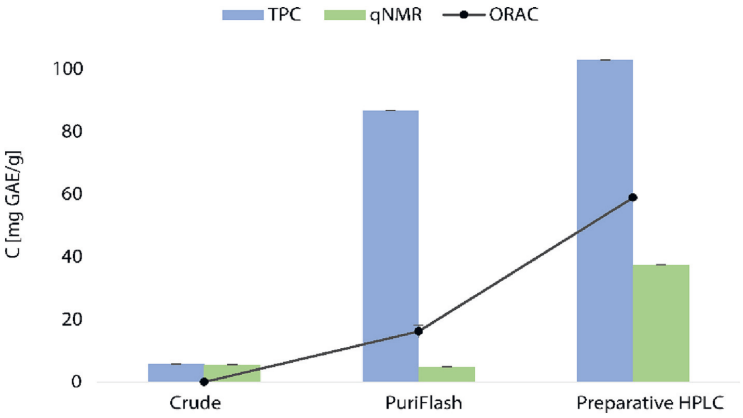

Fig. 1. Total phenolic content (TPC and qNMR reported as gallic acid equivalents (GAE)) in crude methanolic L. *hyperborea* extract, and in selected purified samples (PuriFlash and preparative HPLC) targeting the phenolics. A trendline displays the antioxidant activity for the different samples measured with ORAC reported as Trolox equivalents (TE) [μmol TE/mg] (crude; 0 μmol TE/mg, PuriFlash; 16.20 ± 2.04 μmol TE/mg, preparative HPLC; 58.85 ± 0.30 μmol TE/mg). All values are reported relative to the total mass of the individual sample.

with ethyl acetate, hexane, or both [17,48]. An alternative extraction method was therefore tested, using 70 % aqueous acetone followed by liquid-liquid purification using hexane prior to ethyl acetate (EtOAc) [17,39,54–58]. The resulting extracts were analyzed using TPC, qNMR, and LC-MS.

The LR LC-MS analyses revealed that the 70 % aqueous acetone extract (TPC: 6.51 ± 0.02 mg GAE/g, qNMR: 3.40 ± 0.00 mg GAE/g) and its liquid-liquid purification phases (hexane, EtOAc, H_2O) did not contain any additional phenolic or phlorotannin masses compared to the 60 % aqueous methanol extract (TPC: 6.55 ± 0.08 mg GAE/g, qNMR: 4.51 ± 0.00 mg GAE/g) and its corresponding fractions, based on mass hits in the bibliographic database. Thus, no increased selectivity of phenolics or phlorotannins was observed with this algae biomass using a liquid-liquid extraction operation. Moreover, both crude sample extracts exhibited similar mass distribution trends in the LC-MS analyses, with most of their detected compounds falling within the 200–400 Da range. Therefore, based on the comparable polyphenolic yields obtained by both extraction methods, the methanolic extract and its purified fractions were selected for further analysis.

3.2. Characterization of the alga extract and purified samples

The methanolic crude extract and its purified fractions were analyzed using both low - and high resolution (LR/HR) LC-MS, TPC, ORAC and NMR. The identified polyphenols (Table 3) were detected in the purified fractions from the preparative HPLC, which originated from an early-eluting, polar fraction from PuriFlash (Sections 2.4 and 2.5). The selection of fractions with high polyphenolic content was based on HPLC-DAD analyses. Due to their relatively high concentration in the alga, fucoxanthin and mannitol could be identified by NMR analysis (Section 3.2.5).

3.2.1. Observed mass distribution

The purified samples of L. *hyperborea* were first analyzed with LR LC-MS and the observed masses were compared with a prepared bibliographic database containing about 600 compounds including polyphenols, pigments and aliphatic acids. MS spectra indicated about 1400 different masses in the acquisition area (m/z 100–2000). Around 45 % of these masses were found between 200 and 400 Da (Fig. 2) and of these, 15 % gave a result matching a compound in the bibliographic database. The majority of the tentatively identified compounds (database matches) were also found in the 200–400 Da range (68 %). A limited number of masses were detected above 800 Da (4 %), and of these 1.5 % gave a match with compounds in the database. Of the masses matching the bibliographic database, 6 % (n = 15) were further characterized using LR LC-MS/MS, and HR LC-MS (Tables 5 and 6). Additionally, four non-phenolic compounds were identified using HR LC-MS and NMR (Section 3.2.5).

Table 3
Overview of all identified compounds in side stream methanolic extract and its corresponding fractions.

Compound	Molecular formula	Molecular weight [g/mol]
Hydroxybenzoic acids (HBA)		
Salicylic acid	$C_7H_6O_3$	138.1
Veratric acid	$C_9H_{10}O_4$	182.2
5-Carboxyvanillic acid	$C_9H_8O_6$	212.1
5-Sulfosalicylic acid	$C_7H_6O_6S$	218.2
Vanillic acid 4-sulfate	$C_8H_8O_7S$	247.2
Hydroxycinnamic acids (HCA)		
Sinapic acid	$C_{11}H_{12}O_5$	224.2
Dihydrocaffeic acid 3-sulfate	$C_9H_{10}O_7S$	262.2
Phlorotannins		
Trimer [39,42,59]	$C_{18}H_{14}O_9$	374
Tetramer [39,41,42]	$C_{24}H_{18}O_{12}$	498
Hexamer [41]	$C_{36}H_{26}O_{18}$	746
Sulfated dimer	$C_{12}H_{10}O_9S$	330
Aliphatic acids		
Citric acid	$C_6H_8O_7$	192.1
Ascorbic acid	$C_6H_8O_6$	176.1
Carotenoids		
Fucoxanthin	$C_{42}H_{58}O_6$	658.9
Carbohydrates		
Mannitol	$C_6H_{14}O_6$	182.2

3.2.2. Sulfated phenolics

Several compounds detected in the MS spectra of the purified L. *hyperborea* fractions showed masses just below whole numbers (example: 217.9885), normally seen for sulfur containing compounds (Sulfur: MW = 31.9721 g/mol) [60]. Sulfated conjugates of both phenolic acids and phlorotannins were found in the fractions, either through fragmentation patterns or exact masses (Tables 5 and 6).

Selected purified fractions of L. *hyperborea* were also analyzed with Raman and IR spectroscopy to explore the occurrence of sulfation. The Raman spectrum shows a very strong and characteristic band at 1049 cm^{-1} assigned to symmetric sulphate group stretching, further

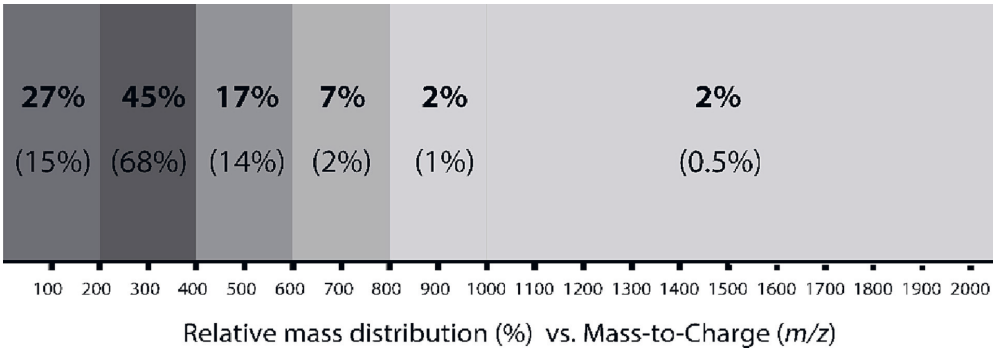

Fig. 2. Relative mass distribution (bold) for the observed masses ($n = 1376$) in the TIC scans of the LR LC-MS analyzed L. *hyperborea* extracts. The mass distribution of masses matching the bibliographic database ($n = 211$) are shown in brackets.

indicating the presence of sulfated polyphenols in L. *hyperborea* (Fig. 3, Table 4) [61–63]. The Raman band at 1456 cm^{-1} strongly suggests the presence of methylation in the compounds [63,64]. IR bands at 1671 cm^{-1} and 1728 cm^{-1} are linked to C═O stretching modes, likely stemming from carboxylic acid groups in phenolic acids.

3.2.3. Phenolic acids

Seven phenolic acids, including three sulfated phenolic acids, were identified in L. *hyperborea*, making up 44 % of the identified compounds. Identification was performed using HR LC-MS and LR LC-MS/MS (Table 5). Phenolic acids exhibit several biological activities, including anti-inflammatory, antioxidant, anti-tumor, and anti-diabetic activities, making them high-value products that can be extracted from *Laminaria hyperborea* [16,69–72].

Salicylic acid (syn.: *p*-hydroxybenzoic acid) was identified in the purified fractions of *L. hyperborea*, with a mass deviation of −0.52 ppm in HR LC-MS (Δppm ≤5). Additionally, its fragmentation pattern showed a peak at *m/z* 93, corresponding to a loss of 44 Da (-CO_2), a common observed fragment loss for acids [73]. By using HPLC and reference standards, Chakraborty et al. (2017) identified phenolic acids

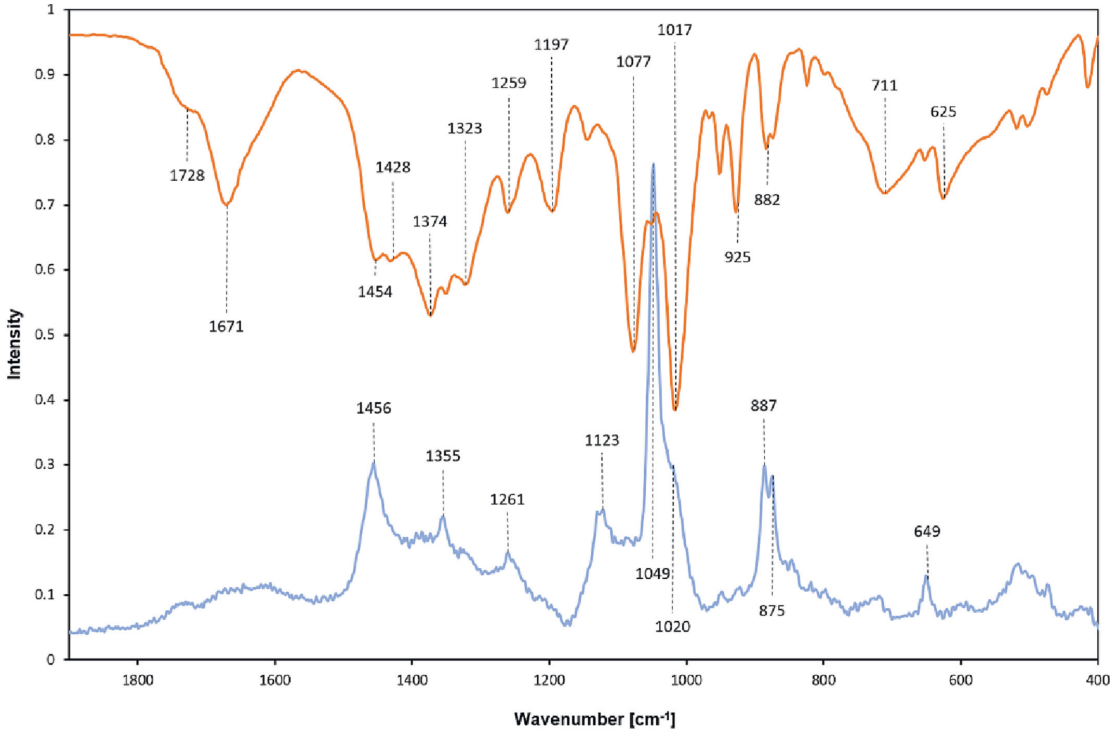

Fig. 3. Overlaid Raman (blue)- and IR- spectra (orange) of a purified fraction of the L. *hyperborea* extract showing indications of sulfated polyphenols. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Vibrational bands assigned in the purified fractions of L. *hyperborea* extracts; δ and ν refers to bending and stretching vibrations, s to symmetrical vibration modes.

Wavenumber [cm^{-1}]	Activity	Vibrational mode	Reference
822	Infrared	ν(C—H)	Grasel et al. (2016) [65]
1017	Infrared	ν(C—O)	Grasel et al. (2016) [65]
1049	Raman	ν_s(O=S=O)	Buzgar et al. (2009), Pereira et al. (2009), Dörschmann et al. (2023) [61–63]
1077	Infrared	ν(C—O)	Grasel et al. (2016) [65]
1259	Infrared	ν(S=O), ν(C—O)	Mathlouthi & Koenig (1987), Glombitza & Knöss (1992) [66,67]
1454	Infrared	ν(C-C)$_{aromatic}$	Nogales-Bueno et al. (2017) [64]
1456	Raman	$\delta_s(CH_3)$	Dörschmann et al. (2023), Nogales-Bueno et al. (2017), da Silva et al. (2008) [63,64,68]
1671	Infrared	ν(C=C), ν(C=O)	Grasel et al. (2016) [65]
1728	Infrared	ν(C=O)	da Silva et al. (2008) [68]
2845	Raman	$\nu_s(CH_2)_{shoulder}$	da Silva et al. (2008) [68]
2939	Raman	ν(C—H)	da Silva et al. (2008) [68]

Table 5
Phenolic acids in *Laminaria hyperborea* identified using HR LC-MS and LR LC-MS/MS.

Compound	Molecular ion [M-H]-	Mass deviation [ppm]	MS/MS ions[a]
Salicylic acid	137.0245	−0.52	**93**
Veratric acid	181.0510	1.93	–
5-Carboxyvanillic acid	211.0252	1.70	151, 107, 83, **65**, 63
5-Sulfosalicylic acid	216.9815	0.72	137, **93**
Vanillic acid 4-sulfate	246.9923	2.33	121, **108**, 93, 80
Sinapic acid	223.0616	1.58	208, 193, **149**, 93
Dihydrocaffeic acid 3-sulfate	261.0082	−1.53	**181**, 166, 122, 81

[a] MS/MS ion marked in bold represent the base peak.

in the brown algae *Anthophycus longifolius*, *Sargassum plagiophyllum*, and *Sargassum polycystum* (formerly *Sargassum myriocystum*), in which salicylic acid was found in the latter [58]. Agregán et al. (2017) identified salicylic acid as a "hydroxybenzoic acid derivative" in the common brown algae *Ascophyllum nodosum,* using LR LC-MS/MS fragmentation patterns. To the author's knowledge this is the first-time salicylic acid has been identified in L. *hyperborea*. Salicylic acid has broad applications and is extensively used in industry, in the production of aspirin, as food preservatives, and as antiseptics [74–79]. Sinapic acid, a hydroxycinnamic acid, was identified with a small mass deviation of 1.58 ppm and a characteristic fragmentation pattern [80]. A fragment at *m/z* 208 represents a loss of one CH_3 group ([M-15-H]$^-$), and a fragment at *m/z* 193 represents a double CH_3 loss (− 2 x CH_3). The base peak at *m/z* 149 (M-74), is most likely the loss of the two methyl groups in addition to the CO_2 loss from the acid group (2 x CH_3 + CO_2). Sinapic acid has previously been identified using LC-MS/MS in the analysis of the green algae Ulva sp., Caulerpa sp., and Grateloupia sp. [81] In the red alga *Gracilaria dura,* Sumayya et al. (2020) also reports the presence of sinapic acid - based on HPLC analyses [82]. To the authors knowledge, sinapic acid has not been identified in L. *hyperborea* prior to this study. 5-Carboxyvanillic acid was identified showing an expected loss of 60 Da ([M-60-H]$^-$), representing the acid group and a methyl group (COOH + CH_3) followed by the typical loss of 44 Da (CO_2). No fragmentation pattern was observed for veratric acid; however, the high-resolution mass deviation was found to be below 5 ppm. Dihydrocaffeic acid 3-sulfate and 5-sulfosalicylic acid both showed the typical loss of a SO_3 group ([M-80-H]$^-$). Vanillic acid 4-sulfate did not show a fragment representing the loss of a SO_3 group; however, a fragment was observed at *m/z* 80 which supports the presence of a sulfate group.

3.2.4. Phlorotannins

Four phlorotannins were identified in L. *hyperborea*, making up 27 % of the identified compounds. All four phlorotannins had different polymerization degrees and one was sulfated. (Table 6). Further three phlorotannins could be identified by their linkages (phlorotannin type) due to characteristic fragmentation patterns.

A quasi-molecular ion peak at *m/z* 373.1196 ([M – H]$^-$) was observed, indicating a trimer phlorotannin. Fragmentation indicated that the trimer may only be connected through ether linkages (C-O-C) due to the observation of a fragment at *m/z* 265 ([M – 108 – H]$^-$), representing a dimer phlorotannin with ether linkages. Alternatively, one of the two ether linkage oxygens might be an extra hydroxyl group, and the bond broken could be a phenyl linkage (C-C). Still, this is rarely observed for smaller phlorotannins, and thus less likely. Lastly, a fragment representing a simple phloroglucinol unit (*m/z* 126) was observed, further indicating that the compound is a phlorotannin. Based to the overall observed fragmentation pattern, the compound was concluded to be a fuhalol type phlorotannin. A tetramer phlorotannin was identified with a quasi-molecular ion peak at *m/z* 497.1867 ([M – H]$^-$). The tetramer mass has also been reported in *Laminaria digitata* and *Ascophyllum nodosum*, but different fragmentation patterns are reported in these analyses [39,41]. An additionally different fragmentation pattern was observed for the same mass in our study of L. *hyperborea,* highlighting the difficulty of identifying phlorotannins. A loss of 44 Da is described as a typical loss for cross-ring cleavage of phlorotannins [17,35]. For the tetramer phlorotannin, two such losses were observed. First between the fragments *m/z* 298 and *m/z* 254 (−44 Da), and between fragments a *m/z* 241 and *m/z* 197 (−44 Da). The fragment at *m/z* 371 represents a loss of a phloroglucinol unit ([M-126-H]$^-$), and the resulting phloroglucinol fragment was also observed at *m/z* 126. A similar fragment at *m/z* 371 was reported for tetramers in both *Laminaria digitata* and *Ascophyllum nodosum* by Vissers et al. (2017) and Sardari et al. (2021), respectively. Different fragmentation patterns will be seen for phlorotannin isomers; however, the HPLC-MS methodology can also influence the fragmentation patterns observed. Therefore, MS/MS experiments were performed at two fragmentation energies (25 eV and 35 eV), and the fragmentation at 35 eV gave some additional unidentified lower-mass ions (*m/z* 130 and *m/z* 83). Based on the fragmentation data obtained, the compound was identified as a phlorotannin tetramer, without further phlorotannin subclass characteristics. A hexamer phlorotannin mass was observed at *m/z* 745.9562 ([M – H]$^-$). This hexamer mass has been reported to be present in four brown algae; *Laminaria digitata*, *Sargassum vulgare*, *Sargassum fusiforme* and *Ascophyllum nodosum* [17,39,41,44]. However, similar to the tetramer phlorotannin, various fragmentation patterns are suggested, as expected for "higher order" phlorotannins [17,39,41,42,44]. From the MS/MS spectrum at 25 eV, a fragment at *m/z* 249 was observed which is typically reported as a dimer (ether linkage), easily lost from a larger

Table 6
Fragmentation pattern observed for phlorotannins in *Laminaria hyperborea* using HPLC-HRMS/MS (fragmentation energy = 25 eV).

Compound	Phlorotannin type	[M – H]$^-$	MS/MS ions[a]
Trimer	Fuhalol	373.1196	265, 229, **126**
Tetramer	Unknown	497.1867	371, 298, 254, 241, 197, **155**, 126
Hexamer	Fucophlorethol	745.9562	**681**, 461, 331, 281, 249, 229
Sulfated dimer	Fuhalol/fucol	328.9978	**249**

[a] MS/MS ion marked in bold represent the base peak.

hexamer structure. Another fragment observed at 25 eV was *m/z* 229, which could represent a different dimer with fucol linkage. Additionally, when using 35 eV as the fragmentation energy, a fragment at *m/z* 461 was observed, also reported by Lopes et al. (2018) for a hexamer structure [35]. Thus, it was concluded that the mass represents a hexamer phlorotannin with both ether- and phenyl linkages; a fucophlorethol.

One early eluting (t_R = 1.46 min) sulfated phlorotannin was identified with a quasi-molecular ion peak at *m/z* 328.9978 $[M - H]^-$. The compound showed indications of being a sulfated phlorotannin due to the mass being just below a whole number (Section 3.2.2). The fragmentation spectrum showed a fragment at *m/z* 249, yielding a loss of −80 Da ($[M\text{-}80\text{-}H]^-$), typical for a SO_3 group. A fragment at *m/z* 249 is often seen for tetramer phlorotannins, representing a dimer with two phloroglucinol units [17,36,41,83]. Therefore, the compound was concluded to be a sulfated dimer phlorotannin, possibly sulfated difucol (C-C) or sulfated diphlorethol (C-O-C) (Fig. 4). The two possible compounds have the same exact mass and therefore also yield the same mass deviation of 3.31 ppm from the observed mass. Accounting for previous results from ^{13}C NMR for L. *hyperborea*, which indicated the presence of more ether linkages than phenyl linkages, it seems most likely that the compound is sulfated diphlorethol [47]. A similar compound was reported by Glombitza and Knöss (1992) in the brown alga *Pleurophycus gardneri* using TLC, IR, and NMR [67]. Additionally, Chouh et al. (2022) report sulfated phlorotannins in *Sargassum vulgare* [17]. To the authors knowledge, sulfated phlorotannins have not previously been reported for *Laminaria hyperborea.*

3.2.5. Fucoxanthin, mannitol, and aliphatic acids

Carotenoids are often extracted alongside phenolics in aqueous alcohols, and fucoxanthin was found in the crude extract of L. *hyperborea* [84]. The pigment was further quantified (qNMR: 16.5 mg/g; ORAC: 40.6 ± 0.9 μmol TE/mg) and identified with ^1H NMR data and a reference standard (Fig. 5) in a late-eluting and less polar PuriFlash fraction. Fucoxanthin is a well-known pigment found to be present in L. *hyperborea* [46,85,86].

^1H NMR spectra of the early-eluting and highly polar fractions from the PuriFlash, revealed the presence of characteristic mannitol chemical shift values alongside the phenolic content. Mannitol is known to be present in brown algae, making up part of the carbohydrate content of L. *hyperborea,* along with alginate [87,88]. The semi-preparative HPLC purification was able to separate between the phenolic content and mannitol.

Two other non-phenolic compounds were identified, both aliphatic acids. Citric acid (MW = 192.12 g/mol) and ascorbic acid (MW = 176.12 g/mol) were observed in HR LC-MS spectra and had mass defects of 2.27 ppm and 1.93 ppm, respectively. Aliphatic acids have been widely reported in brown algae [17,18,46,89].

4. Conclusion

Aqueous alcoholic (60 % methanol/ethanol) maceration of L. *hyperborea* leaves gave similar phenolic extraction yields as UAE and ASE facilitated extractions (TPC: 5.72 ± 0.07 mg GAE/g; qNMR: 5.51 ± 0.013 mg GAE/g). No antioxidant activity was observed for the crude extract. However, for polar preparative-HPLC purified fractions, a high phenolic recovery was obtained (TPC: 102.34 ± 0.09 mg GAE/g; qNMR: 37.34 ± 0.017 mg GAE/g), and the increased purity was confirmed with higher antioxidant activity (ORAC: 58.85 ± 0.30 μmol TE/mg). Alternative methods were applied to increase the phenolic recovery, including phlorotannin selectivity. This included manipulation of extraction solvent polarity and purification with liquid-liquid partitioning with solvents of decreasing polarity. However, no high molecular weight phlorotannins were observed. This may be due to the natural polyphenolic composition of L. *hyperborea* leaves, primarily consisting of soluble low molecular weight compounds (< 800 Da). Several hydroxybenzoic- and hydroxycinnamic acids were identified, and the phenolic acids make up the majority of the phenolic contents. A general high occurrence of sulfated phenolic compounds was observed in LC-MS and a dimeric sulfated difucol/diphloroethol was characterized for the first time. To the author's knowledge, this is the first study reporting characterization of individual polyphenols in *Laminaria hyperborea.*

The low heterogeneity and seemingly higher solubility of the polyphenolic compounds found in the leaf fraction of L. *hyperborea* allows for

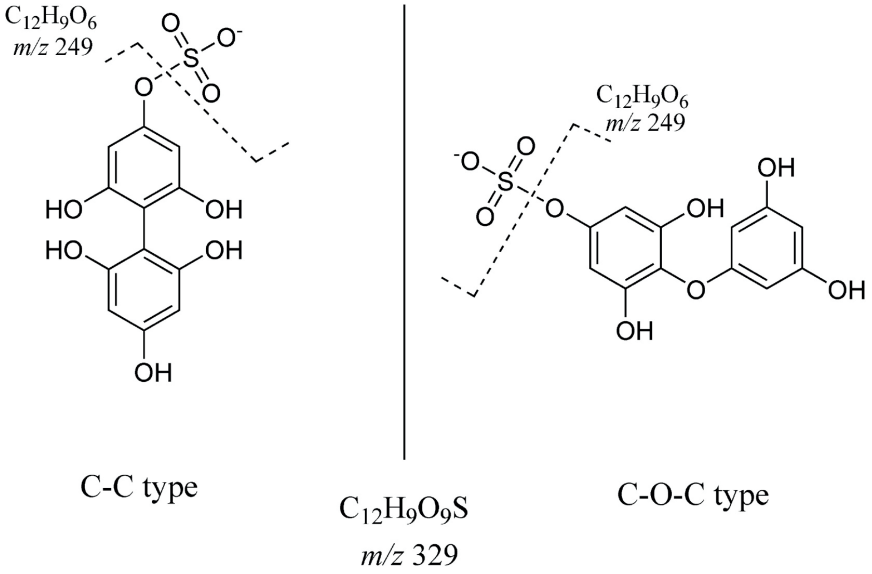

Fig. 4. Isomeric structures of sulfated difucol/diphlorethol at $[M - H]^-$ = 328.9978 (330 g/mol) with fragmentation (*m/z* 249) and neutral loss of the sulfate group ($O_3S^\bullet$, 80 Da). The position of the sulfate group substitution is unknown and arbitrary placed in the figure.

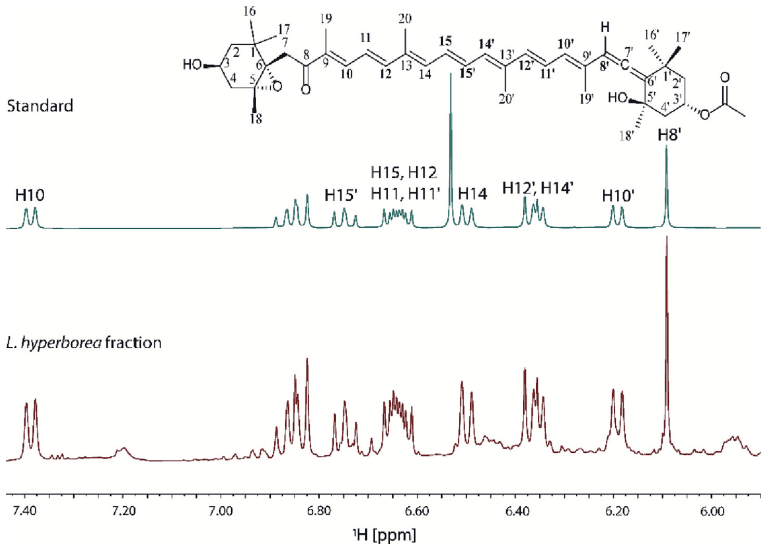

Fig. 5. Stacked ^{1}H NMR spectra of a fucoxanthin reference standard (blue) and isolated fucoxanthin in a late-eluting, less polar PuriFlash L. *hyperborea* fraction (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extraction of high value products from both solid and liquid side streams of commercial alginate production. Additionally, other high value compounds and fucoxanthin and mannitol were successfully separated from the phenolic fractions. The results will contribute to increasing the value of the leaf fraction and further improving the sustainability of alginate production and seaweed biorefinery.

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CRediT authorship contribution statement

Marie Emilie Wekre: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **Bjarte Holmelid:** Investigation, Resources, Methodology. **Jarl Underhaug:** Methodology, Supervision, Resources. **Bjørn Pedersen:** Investigation. **Georg Kopplin:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Monica Jordheim:** Conceptualization, Methodology, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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