RESEARCH ARTICLE

Phytochemical Analysis WILEY

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

Marie Emilie Wekre[1,2] | Sondre Hellesen Brunvoll[1] | Monica Jordheim[1]

[1]Department of Chemistry, University of Bergen, Bergen, Norway

[2]Alginor ASA, Haugesund, Norway

Correspondence
Monica Jordheim, Department of Chemistry, University of Bergen, Allégt. 41, N-5007 Bergen, Norway.
Email: monica.jordheim@uib.no.

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, $A = \varepsilon c l$). 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)
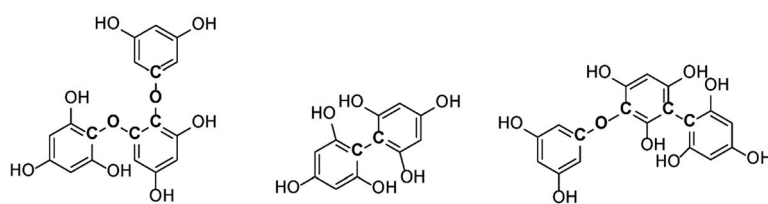
Example:	Triphlorethol A	Difucol	Fucophlorethol
Linkage:	**C-O-C**	**C-C**	**C-C / C-O-C**
	Ether	Phenyl	Ether and phenyl
Type:	Phlorethol	Fucol	Fucophlorethol

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.

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

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

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

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

Species	Intensity ratio measured		
	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.

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]

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. [Colour figure can be viewed at wileyonlinelibrary.com]
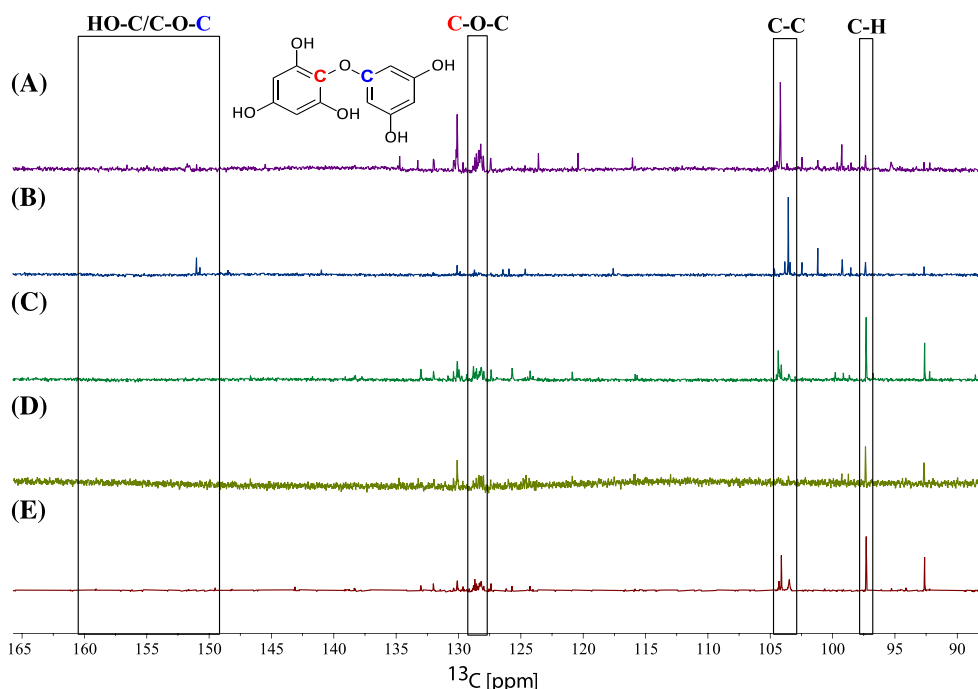
a possibility. Although Ford et al. (2020) did not report a fucol-to-phlorethol ratio, calculations based on the data provided in their study yield the fucol-to-phlorethol ratio is 1:7 for *A. nodosum* and 1:2 for *F. serratus*. The linkage ratios are, to some extent, sample specific because the intensities 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 Fucaceace 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. [Colour figure can be viewed at wileyonlinelibrary.com]
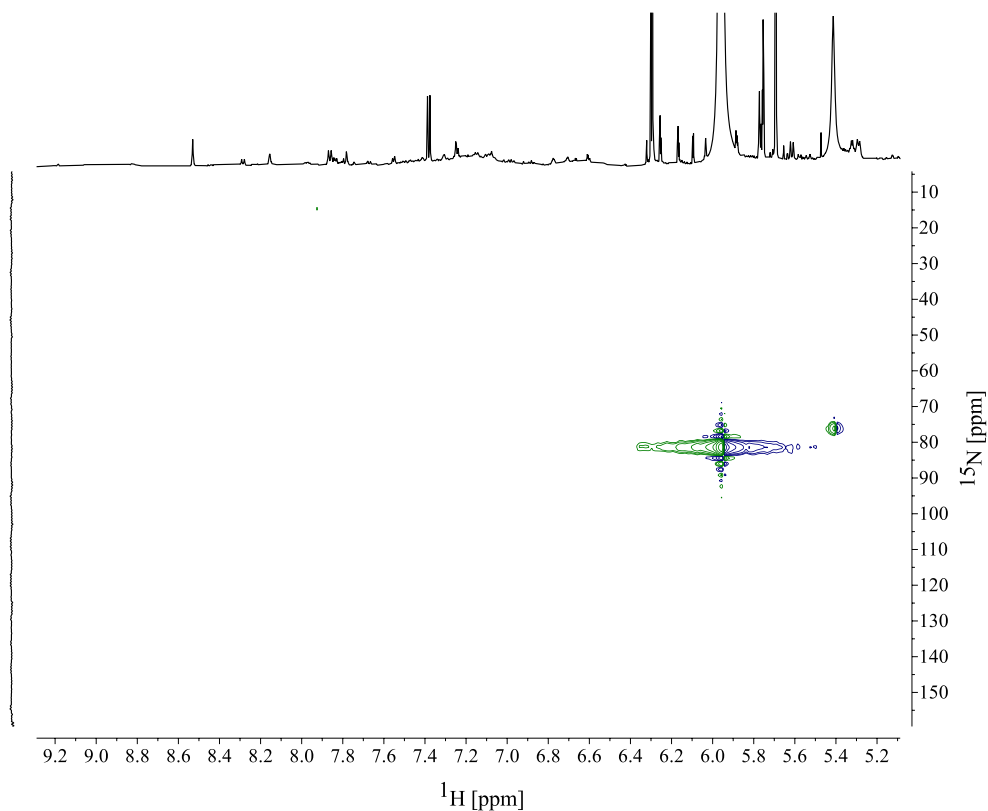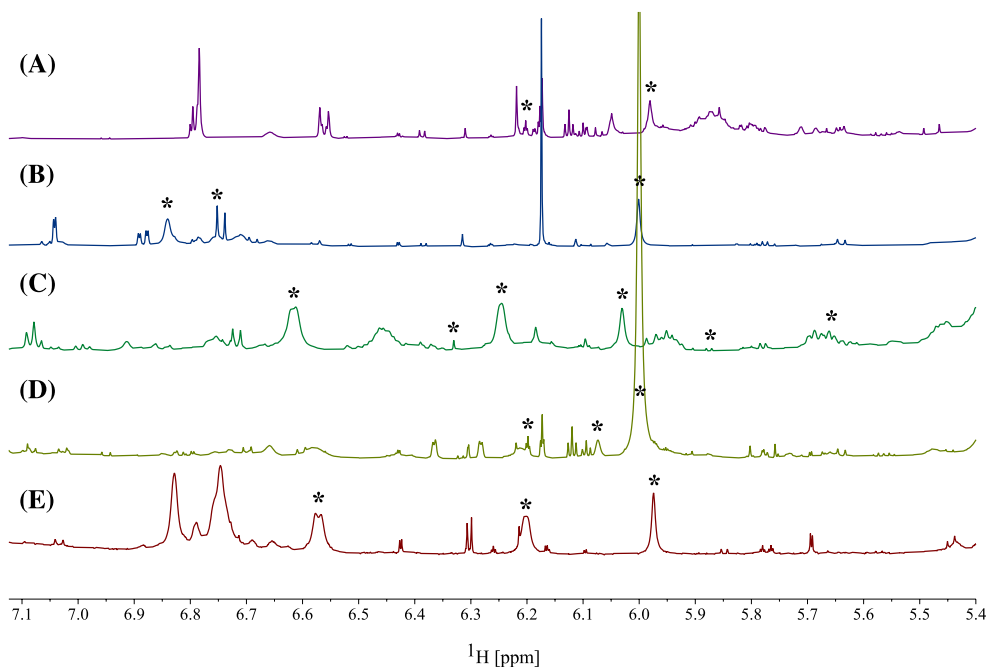

FIGURE 4 ^{1}H NMR spectra displaying the polyphenolic region (7.0–5.5 ppm) used for quantification of *Fucus vesiculosus* (A), *Ascophyllum nodosum* (B), *Saccharina latissima* (C), *Laminaria digitata* (D), and *Laminaria hyperborea* M20 (E). Signals labelled with asterisk (*) were deselected based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

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]
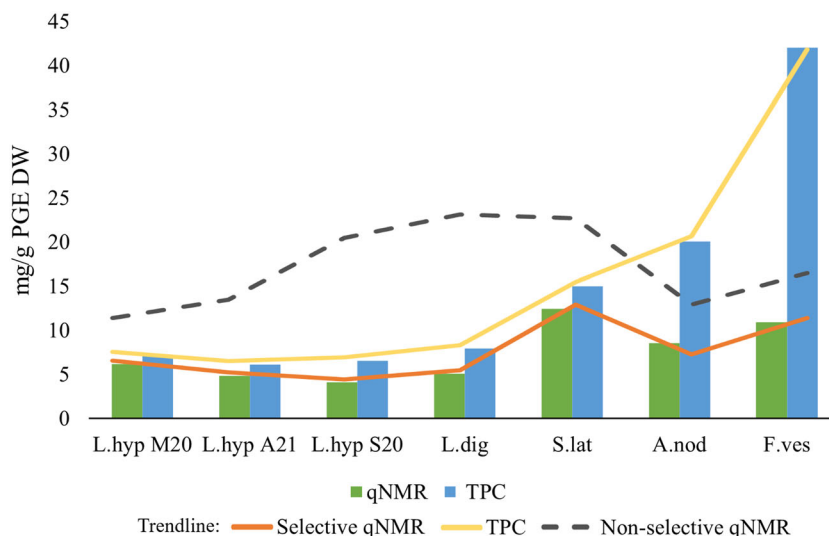
3.3 | Comparing the selective qNMR method with the TPC assay

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).

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.

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.

ORCID

Marie Emilie Wekre https://orcid.org/0000-0001-7242-0723
Monica Jordheim https://orcid.org/0000-0002-6804-921X

REFERENCES

1. Langton R, Augyte S, Price N, et al. An Ecosystem Approach to the Culture of Seaweed. 24 (2019).
2. Collins JE, Vanagt T, Huys I, Vieira H. Marine Bioresource Development – Stakeholder's Challenges, Implementable Actions, and Business Models. *Front Mar Sci.* 2020;7:62. doi:10.3389/fmars.2020. 00062

3. Stévant P, Rebours C, Chapman A. Seaweed aquaculture in Norway: recent industrial developments and future perspectives. *Aquac Int.* 2017;25:1373-1390.

4. Holdt SL, Kraan S. Bioactive compounds in seaweed: functional food applications and legislation. *J Appl Phycol.* 2011;23:543-597.

5. Domínguez H. Algae as a source of biologically active ingredients for the formulation of functional foods and nutraceuticals. In: *Functional Ingredients from Algae for Foods and Nutraceuticals.* Elsevier; 2013:1-19. doi:10.1533/9780857098689.1

6. Mahadevan K. Seaweeds: a sustainable food source. In: *Seaweed Sustainability.* Elsevier; 2015:347-364. doi:10.1016/B978-0-12-418697-2.00013-1

7. Mahalik NP, Kim K. Aquaculture Monitoring and Control Systems for Seaweed and Fish Farming. *World J Agric Res.* 2014;2:176-182.

8. Ktari L, Chebil Ajjabi L, De Clerck O, Gómez Pinchetti JL, Rebours C. Seaweeds as a promising resource for blue economy development in Tunisia: current state, opportunities, and challenges. *J Appl Phycol.* 2021;34(1):489-505. doi:10.1007/s10811-021-02579-w

9. Ford L, Theodoridou K, Sheldrake GN, Walsh PJ. A critical review of analytical methods used for the chemical characterisation and quantification of phlorotannin compounds in brown seaweeds. *Phytochem Anal.* 2019;30:587-599.

10. Hudek K, Davis LC, Ibbini J, Erickson L. Commercial Products from Algae. In: Bajpai R, Prokop A, Zappi M, eds. *Algal Biorefineries.* Springer Netherlands; 2014:275-295. doi:10.1007/978-94-007-7494-0_11

11. Boukid F, Castellari M. Food and Beverages Containing Algae and Derived Ingredients Launched in the Market from 2015 to 2019: A Front-of-Pack Labeling Perspective with a Special Focus on Spain. *Foods.* 2021;10(1):173. doi:10.3390/foods10010173

12. Murray M, Dordevic AL, Ryan L, Bonham MP. An emerging trend in functional foods for the prevention of cardiovascular disease and diabetes: Marine algal polyphenols. *Crit Rev Food Sci Nutr.* 2018;58:1342-1358.

13. Cajnko MM, Novak U, Likozar B. Cascade valorization process of brown alga seaweed Laminaria hyperborea by isolation of polyphenols and alginate. *J Appl Phycol.* 2019;31:3915-3924.

14. Goleniowski M, Bonfill M, Cusido R, Palazón J. Phenolic Acids. In: Ramawat KG, Mérillon J-M, eds. *Natural Products.* Springer Berlin Heidelberg; 2013:1951-1973. doi:10.1007/978-3-642-22144-6_64

15. Jiménez-Escrig A, Jiménez-Jiménez I, Pulido R, Saura-Calixto F. Antioxidant activity of fresh and processed edible seaweeds: Antioxidant activity of seaweeds. *J Sci Food Agric.* 2001;81:530-534.

16. Matsukawa R, Dubinsky Z, Kishimoto E, et al. A comparison of screening methods for antioxidant activity in seaweeds. *J Appl Phycol.* 1997;9(1):29-35. doi:10.1023/A:1007935218120

17. Nasseri MA, Behravesh S, Allahresani A, Kazemnejadi M. Phytochemical and antioxidant studies of Cleome heratensis (Capparaceae) plant extracts. *Bioresour Bioprocess.* 2019;6(1):5. doi:10.1186/s40643-019-0240-1

18. Cotas J, Leandro A, Monteiro P, et al. Seaweed Phenolics: From Extraction to Applications. *Mar Drugs.* 2020;18(8):384. doi:10.3390/md18080384

19. Svensson CJ, Pavia H, Toth GB. Do plant density, nutrient availability, and herbivore grazing interact to affect phlorotannin plasticity in the brown seaweed Ascophyllum nodosum. *Mar Biol.* 2007;151:2177-2181.

20. Pavia H, Cervin G, Lindgren A, Åberg P. Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga Ascophyllum nodosum. *Mar Ecol Prog Ser.* 1997;157:139-146.

21. Rönnberg O, Ruokolahti C. Seasonal variation of algal epiphytes and phenolic content of Fucus vesiculosus in a northern Baltic archipelago. *Ann Bot Fenn.* 1986;23:317-323.

22. Pangestuti R, Kim S-K. Biological activities and health benefit effects of natural pigments derived from marine algae. *J Funct Foods.* 2011;3:255-266.

23. Li Y-X, Wijesekara I, Li Y, Kim S-K. Phlorotannins as bioactive agents from brown algae. *Process Biochem.* 2011;46:2219-2224.

24. Heo S, Park E, Lee K, Jeon Y. Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresour Technol.* 2005;96:1613-1623.

25. Wijesekara I, Yoon NY, Kim S-K. Phlorotannins from Ecklonia cava (Phaeophyceae): Biological activities and potential health benefits. *Biofactors.* 2010;36(6):408-414. doi:10.1002/biof.114

26. Besednova NN, Andryukov BG, Zaporozhets TS, et al. Antiviral Effects of Polyphenols from Marine Algae. *Biomedicine.* 2021;9(2):200. doi:10.3390/biomedicines9020200

27. Kwon H-J, Ryu YB, Kim YM, et al. In vitro antiviral activity of phlorotannins isolated from Ecklonia cava against porcine epidemic diarrhea coronavirus infection and hemagglutination. *Bioorg Med Chem.* 2013;21(15):4706-4713. doi:10.1016/j.bmc.2013.04.085

28. Catarino MD, Amarante SJ, Mateus N, Silva AMS, Cardoso SM. Brown Algae Phlorotannins: A Marine Alternative to Break the Oxidative Stress, Inflammation and Cancer Network. *Foods.* 2021;10(7):1478. doi:10.3390/foods10071478

29. Ahn M-J, Yoon KD, Min SY, et al. Inhibition of HIV-1 Reverse Transcriptase and Protease by Phlorotannins from the Brown Alga Ecklonia cava. *Biol Pharm Bull.* 2004;27(4):544-547. doi:10.1248/bpb.27.544

30. Artan M, Li Y, Karadeniz F, Lee SH, Kim MM, Kim SK. Anti-HIV-1 activity of phloroglucinol derivative, 6,6′-bieckol, from Ecklonia cava. *Bioorg Med Chem.* 2008;16(17):7921-7926. doi:10.1016/j.bmc.2008.07.078

31. Kong C-S, Kim J-A, Yoon N-Y, Kim S-K. Induction of apoptosis by phloroglucinol derivative from Ecklonia Cava in MCF-7 human breast cancer cells. *Food Chem Toxicol.* 2009;47:1653-1658.

32. Park J-Y, Kim JH, Kwon JM, et al. Dieckol, a SARS-CoV 3CLpro inhibitor, isolated from the edible brown algae Ecklonia cava. *Bioorg Med Chem.* 2013;21(13):3730-3737. doi:10.1016/j.bmc.2013.04.026

33. Ganesan AR, Tiwari U, Rajauria G. Seaweed nutraceuticals and their therapeutic role in disease prevention. *Food Sci Human Wellness.* 2019;8:252-263.

34. Zhou N, Gu X, Zhuang T, Xu Y, Yang L, Zhou M. Gut Microbiota: A Pivotal Hub for Polyphenols as Antidepressants. *J Agric Food Chem.* 2020;68(22):6007-6020. doi:10.1021/acs.jafc.0c01461

35. Tong T, Liu X, Yu C. Extraction and Nano-Sized Delivery Systems for Phlorotannins to Improve Its Bioavailability and Bioactivity. *Mar Drugs.* 2021;19(11):625. doi:10.3390/md19110625

36. Wekre ME, Kåsin K, Underhaug J, Holmelid B, Jordheim M. Quantification of Polyphenols in Seaweeds: A Case Study of Ulva intestinalis. *Antioxidants.* 2019;8(12):612 doi:10.3390/antiox8120612

37. Zenthoefer M, Geisen U, Hofmann-Peiker K, et al. Isolation of polyphenols with anticancer activity from the Baltic Sea brown seaweed Fucus vesiculosus using bioassay-guided fractionation. *J Appl Phycol.* 2017;29(4):2021-2037. doi:10.1007/s10811-017-1080-z

38. Koivikko R, Loponen J, Honkanen T, Jormalainen V. CONTENTS OF SOLUBLE, CELL-WALL-BOUND AND EXUDED PHLOROTANNINS IN THE BROWN ALGA Fucus vesiculosus, WITH IMPLICATIONS ON THEIR ECOLOGICAL FUNCTIONS. *J Chem Ecol.* 2005;31(1):195-212. doi:10.1007/s10886-005-0984-2

39. Steevensz AJ, MacKinnon SL, Hankinson R, et al. Profiling Phlorotannins in Brown Macroalgae by Liquid Chromatography-High Resolution Mass Spectrometry: Phlorotannins in Seaweed by LC-HRMS. *Phytochem Anal.* 2012;23(5):547-553. doi:10.1002/pca.2354

40. Mwangi HM, Njue WM, Onani MO, Thovhoghi N, Mabusela WT. Phlorotannins and a sterol isolated from a brown alga Ecklonia maxima, and their cytotoxic activity against selected cancer cell lines HeLa, H157 and MCF7. *Interdiscip J Chem.* 2017;2(2):1-6. doi:10.15761/IJC.1000120

41. Sardari RRR, Prothmann J, Gregersen O, Turner C, Nordberg Karlsson E. Identification of Phlorotannins in the Brown Algae, Saccharina latissima and Ascophyllum nodosum by Ultra-High-Performance Liquid Chromatography Coupled to High-Resolution Tandem Mass Spectrometry. *Molecules*. 2020;26(1):43. doi:10.3390/molecules26010043

42. Fernando IPS, Lee W, Ahn G. Marine algal flavonoids and phlorotannins; an intriguing frontier of biofunctional secondary metabolites. *Crit Rev Biotechnol*. 2022;42(1):23-45. doi:10.1080/07388551.2021.1922351

43. Singh IP, Sidana J. Phlorotannins. In: *Functional Ingredients from Algae for Foods and Nutraceuticals*. Elsevier; 2013:181-204. doi:10.1533/9780857098689.1.181

44. Vissers AM, Caligiani A, Sforza S, Vincken J-P, Gruppen H. Phlorotannin Composition of *Laminaria digitata*: Phlorotannin composition of Laminaria digitata. *Phytochem Anal*. 2017;28:487-495.

45. Ford L, Stratakos AC, Theodoridou K, et al. Polyphenols from Brown Seaweeds as a Potential Antimicrobial Agent in Animal Feeds. *ACS Omega*. 2020;5(16):9093-9103. doi:10.1021/acsomega.9b03687

46. Monbet P, Worsfold P, McKelvie I. Advances in marine analytical chemistry. *Talanta*. 2019;202:610. doi:10.1016/j.talanta.2019.03.097

47. Wang Z-F, You Y-L, Li F-F, Kong W-R, Wang S-Q. Research Progress of NMR in Natural Product Quantification. *Molecules*. 2021;26(20):6308. doi:10.3390/molecules26206308

48. Ferdouse F, Holdt SL, Smith R, Murúa P, Yang Z. *The global status of seaweed production, trade and utilization*GLOBEFISH Res. Programme. Vol. 124I; 2018.

49. Murray M, Dordevic AL, Ryan L, Bonham MP. Phlorotannins and Macroalgal Polyphenols: Potential As Functional Food Ingredients and Role in Health Promotion. In: Rani V, Yadav UC, eds. *Functional Food and Human Health*. Springer Singapore; 2018:27-58.

50. Williamson G, Manach C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr*. 2005;81(1):243S-255S. doi:10.1093/ajcn/81.1.243S

51. Stern JL, Hagerman AE, Steinberg PD, Winter FC, Estes JA. A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *J Chem Ecol*. 1996;22:1273-1293.

52. Farvin KHS, Jacobsen C. Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chem*. 2013;138:1670-1681.

53. Parys S, Rosenbaum A, Kehraus S, Reher G, Glombitza KW, König GM. Evaluation of Quantitative Methods for the Determination of Polyphenols in Algal Extracts. *J Nat Prod*. 2007;70(12):1865-1870. doi:10.1021/np070302f

54. O'Sullivan AM, O'Callaghan YC, O'Grady MN, et al. In vitro and cellular antioxidant activities of seaweed extracts prepared from five brown seaweeds harvested in spring from the west coast of Ireland. *Food Chem*. 2011;126(3):1064-1070. doi:10.1016/j.foodchem.2010.11.127

55. Zhang Q, Zhang J, Shen J, Silva A, Dennis DA, Barrow CJ. A Simple 96-Well Microplate Method for Estimation of Total Polyphenol Content in Seaweeds. *J Appl Phycol*. 2006;18(3–5):445-450. doi:10.1007/s10811-006-9048-4

56. Wang T, Jónsdóttir R, Ólafsdóttir G. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chem*. 2009;116:240-248.

57. Heffernan N, Smyth TJ, FitzGerald RJ, Soler-Vila A, Brunton N. Antioxidant activity and phenolic content of pressurised liquid and solid-liquid extracts from four Irish origin macroalgae. *Int J Food Sci Technol*. 2014;49:1765-1772.

58. Tierney MS, Smyth TJ, Hayes M, Soler-Vila A, Croft AK, Brunton N. Influence of pressurised liquid extraction and solid-liquid extraction methods on the phenolic content and antioxidant activities of Irish macroalgae. *Int J Food Sci Technol*. 2013;48(4):860-869. doi:10.1111/ijfs.12038

59. Gisbert M, Barcala M, Rosell CM, Sineiro J, Moreira R. Aqueous extracts characteristics obtained by ultrasound-assisted extraction from Ascophyllum nodosum seaweeds: effect of operation conditions. *J Appl Phycol*. 2021;33(5):3297-3308. doi:10.1007/s10811-021-02546-5

60. Parys S, Kehraus S, Pete R, Küpper FC, Glombitza KW, König GM. Seasonal variation of polyphenolics in *Ascophyllum nodosum* (Phaeophyceae). *Eur J Phycol*. 2009;44(3):331-338. doi:10.1080/09670260802578542

61. Anaëlle T, Serrano Leon E, Laurent V, et al. Green improved processes to extract bioactive phenolic compounds from brown macroalgae using Sargassum muticum as model. *Talanta*. 2013;104:44-52. doi:10.1016/j.talanta.2012.10.088

62. Li Y, Fu X, Duan D, Liu X, Xu J, Gao X. Extraction and Identification of Phlorotannins from the Brown Alga, Sargassum fusiforme (Harvey) Setchell. *Mar Drugs*. 2017;15(2):49. doi:10.3390/md15020049

63. Jégou C, Kervarec N, Cérantola S, Bihannic I, Stiger-Pouvreau V. NMR use to quantify phlorotannins: The case of Cystoseira tamariscifolia, a phloroglucinol-producing brown macroalga in Brittany (France). *Talanta*. 2015;135:1-6. doi:10.1016/j.talanta.2014.11.059

64. Leyton A, Pezoa-Conte R, Barriga A, et al. Identification and efficient extraction method of phlorotannins from the brown seaweed Macrocystis pyrifera using an orthogonal experimental design. *Algal Res*. 2016;16:201-208. doi:10.1016/j.algal.2016.03.019

65. Roleda MY, Marfaing H, Desnica N, et al. Variations in polyphenol and heavy metal contents of wild-harvested and cultivated seaweed bulk biomass: Health risk assessment and implication for food applications. *Food Control*. 2019;95:121-134. doi:10.1016/j.foodcont.2018.07.031

66. Folin O, Ciocalteu V. ON TYROSINE AND TRYPTOPHANE DETERMINATIONS IN PROTEINS. *J Biol Chem*. 1927;73(2):627-650. doi:10.1016/S0021-9258(18)84277-6

67. Ikawa M, Schaper TD, Dollard CA, Sasner JJ. Utilization of Folin−Ciocalteu Phenol Reagent for the Detection of Certain Nitrogen Compounds. *J Agric Food Chem*. 2003;51:1811-1815.

68. Jacobsen C, Sørensen A-DM, Holdt SL, Akoh CC, Hermund DB. Source, Extraction, Characterization, and Applications of Novel Antioxidants from Seaweed. *Annu Rev Food Sci Technol*. 2019;10:541-568.

69. van Alstyne KL. Comparison of three methods for quantifying brown algal polyphenolic compounds. *J Chem Ecol*. 1995;21:45-58.

70. Pękal A, Pyrzynska K. Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. *Food Anal Methods*. 2014;7:1776-1782.

71. Jordheim M, Aaby K, Fossen T, Skrede G, Andersen ØM. Molar Absorptivities and Reducing Capacity of Pyranoanthocyanins and Other Anthocyanins. *J Agric Food Chem*. 2007;55:10591-10598.

72. Pauli GF, Gödecke T, Jaki BU, Lankin DC. Quantitative ^{1}H NMR. Development and Potential of an Analytical Method: An Update. *J Nat Prod*. 2012;75:834-851.

73. Pauli GF, Jaki BU, Lankin DC. Quantitative1H NMR: Development and Potential of a Method for Natural Products Analysis. *J Nat Prod*. 2005;68(1):133-149. doi:10.1021/np0497301

74. Nerantzaki AA, Tsiafoulis CG, Charisiadis P, Kontogianni VG, Gerothanassis IP. Novel determination of the total phenolic content in crude plant extracts by the use of 1H NMR of the –OH spectral region. *Anal Chim Acta*. 2011;688:54-60. doi:10.1016/j.aca.2010.12.027

75. Sævdal Dybsland C, Bekkby T, Hasle Enerstvedt K, Kvalheim OM, Rinde E, Jordheim M. Variation in Phenolic Chemistry in Zostera marina Seagrass along Environmental Gradients. *Plan Theory*. 2021;10(2):334. doi:10.3390/plants10020334

76. Ummat V, Tiwari BK, Jaiswal AK, et al. Optimisation of Ultrasound Frequency, Extraction Time and Solvent for the Recovery of Polyphenols, Phlorotannins and Associated Antioxidant Activity from Brown Seaweeds. *Mar Drugs*. 2020;18(5):250 doi:10.3390/md18050250

77. Singleton VL, Orthofer R, Lamuela-Raventós RM. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In: *Methods in Enzymology*, Vol. 299. Elsevier; 1999:152-178. doi:10.1016/S0076-6879(99)99017-1

78. Singleton VL, Rossi JA. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am J Enol Vitic*. 1965;16:144.

79. Ün İ, Ün ŞŞ, Tanrıkulu N, Ünlü A, Ok S. Assessing the concentration of conjugated fatty acids within pomegranate seed oil using quantitative nuclear magnetic resonance (qNMR). *Phytochem Anal*. 2021;33:452-459. doi:10.1002/pca.3101

80. Piotto M, Bourdonneau M, Elbayed K, Wieruszeski J-M, Lippens G. New DEFT sequences for the acquisition of one-dimensional carbon NMR spectra of small unlabelled molecules. *Magn Reson Chem*. 2006;44:943-947.

81. Zhang K, Tieke B, Forgie JC, Vilela F, Parkinson JA, Skabara PJ. Cross-linked polymers based on 2,3,5,6-tetra-substituted pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (DPP): Synthesis, optical and electronic properties. *Polymer*. 2010;51(26):6107-6114. doi:10.1016/j.polymer.2010.10.054

82. Pavia H, Toth GB. Influence of light and nitrogen on the phlorotannin content of the brown seaweeds Ascophyllum nodosum and Fucus vesiculosus. *Hydrobiologia*. 2000;440(1/3):299-305. doi:10.1023/A:1004152001370

83. Tabassum MR, Xia A, Murphy JD. Seasonal variation of chemical composition and biomethane production from the brown seaweed Ascophyllum nodosum. *Bioresour Technol*. 2016;216:219-226. doi:10.1016/j.biortech.2016.05.071

84. Schiener P, Black KD, Stanley MS, Green DH. The seasonal variation in the chemical composition of the kelp species Laminaria digitata, Laminaria hyperborea, Saccharina latissima and Alaria esculenta. *J Appl Phycol*. 2015;27(1):363-373. doi:10.1007/s10811-014-0327-1

85. Lopes G, Barbosa M, Vallejo F, et al. Profiling phlorotannins from Fucus spp. of the Northern Portuguese coastline: Chemical approach by HPLC-DAD-ESI/MS and UPLC-ESI-QTOF/MS. *Algal Res*. 2018;29:113-120. doi:10.1016/j.algal.2017.11.025

86. Ragan MA, Jamieson WD. Oligomeric polyphloroglucinols from Fucus vesiculosus: Photoplate mass spectrometric investigation. *Phytochemistry*. 1982;21(11):2709-2711. doi:10.1016/0031-9422(82)83103-8

87. Hermund DB, Plaza M, Turner C, et al. Structure dependent antioxidant capacity of phlorotannins from Icelandic Fucus vesiculosus by UHPLC-DAD-ECD-QTOFMS. *Food Chem*. 2018;240:904-909. doi:10.1016/j.foodchem.2017.08.032

88. Heffernan N, Brunton NP, FitzGerald RJ, Smyth TJ. Profiling of the Molecular Weight and Structural Isomer Abundance of Macroalgae-Derived Phlorotannins. *Mar Drugs*. 2015;13(1):509-528. doi:10.3390/md13010509

89. Montero L, Sánchez-Camargo AP, García-Cañas V, et al. Anti-proliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga Sargassum muticum collected on North-Atlantic coasts. *J Chromatogr a*. 2016;1428:115-125. doi:10.1016/j.chroma.2015.07.053

90. Caro Y, Anamale L, Fouillaud M, Laurent P, Petit T, Dufosse L. Natural hydroxyanthraquinoid pigments as potent food grade colorants: an overview. *Nat Prod Bioprospecting*. 2012;2(5):174-193. doi:10.1007/s13659-012-0086-0

91. Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. *J Agric Food Chem*. 2005;53(6):1841-1856. doi:10.1021/jf030723c

92. Castro-Alves VC, Cordenunsi BR. Total Soluble Phenolic Compounds Quantification Is Not As Simple As It Seems. *Food Anal Methods*. 2015;8:873-884.

93. Prior RL, Wu X, Schaich K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *J Agric Food Chem*. 2005;53:4290-4302.

94. López-Hidalgo C, Meijón M, Lamelas L, Valledor L. The rainbow protocol: A sequential method for quantifying pigments, sugars, free amino acids, phenolics, flavonoids and MDA from a small amount of sample. *Plant Cell Environ*. 2021;44(6):1977-1986. doi:10.1111/pce.14007

95. Sanoner P, Guyot S, Marnet N, Molle D, Drilleau J-F. Polyphenol Profiles of French Cider Apple Varieties (*Malus domestica* sp.). *J Agric Food Chem*. 1999;47(12):4847-4853. doi:10.1021/jf990563y

96. Wong MC, Griffiths G, Vercaemer B. Seasonal Response and Recovery of Eelgrass (Zostera marina) to Short-Term Reductions in Light Availability. *Estuaries Coast*. 2020;43(1):120-134. doi:10.1007/s12237-019-00664-5