

## RESEARCH ARTICLE

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

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## Funding information

Bergen Research Foundation, Grant/Award Number: BFS-NMR-1; Norges Forskningsråd, Grant/Award Number: 297507; Sparebankstiftinga Sogn og Fjordane, Grant/Award Number: 509-42/16; Research Council of Norway through the Norwegian NMR Platform, NNP, Grant/Award Number: 226244/F50

## 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 <sup>1</sup>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 <sup>1</sup>H signals was based on information from 2D (<sup>1</sup>H–<sup>13</sup>C, <sup>1</sup>H–<sup>15</sup>N) NMR spectra. <sup>13</sup>C NMR phlorotannin characterisation facilitated the average number of protons expected to be found per aromatic ring used for the <sup>1</sup>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 <sup>13</sup>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

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## 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.<sup>1-4</sup> 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.<sup>1,5-9</sup> Several products, for various applications, can be extracted from macroalgae including alginate, fucoidan, mannitol, cellulose, proteins, carotenoids, and polyphenols.<sup>10,11</sup>

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

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).<sup>9,12,18,43-45</sup>

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.<sup>36,46,47</sup> Furthermore, the diversity of seaweed polyphenols complicates data collection and standardisation of procedures.<sup>9</sup> Being able to quantify the polyphenolic content in crude seaweed materials with higher accuracy is important in order to fully explore seaweeds' potential applications.<sup>48-51</sup> 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.<sup>66</sup> However, the method

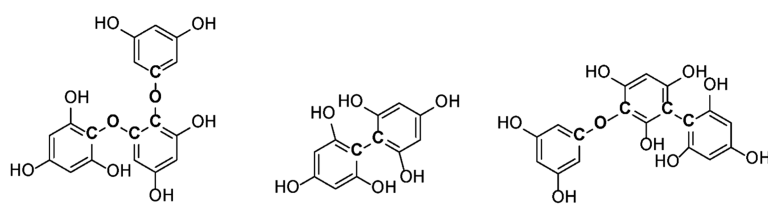
depends on a non-selective redox reaction and has been evaluated to yield only estimates of polyphenol content.<sup>36,67-70</sup> High performance liquid chromatography (HPLC) with UV-visible diode array detection (DAD) is also used for polyphenol quantification but rarely for quantification of the total polyphenolic content. This is due to the quantification method being based on molar absorptivity ( $\epsilon$ ; Beer-Lambert's law,  $A = \epsilon cl$ ). Molar absorptivity ( $\epsilon$ ) values vary greatly, even within polyphenol classes, making a precise "one standard" total polyphenolic quantification with HPLC-DAD impossible.<sup>71</sup> Quantitative NMR is a quantification method independent of colorimetric changes, molar absorptivity, and calibration curves.<sup>36,44,45,53,72-74</sup> 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.<sup>36</sup>

In this study, we continue our examination of total phenolic quantification methods for seaweeds by optimising the quantitative <sup>1</sup>H NMR method and compare the results with the FC TPC assay.<sup>36</sup> 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.<sup>75</sup> 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. <sup>13</sup>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 ( $\geq 99.9\%$ ), ethanol (absolute), ethyl acetate ( $\geq 99.5\%$ ), DMSO<sub>2</sub> (TraceCERT<sup>®</sup>), and DMSO-*d*<sub>6</sub> (0.03% TMS)



Example: Triphlorethol A  
Linkage: C-O-C  
Ether  
Type: Phlorethol

Difucol  
Linkage: C-C  
Phenyl  
Type: Fucol

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

\*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 Storåkvika, 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).<sup>76</sup> 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.<sup>77,78</sup> Briefly, in the method 0.2 mL sample, blank or standard, 1.59 mL FC reagent, and 4.0 mL 20% (w/v) Na<sub>2</sub>CO<sub>3</sub> 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*<sub>6</sub> (0.03% TMS) containing the internal standard DMSO<sub>2</sub> ( $C = 10$  mM). Quantification using <sup>1</sup>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  $5 \times T_1$  for all <sup>1</sup>H spectra obtained for quantitative NMR (qNMR) analysis.<sup>36,79</sup>

The one-dimensional (1D) <sup>1</sup>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  $5 \times T_1$  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<sub>2</sub> (10 mM, No. H = 6, MW = 94.13 g/mol) as the internal standard.

$$C_{\text{sample}} [\text{M}] = \frac{I_{\text{sample}} \times n_{\text{DMSO}_2} \times C_{\text{DMSO}_2}}{I_{\text{DMSO}_2} \times n_{\text{sample}}} \quad (1)$$

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) <sup>1</sup>H-<sup>13</sup>C and <sup>1</sup>H-<sup>15</sup>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).

<sup>1</sup>H-<sup>13</sup>C HMBC spectra were acquired using the *hmbcetgp13nd* pulse sequence with non-uniform sampling (50%), 352 scans, 16 dummy scans, <sup>1</sup>H sweep width of 13.02 ppm, <sup>13</sup>C sweep width of 220.0 ppm, and a relaxation time of 2.0 seconds.

<sup>1</sup>H-<sup>13</sup>C HSQC spectra used the *hsqcetgpsi2.3* pulse sequence with 128 scans, 32 dummy scans, <sup>1</sup>H sweep width of 13.02 ppm, and <sup>13</sup>C sweep width of 200.0 ppm.

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

1D <sup>13</sup>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 measured with two standards, gallic acid and phloroglucinol, with known concentrations ( $C_{\text{known}}$ ) using DMSO<sub>2</sub> as the internal reference

Standard	$C_{\text{known}}$ [M]	Chemical shift [ppm]	Integral	Number of protons ( $n$ )	$C_{\text{measured}}$ [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  $^{13}\text{C}$  spectra with maximised signal-to-noise ratio could be acquired in shorter time.<sup>80,81</sup> Signals of 95–160 ppm were used to obtain carbon ratios distinguishing characteristic phlorotannin carbons.

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Phlorotannin characterisation with $^{13}\text{C}$ NMR

As phlorotannins are the dominating polyphenolic compounds in brown seaweeds,  $^{13}\text{C}$  NMR was used to assess the linkage profiles of the phlorotannins in the five seaweeds examined (Table 4).  $^{13}\text{C}$  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.<sup>44,45</sup> Figure 2 displays the  $^{13}\text{C}$  NMR spectra of the examined species (A-E) and indicates the characteristic signal regions

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

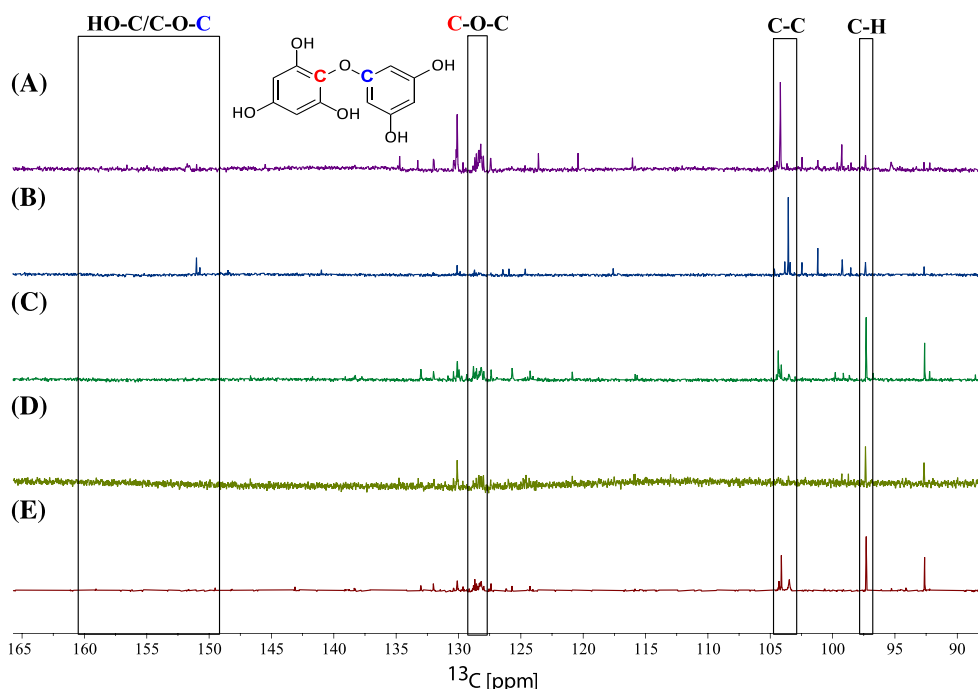
Species	Intensity ratio measured		
	C-O-C	C-C	C-H <sup>a</sup>
<i>Laminaria hyperborea</i> M20	3.08	1.00	1.00
<i>Laminaria digitata</i>	0.94	0.32	1.00
<i>Saccharina latissima</i>	2.73	0.68	1.00
<i>Fucus vesiculosus</i>	20.1	1.11	1.00
<i>Ascophyllum nodosum</i>	4.95	5.67	1.00

<sup>a</sup>(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  $^{13}\text{C}$  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  $^{13}\text{C}$  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).<sup>44,45</sup>

Ford et al. (2020) and Vissers et al. (2017) report on characterisation of phlorotannins in brown seaweeds using  $^{13}\text{C}$  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.<sup>45</sup> Vissers et al. (2017) report a higher abundance of ether linkages compared with phenyl linkages in *L. digitata*.<sup>44</sup> 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**  $^{13}\text{C}$  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.<sup>82–84</sup> 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.<sup>41,85,86</sup>

Furthermore, the phlorotannin <sup>13</sup>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 <sup>1</sup>H qNMR.<sup>44,53</sup> Phlorethols (Figure 1) generally consist of one terminal aromatic ring containing three aromatic hydrogens, yielding signals in the characteristic polyphenol region of the <sup>1</sup>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.<sup>44</sup> 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.<sup>41,85,87,88</sup> 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.<sup>89</sup> 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 <sup>13</sup>C ratios and the knowledge of the expected structures, the average number of protons per

aromatic ring was set to be 2H ( $n_{\text{sample}} = 2$ , 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).

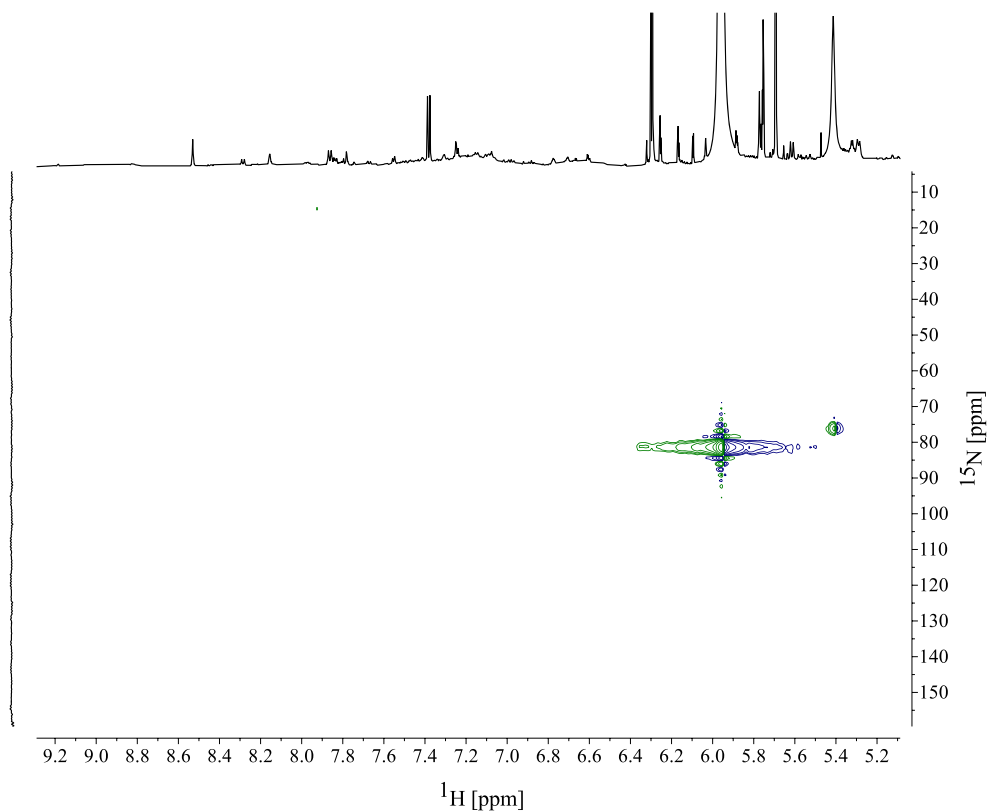
### 3.2 | Total quantification of polyphenols

<sup>1</sup>H qNMR can be performed by integrating the -OH spectral region (14–8 ppm), as proposed by Nerantzaki et al. (2011).<sup>74</sup> More conventional methods, however, integrate the aromatic region (8–6 ppm).<sup>36,44,53,60,63,72,73</sup> Due to possible H-D exchange with aromatic-OH groups, leading to loss of intensity and broad peaks, the aromatic <sup>1</sup>H-region was selected for polyphenolic quantification. Based on knowledge of chemical <sup>1</sup>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.<sup>44,45,53</sup> 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). <sup>1</sup>H-<sup>15</sup>N HSQC spectra indicated that this <sup>1</sup>H peak was coupled to a nitrogen δ 5.97/81.8 (<sup>1</sup>H/<sup>15</sup>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 <sup>1</sup>H-<sup>13</sup>C HMBC spectra for each alga. Figure 4 displays the quantified region of the <sup>1</sup>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 ( $n_{\text{sample}}$ ). Increasing this value will decrease the molar concentration calculated; however, using <sup>13</sup>C NMR to estimate this value provides a more accurate quantification.<sup>44</sup> 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.

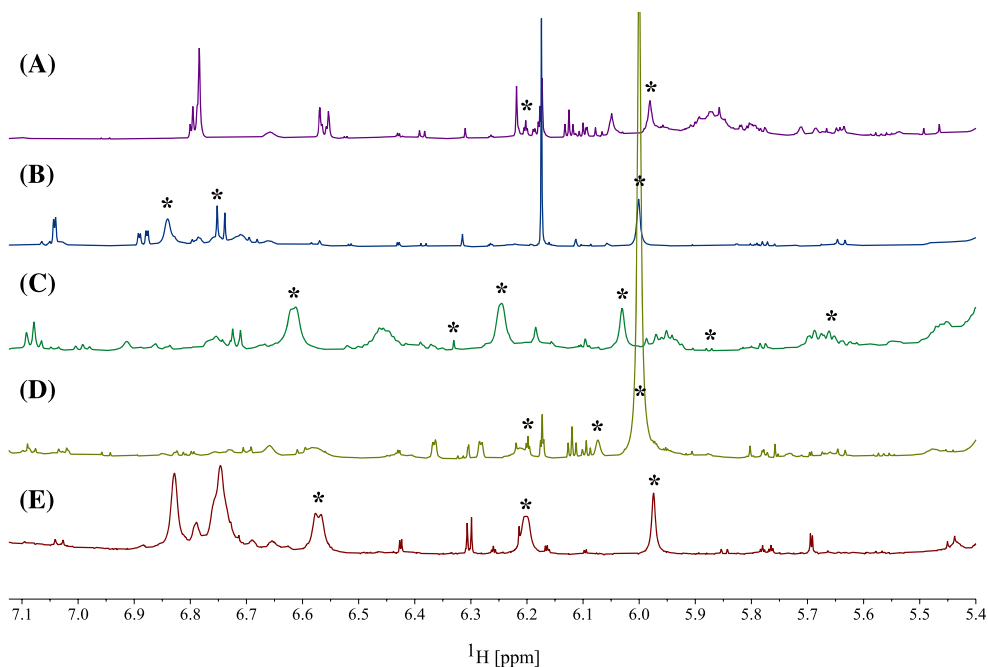
Sample	Family	Zone	C [mg GAE/g DW]	C [mg PGE/g DW]
<i>L. hyperborea</i> M20	Laminariaceae	Sublittoral	8.32 ± 0.00	6.17 ± 0.01
<i>L. hyperborea</i> S20	Laminariaceae	Sublittoral	5.51 ± 0.00	4.08 ± 0.01
<i>L. hyperborea</i> A21	Laminariaceae	Sublittoral	6.57 ± 0.00	4.87 ± 0.01
<i>L. digitata</i>	Laminariaceae	Sublittoral	6.86 ± 0.00	5.09 ± 0.01
<i>S. latissima</i>	Laminariaceae	Sublittoral	16.8 ± 0.0	12.4 ± 0.0
<i>A. nodosum</i>	Fucaceae	Eulittoral	11.57 ± 0.0	8.57 ± 0.0
<i>F. vesiculosus</i>	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**  $^1\text{H}$ - $^{15}\text{N}$  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**  $^1\text{H}$  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.<sup>78</sup> 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.<sup>36,44,45,53</sup> 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).<sup>44,45,53,60</sup> 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.<sup>9,36,53,60</sup> 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).<sup>45,53</sup> 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 ( $n_{\text{sample}} = 1.7$ ).<sup>45,53</sup> 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).<sup>63,65</sup>

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).<sup>52–55,57</sup> 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%).<sup>56</sup>

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

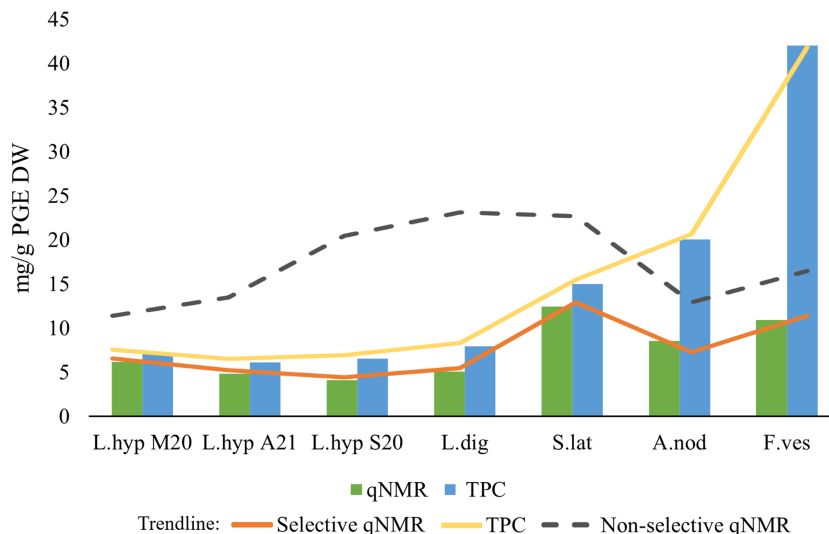
In Figure 5 the quantified polyphenolic content of *L. hyperborea*, harvested in various seasons, *L. digitata*, *S. latissima*, *A. nodosum*, and *F. vesiculosus* using the selective qNMR method and the TPC assay are shown—including a trendline for the two methods compared with a non-selective qNMR 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.<sup>9,45,60,65,90</sup> 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 times 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*.<sup>45</sup> 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*.<sup>60</sup> 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]
<i>L. hyperborea</i> M20	Laminariaceae	Sublittoral	6.23 ± 0.11	7.15 ± 0.15
<i>L. hyperborea</i> S20	Laminariaceae	Sublittoral	5.72 ± 0.07	6.56 ± 0.08
<i>L. hyperborea</i> A21	Laminariaceae	Sublittoral	5.35 ± 0.04	6.14 ± 0.05
<i>L. digitata</i>	Laminariaceae	Sublittoral	6.94 ± 0.09	7.93 ± 0.09
<i>S. latissima</i>	Laminariaceae	Sublittoral	13.1 ± 0.04	15.0 ± 0.05
<i>A. nodosum</i>	Fucaceae	Eulittoral	17.6 ± 0.04	20.1 ± 0.05
<i>F. vesiculosus</i>	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 latissima*, *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.<sup>69,70,91,92</sup> 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.<sup>68,91,93</sup> 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.<sup>9,36,75,77,78,90,94–96</sup> 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 <sup>13</sup>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.

#### ACKNOWLEDGMENTS

M.E.W. gratefully acknowledges the Norwegian Research Council (project NFR297507) and Alginor ASA for their fellowship and Georg Kopplin for guidance. The authors acknowledge Jarl Underhaug for great guidance and support when performing NMR experiments at the Norwegian NMR Platform (NNP). The authors acknowledge the Ocean Forest project at Lerøy AS for providing *Saccharina latissima* material. This work was partly supported by the Bergen Research Foundation (BFS-NMR-1), Sparebankstiftinga Sogn og Fjordane (509-42/16), and the Research Council of Norway through the Norwegian NMR Platform, NNP (226244/F50).

#### 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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**How to cite this article:** Wekre ME, Hellesen Brunvoll S, Jordheim M. Advancing quantification methods for polyphenols in brown seaweeds—applying a selective qNMR method compared with the TPC assay. *Phytochemical Analysis*. 2022;33(7):1099-1110. doi:10.1002/pca.3162