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Rapid and cost-efficient microplate assay for the accurate quantification of total phenolics in seaweeds

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ABSTRACT

Brown seaweeds (*Phaeophyceae*) are a rich source of polyphenols (up to 20% dry weight) with a structure based on phloroglucinol (1,3,5-trihydroxybenzene). To-date the determination of total phenolics content (TPC) involves a redox reaction with the Folin-Ciocalteu (FC) reagent. However, side reactions with other reducing substances preclude accurate, direct measurement of TPC. This research reports a novel microplate assay involving a coupling reaction between phloroglucinol with Fast Blue BB (FBBB) diazonium salt, at basic pH, to form a stable tri-azo complex with maximum absorbance at 450 nm. Linear regression correlation values (R^2) were ≥0.99 with phloroglucinol as standard. Direct quantification of TPCs (phloroglucinol equivalents, PGEs) in crude aqueous and ethanolic extracts from *A. nodosum* demonstrated that the new FBBB assay is not subject to side-redox interference and provides a more accurate estimate of TPC (1.2–3.9-fold lower than with the FC assay) in a relatively rapid (30 min), cost-effective (0.24€/test) microplate format.

1. Introduction

Polyphenolics are diverse naturally occurring compounds commonly found in higher plants, marine organisms and microorganisms that have many reported biological activities (Cotas et al., 2020). In macroalgae they are responsible for colouration and their known endogenous roles include preserving the physiological integrity of algae, providing chemical defence against bacteria, epiphytes and hydroids, and giving protection against oxidative stress and changes in nutrient availability (Handique & Baruah, 2002; Rosa, Tavares, Sousa, Pagès, Seca, & Diana, 2020). Another important characteristic of polyphenols are the availability of multiple polar functional groups for selective and unselective binding with biologically important molecules such as proteins (Handique & Baruah, 2002).

Seaweeds in the class *Phaeophyceae* (*Phylum Ochrophyta*; brown seaweeds) have been identified as a source of polyphenols with unique structural properties (Apostolidis & Lee, 2010). Phlorotannins display very similar characteristics to the tannins found in terrestrial plants, but both are structurally very different. The most common polyphenols in brown seaweeds are derivatives of phloroglucinol (1,3,5-trihyroxybenzene; PG) subunits, namely phlorotannins (PTs). They are formed by oxidative C-C and C-O coupling reactions of phloroglucinol monomers (Audibert, Fauchon, Blanc, Hauchard, & Gall, 2010; Handique & Baruah, 2002; Rosa et al., 2020). PTs are polyhydroxy compounds that are structurally very diverse, range in molecular weight from 126 Da to 650 kDa (Besednova et al., 2020), and can be readily oxidized. The antioxidant activity of these compounds is the most reported bioactivity (Craft, Kerrihard, Amarowicz, & Pegg, 2012; Gómez-Mascaraque et al., 2021; Guendouze-Bouchefa et al., 2015). However, in addition, polyphenols from brown algae have been shown to exhibit bactericidal and prebiotic properties (Gómez-Mascaraque et al., 2021; Guendouze-Bouchefa et al., 2015), alpha-glucosidase inhibitory activity (Nwosu et al., 2011; Zhang et al., 2007), anti-inflammatory activity (Kim & Kim, 2010) among others and have been investigated for their applications in the food industry (Nagai & Yukimoto, 2003) aquafeed and veterinary care industries (Gunathilake et al., 2022). In seaweeds, PTs exist in membrane-bound vesicles (c.90%) and in complex structures (c.10%) with other cell wall components (e.g. alginic acids), which complicates their extraction and quantification. Therefore, various methods have been investigated including the use of polar organic solvent systems to increase the solubilization or recovery of PTs (Erpel, Mateos, Pérez-Jiménez, & Pérez-Correa, 2020; Mekinić et al., 2019). Although PTs can account for up to 20% of the dry thallus weight of species of Fucales (Connan, Goulard, Stiger, Deslandes, & Gall, 2004; van Hees, Olsen,

Abbreviations: FBBB, fast blue bb salt; F-C, Folin-Ciocalteu; F-D, Folin-Denis; PGE, phloroglucinol equivalents.

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Wernberg, van Alstyne, & Kendrick, 2017) reported levels are dependent on seasonal and nutritional factors, extraction methods and experimental methods for quantification (Mekinić et al., 2019).

The most common wet chemistry assays used to quantify total polyphenol content (TPC) include assays, and modifications thereof, based on reaction of oxidizable phenolic groups with a reducing reagent to form a coloured reaction product that has a characteristic absorbance maximum (λmax) at a given wavelength in the UV–visible region of the light spectrum. These assays include methods based on use of Prussian blue (Price & Butler 1969) 2,4-dimethoxybenzaldehyde (DMBA) (Stern et al., 1996) and the Folin-Denis method (Folin & Denis, 1912)The Folin-Denis (F-D) method (Folin & Denis, 1912) is the most commonly used and is based on a colour reaction between easily oxidized polyphenols or hydroxylated aromatic compounds and phosphotungsten-polymolybdic acid. This method was improved later by Folin and Ciocalteu (1927) by including lithium sulphate to prevent precipitation in the reaction and consequently increase the sensitivity and reproducibility of the method. Nowadays, the Folin-Ciocalteu (1 N) and Folin-Denis (2 N) reagents are commercially available for polyphenol quantification, and they are still found to be the preferred assays for polyphenolic quantification. Modifications to optimize these assays have been reported for estimating phlorotannin content of algal extracts and samples (van Alstyne, 1995; Zhang et al., 2006). The continued use of the F-C method as the reference method for the colorimetric quantification of total phenolics, almost a century after its development, is due to its ease of use, relative speed, and low cost.

The F-C method is based on the reduction of the heteropoly-phosphotungstates/phosphomolybdates in alkaline medium and subsequent oxidation of phenolates to quinones (Folin & Ciocalteu, 1927; Zhang et al., 2006). Nevertheless, the downside of the method is its lack of specificity and cross reactivity with other reducing substances in crude algal samples, most notably proteins, ascorbic acid, amino acids, reducing sugars and other monosaccharides (Holdt & Kraan, 2011; Pereira, Morrison, Shukla, & Critchley, 2020; Pico, Pismag, Laudouze, & Martinez, 2020). Along with these, other possible interferences that are normally present in majority of food, plants and seaweeds biomasses, include aromatic amines, nucleotides, sulphur dioxide, thiols, unsaturated fatty acids, Fe^{2+}, vitamins, aldehydes, ketones and organic acids (Pico et al., 2020).

Different approaches to improve the specificity of F-C method have been proposed over the years, mainly through the physical removal of interferences (Hinojosa-Nogueira, Muros, Rufián-Henares, & Pastoriza, 2017). Such purification or clean-up protocols are time consuming, add to the overall cost and they can lead to the degradation or loss of the phenolic compounds. Consequently, there is need for a sensitive, accurate, reproducible method that reacts selectively with PG and PTs for direct quantification of PTs in crude seaweed extracts. While other methods, such as the DMBA method (Stern, I' et al. 1996) react with PG units in PTs, sensitivity can be lower and side-reactions with some non-polar compounds can occur that cause a shift in absorbance. Furthermore, while selective spectroscopic methods like quantitative 1H NMR (qNMR) are highly selective and accurate, sample complexity is an issue and experience and some prior knowledge of the phenolics composition of samples is needed for robust signal interpretation and quantification (Wekre, Hellesen Brunvoll, & Jordheim 2022). Moreover, NMR instrumentation may not be available universally for routine sample analysis.

In this research, we investigate the potential to adapt and optimise a phenolic selective wet chemistry assay developed previously by Medina (Medina, 2011b) for the quantification of phenolics in grains, fruit and fruit beverages. This method uses the Fast Blue BB dye (FBBB), also known as Cl Azoic diazo 20 and involves specific coupling of the diazonium group (-$^{+}$N = N-) of FBBB with an aromatic ring containing reactive hydroxyl (–OH) groups (e.g. phenolic acid) to quantify the total phenolic content by reference to a gallic acid standard (Medina, 2011a, 2011b). The selectivity and specificity of the FBBB-phenolic acid reaction reduces the likelihood of reaction with common interferences found in foodstuffs and edible products such as reducing sugars and ascorbic acid, thus avoiding the need to include sample clean-up steps. The FBBB method has been used to quantify total phenolics, with gallic acid as reference standard, of fruits (Lester, Lewers, Medina, & Saftner, 2012), fruit derivatives, tea, coffee samples (Medina 2011b, 2011a) and urine samples (Hinojosa-Nogueira et al., 2017), where the main interferences were ascorbic acid and reducing sugar.

To the authors' best knowledge, the FBBB method has not been used for quantification of seaweed TPCs, and the potential reaction of FBBB with PG has not been shown. Therefore, the scope of this research was to develop a reliable, accurate, cost-effective microplate method using FBBB for the selective detection and quantification of PG-rich PTs, to obtain information on the reaction between FBBB and PG and potential formation of an azo-complex using UV–visible spectroscopy and Liquid chromatography in combination with Mass spectrometry (LC-MS), to optimize the concentrations of the Fast Blue BB and alkali required in the assay, and determine the timeframe to complete the reaction. Finally, we aimed to compare the new method for seaweed PT quantification with the FC method and thereby demonstrate the suitability of the new method adaptation for selective quantification of PTs in crude aqueous and ethanolic seaweed extracts from *A. nodosum*, where reducing compounds (e.g. carbohydrates) are present.

2. Materials and methods

2.1. Algal material

A. nodosum was harvested from Dungloe Bay, Co. Donegal, Ireland, in September 2018. The seaweed was washed briefly with fresh water to remove sand and epiphytes, cut into pieces (ca. 1 mm2) and stored immediately at −20 °C until further use.

2.2. Reagents and equipment

Thermo Scientific™ (formerly Alfa Aesar™) Fast Blue BB salt (4-benzoylamino- 2,5-dimethoxybenzenediazonium chloride hemi[zinc chloride]) was purchased from Fischer Scientific Ireland (Dublin, Ireland). Folin-Ciocalteu phenol reagent (2 N; F9252), Gallic acid (3,4,5-trihydroxybenzoic acid; G7384), and Phloroglucinol (1,3,5-trihydroxybenzene; #1070690025), were from Sigma-Aldrich Ireland Ltd (Wicklow, Ireland). Microcentrifuge tubes (2.0 mL brown) and high optical quality 96-well microplates well (flat-bottomed; F-bottom) were purchased from Sarstedt Ireland Ltd (Wexford, Ireland). A PowerWave XS2 Microplate reader equipped with Gen5 software (BioTek; Mason Technology, Dublin, Ireland), was used to record end-point absorbance measurements at specific wavelengths (λ) in assays, and to conduct spectral scans (280–700 nm or 300–700 nm) of reaction mixtures over the UV–visible range of the spectrum.

2.3. Initial evaluation of the Fast Blue BB assay for quantification of phloroglucinol and optimisation of FBBB concentration

For the initial modification of the method, Phloroglucinol and Gallic acid standards (1 mL) with concentrations 0, 200, 400, 600, 800 and 1000 μg/mL were transferred (in triplicate) to dark microtubes (2 mL) and 100 μL of 0.1% (w/v), 0.25% (w/v) or 0.5% (w/v) of Fast Blue BB were added. The samples were strongly vortexed, then 100 μL of 5% (w/v) NaOH were added to each tube to start the reaction. Subsequently, 25 μL of the reaction mixtures were transferred to individual wells in a 96-well microplate and diluted with 200 μL of distilled water. The OD was measured at 420 nm and 450 nm and a spectral scan from 280 nm to 700 nm was also conducted to determine the precise λ_{max} for the absorbance of the phloroglucinol-FBBB reaction product, using the BioTek PowerWave XS2 Microplate reader equipped with Gen5 software.

2.4. Evaluation of the effect of acid and base concentrations on the reaction between phloroglucinol and Fast Blue BB

Phloroglucinol standard (1 mL) at concentrations of 0, 40, 80, 120, 160 and 200 μg/mL was transferred (in triplicate) to individual dark microtubes (2 mL), followed by 100 μL of 0.1 M or 0.2 M HCl (or distilled water). Then, 100 μL to 300 μL of 0.1% (w/v), 0.25% (w/v) or 0.5% (w/v) Fast Blue BB in distilled water was added. The samples were strongly vortexed and 100 μL of 2%, 5% or 10% (w/v) NaOH were added to each tube to start the reaction. Finally, 25 μL of the reaction mixtures were transferred to individual wells in a 96-well microplate and diluted with 200 μL of distilled water. The absorbance was measured at 420 nm and 450 nm and a spectral scan (280 nm-700 nm), as outlined previously.

2.5. Investigation of the effects of solvent and reaction time on the reaction between phloroglucinol and Fast Blue BB

For the last modification of the method, phloroglucinol standard (20 μL), at concentrations of 0, 40, 80, 120, 160 and 200 ppm (or μg/mL) in distilled water or 70% (v/v) ethanol, were transferred (in triplicate) to individual wells in a 96-well microplate. Next, 20 μL of 20 mM HCl were added to each reaction well, followed by 30 μL of 0.25% (w/v) FBBB, 20 μL 1% NaOH and 220 μL of distilled water. The microplate was covered with an acetate foil to prevent evaporation and incubated in the dark at 25 °C for either 30, 60 or 90 min. Finally, 100 μL from each reaction well were transferred to a new microplate and after the addition of 200 μL of distilled water (or ethanol) absorbance values were read at 420 nm and 450 nm, followed by a spectral scan from 300 nm to 700 nm.

2.6. Chromatography and mass spectrometry (LC-MS) analysis of reaction products

Concentrations of phloroglucinol (and unknown) reaction mixtures were adjusted to 1.0 mg/mL prior to LC separation and analysis by mass spectrometry. The Accurate-Mass Q-TOF LC/MS from Agilent Technologies was equipped with an Agilent Extend C18 column (2.1×50 mm × 1.8 μm) and a pump for reaction product separation, an auto sampler, a column oven and a UV/Vis detector for the chromatographic separation. The sample components were separated using Nitrogen as carrier gas at the flow rate of 0.4 mL/min. The injector temperature was set at 300 °C during each run. Two microlitres of phloroglucinol standard or sample extract were injected into the LC-MS instrument as follows: the mobile phases consisted of water (A) and acetonitrile (B). The flow rate was 0.4 mL/min, and the gradient program was as follows: 5–95% B (03 min).

Ionization (of separated reaction products) was achieved using electrospray ionization (ESI) in positive mode with selected reaction monitoring (SRM). Mass spectrometry was performed with an ESI source in the positive-ionization mode and a sheath gas flow of 3 to 8 L/min. The ion spray voltage was set at 3500 V and the source temperature was 350 °C.

2.7. Production of the seaweed samples

The seaweed (algal) extracts were prepared by extraction in hot or cold water, or 70% (v/v) aqueous ethanol (EtOH). Finely-cut *A. nodosum* pieces were extracted at different ratios of extractant to seaweed (E:S, solvent volume:wet weight) at 25°C, 50°C or 70 °C for 1, 4 or 6 h. The liquid fraction was separated from residual solids by centrifugation (20 min, 3500 rpm, 4 °C). The extractions conditions are summarized in Table 1 The supernatant from aqueous extracts were freeze-dried and the solid fine powder was resuspended extraction at 3% (w/v) using distilled water. The ethanolic supernatant was analysed directly at 1:2, 1:5 and 1:10 dilutions in triplicate at least.

2.8. Total phenolic assay by the Folin-Ciocalteu method

The procedure used for total phenolic quantification was a modification of the method developed by Zhang and colleagues (Zhang et al., 2006), which previously adapted the F-C method to a microplate format (to reduce the reagent volumes, minimize the use of material and shorten the process). This procedure was evaluated with phloroglucinol as standard at concentrations of at 0, 200, 400, 600, 800 and 1000 μg/mL. In brief, 20 μL of standard or sample were added to each individual well (in triplicate) and mixed with 10 μL of Folin-Denis reagent (2 N) prior to the addition of 40 μL of 20% (w/v) of Na_2CO_3. To complete the reaction, 130 μL of distilled water was added to each reaction well and the plate was incubated in darkness at 37ºC for 40 min. Finally, the absorbance was read at 765 nm. The TPCs of seaweed extract samples were calculated by reference to phloroglucinol as standard.

3. Results and discussion

3.1. Investigation of the Fast Blue BB triazonium method for quantification of phloroglucinol

The FBBB method has been shown previously to be a more accurate and alternative method to the F-C assay for quantification of total phenolics in different food, grains and beverages (Medina 2011b, 2011a). This wet chemistry assay method relies on the coupling capacity of the FBBB diazonium salt that enables formation of azo compounds through reaction with the electron reactive group (OH) of phenols in an electrophilic aromatic substitution reaction with release of a proton. The electrophilic aromatic substitution happens in conditions of alkaline pH. It has been reported that this coupling reaction occurs preferentially with the OH at the para position of the activating phenolic group of gallic acid, or with the OH at the *ortho* position, if the *para* position is occupied (Medina, 2011b). The F-C assay has also been used widely for quantification of TPC levels in seaweed extracts. However, as with plant extracts/plant-derived samples, the reliability of this method for direct and accurate TPC quantification for crude seaweed extracts is influenced strongly by the likely presence of other reducing substances in these samples. Consequently, there is a need for a reliable, easy-to-use microplate (wet chemistry) assay for direct and accurate quantification of seaweed extract TPCs.

To the authors' best knowledge, the FBBB method has not been investigated previously for the detection and quantification of phloroglucinol and phloroglucinol-rich seaweed phlorotannins (PTs). Therefore, we initially investigated the protocol developed by (Medina 2011b, 2011a) and compared reactivity of FBBB with both phloroglucinol and gallic standards. The absorbance of reaction of FBBB with gallic acid and plant-derived samples was maximal at 420 nm, after 60 min reaction (Medina, 2011a). We observed an absorbance maximum at 450 nm

Table 1
Conditions to produce the different seaweed samples using different solvents, extractant to seaweed ratio, temperature and extraction time to study the total phenolic content using the traditional Folin-Denis (F-C) method and the new adapted and optimised Fast Blue BB method.

Extractant	H_2O	H1	H2	H3	H4	H5	H6	H7	H8	H9
	EtOH	E1	E2	E3	E4	E5	E6	E7	E8	E9
Extraction Temperature (°C)		25	25	70	70	25	25	70	70	50
Extractant to Seaweed ratio (E:S, v:w)		2:1	5:1	2:1	5:1	2:1	5:1	2:1	5:1	3.5:1
Extraction time (h)		1	1	1	1	6	6	6	6	4

following reaction of phloroglucinol with FBBB, while unreacted FBBB absorbed light maximally at 350 nm. The results also showed that the intensity of the azo complex formed between phloroglucinol and FBBB was higher (higher absorbance) than the azo complex formed between gallic acid (di-azo complex) and the FBBB salt (Fig. S2A and B). As phloroglucinol has an additional OH that may act as a reactive group, the results suggest that the intensity may be due to the additional substitution. Additionally, the linearity of the reaction between phloroglucinol and FBBB was observed only up to concentrations of 200 μg/mL under the initial assay conditions (100 μL of 0.25% or 0.5% Fast BB blue salt and 100 μL of 5% NaOH) and absorbance intensity was higher when 0.5% FBBB was used. In contrast, the linearity was maintained ($R^2_{(0.5\%)} = 0.9945$ and $R^2_{(0.25\%)} = 0.9963$) for gallic acid at concentrations up to 1000 μg/mL and the absorbance intensity was similar for both dye concentrations. To optimize the dye to phloroglucinol ratio, the assay was repeated for a standard concentration of 0–100 μg/mL phloroglucinol, and the response showed good linearity ($R^2 = 0.9923$) when the concentration of FBBB was 0.5% (w/v) (Fig. S2C, Supplementary material). The reaction was not linear at 0.25% (w/v) FBBB, where the absorbance remained constant above 0–50 μg/mL phloroglucinol suggesting that insufficient FBBB was present to react with all of the phloroglucinol present at concentrations ≥0–50 μg/mL. The spectral scans of the 100 μg/mL phloroglucinol with 0.25% (w/v) FBBB and 0.5% (w/v) FBBB reaction mixtures showed a higher absorbance intensity at λ_{max} when the concentration of the dye was higher in the reaction. The peak at 350 nm associated with unreacted phloroglucinol, disappeared correspondingly.

Heating the reaction mixture did not provide any improvement to the reaction (*data not shown*). Therefore, all experiments conducted to optimize the assay were performed at 25 °C using either a water bath or a microplate incubator to ensure constant temperature along the process, and in the dark to minimize light exposure.

In this study, LC-MS was used to investigate the type of aromatic substitution reaction between FBBB and phloroglucinol. Formation of a tri-azo complex was confirmed by mass spectrometry (see Fig. S2, Supplementary material). Therefore, we propose the mechanism shown in Fig. 1 for the interaction of phloroglucinol and Fast Blue BB. Fig. 2.

The suggested reading absorbance by Medina (2011) was 420 nm as the absorbance produced a lower background reading and at the same

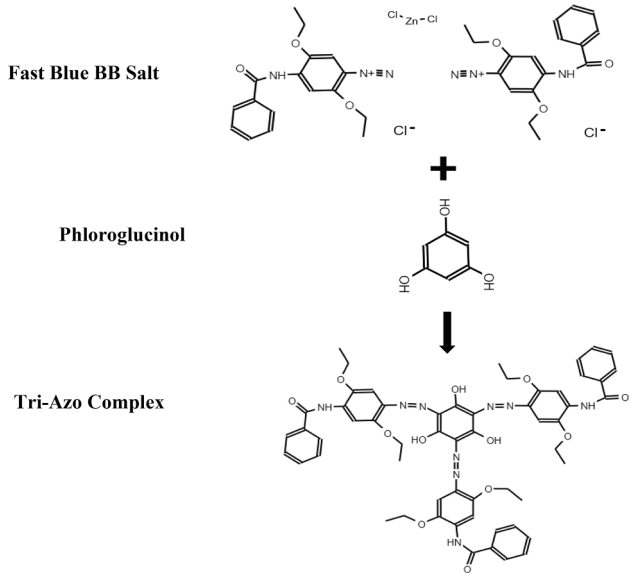

Fig. 1. Proposed interaction between phloroglucinol and the Fat Blue BB diazonium salt at alkaline pH to form tri-azo complex. Diagrams of chemical compounds were created using the software developed by Bienfait and Ertl, 2013 (Bienfait & Ertl, 2013).

time provided the highest absorbance values. Nevertheless, in this study, the readings at 450 nm provided the highest absorbance value for the tri-azo complex formed between Phloroglucinol and FBBB with a minimum background reading. The OD values at 450 nm, were 20% greater than those obtained at 420 nm.

Interactions of Fast Blue BB with various concentrations of HCl (0.1 M and 0.2 M) and NaOH (2%, 5% and 10%) were studied with the absorbance registered at 420 and 450 nm after 60 min of reaction. Concentrations below 5% (w/v) NaOH yielded worst linearity as the regression correlations (R^2) were reduced from 0.99 to 0.97. The acidification of the mixture prior the alkalinisation was intended to create uniform conditions by ensuring accurate reaction even if the nature of the sample or standard caused a pH drop. While the addition of 100 μL of 0.1 M HCl resulted in reduced absorbance values and the linearity remained above 0.99 ($R^2 = 0.9901$), addition of 100 μL of 0.2 M HCl seemed to slightly improve the linearity ($R^2 = 0.9911$).

To further refine the method, increasing concentrations of Fast Blue BB salt were studied (0.25% (w/v) and 0.5% (w/v)). Increasing concentrations of FBBB improved linearity and, therefore, permitted the extension of the concentration range of phloroglucinol for quantification. The increment of the FBBB into the reaction mixture was conducted by adding increasing concentrations of the dye or adding more volume to the reaction. Addition of 400 μL of 0.25% FBBB resulted into higher absorbance values and yielded a better correlation factor ($R^2 = 0.9901$) when compared to the addition of 200 μL of 0.5% FBBB ($R^2 = 0.9883$). This may be due to the solubility limitations of the reagents. As concentrations increased solubility decreased, meaning that to introduce the mass of dye and phloroglucinol desired in the reaction, it is preferable to increase the reaction volume to improve solubilisation of the reagents and consequently the reaction conditions.

Fast Blue BB dissolved in water and ethanol (70% (v/v)) provided similar results, either ethanol (70% (v/v)) ($R^2 = 0.9953$) or distilled water ($R^2 = 0.9985$) could be used as solvent as the absorbance values obtained at 420 or 450 nm were linear and both were used for the later sample analysis. The best linearity was obtained by mixing in a microplate 20 μL of sample or standard up to 0.2 mg/mL (in either water or 70% (v/v) EtOH) with 20 μL of 20 mM HCl, 30 μL of 0.25% (w/v) FBBB, 20 μL of 1% NaOH and 240 μL of solvent. The colour development for these standard curves can be observed in Fig. S3 of the supplementary material. These optimized conditions were tested to evaluate linearity over time after 30, 60 and 90 min and results can be observed in Fig. 3. It was observed that linearity decreased over time yielding the highest regression correlations after 30 min of incubation in darkness at 25 °C ($R^2_{(Water)} = 0.9967$ and $R^2_{(EtOH)} = 0.9932$).

3.2. Analysis of water extracts from Ascophyllum nodosum

Marine brown macroalgae (*Phaeophyta*) have been recognized as one of the main natural sources for biologically active polyphenols (Pádua, Rocha, Gargiulo, & Ramos, 2015; Vo & Kim, 2013). Among the most common methods for extraction of polyphenols (and other bioactive molecules such as polysaccharides) is the extraction using hydrophilic solvents being water and ethanol two of the most common (Jacobsen, Sørensen, Holdt, Akoh, & Hermund, 2019). Solvent extraction methods are usually companied by the direct quantification of phenols via Folin-Ciocalteau (or modifications of this method) (Sardari, Prothmann, Gregersen, Turner, & Karlsson, 2021) and therefore, assessing the consistency of the FBBB method in solvent extracted samples is critical for the implementation of the FBBB method. The total phenolic content in aqueous and ethanolic extracts from *A.nodosum* was assessed aiming to test the consistency of the proposed method based on the coupling property of the FBBB salt with phloroglucinol to form tri-azo compounds that have a peak of absorbance at 450 nm. The results from the analysis of the water extracts from *Ascophyllum nodosum* as per the conditions indicated in Table 1 are presented in Table 2. The seaweed extracts with highest phenolic content were those obtained using two parts of water

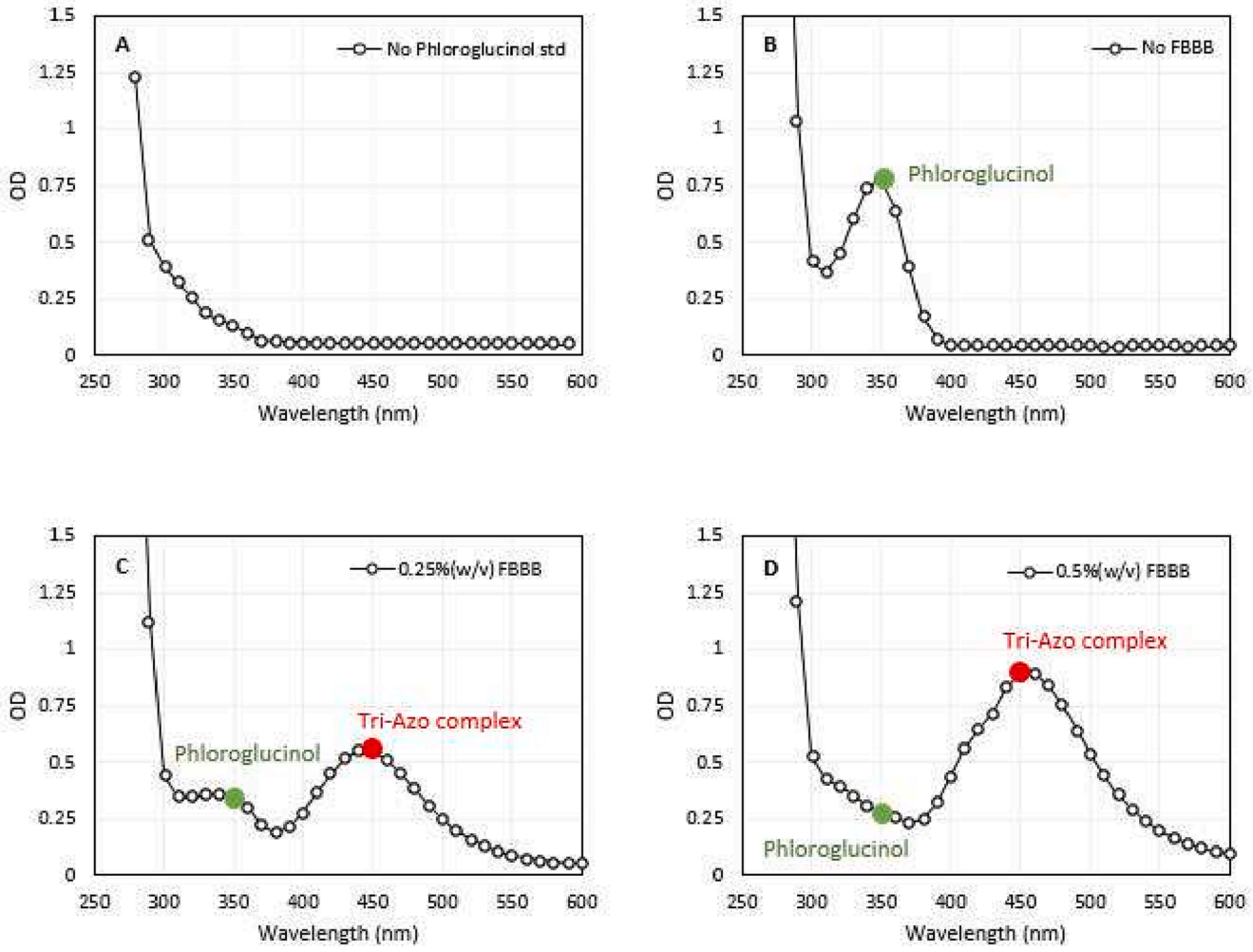

Fig. 2. **(A)** Spectroscan of the reagent blank reaction mixture (0 mg/ml of PG, 0.5% FBBB and 5% (w/v) NaOH). **(B)** Spectroscan of the phloroglucinol standard blank mixture (0.1 mg/ml of PG and 5% (w/v) NaOH). **(C)** Spectroscan of the reaction mixture (0.1 mg/ml of PG and 0.25% FBBB) indicating the peak associated with the formation of the tri-azo complex at 450 nm (●) and the peak associated with the PG that did not react (●) at 350 nm. **(D)** Spectroscan of the reaction mixture of 0.1 mg/ml of phloroglucinol and 0.5% FBBB indicating that the peak associated with the formation of the tri-azo complex at 450 nm (●) and the peak associated to the remaining PG (●) at 350 nm.

extract to one part of wet seaweed (H1, H3, H5 and H7) using either hot or cold water extractions. It has been reported that highest extraction yields are achieved for greater seaweed to extractant ratios, which is, at the same time beneficial to reduce operational volumes, for increasing temperatures (but not higher than 50 °C-60 °C), as well as for longest extraction processes (Boi et al., 2016; Sardari et al., 2021). The extraction time seemed to be determinant for the extraction of phenolics using water as extractant. The seaweed extracts obtained after 6 h (H5 to H8) were richest in phenolics ranging from 14.45 to 20.94 g PGE/100 g dry extract using the FBBB method (C) and from 14.54 to 24.69 g PGE/100 g dry extract using the F-C method. The ratios of Folin-Ciocalteu to Fast Blue BB were 1.0–1.4 for dilutions of 1:25 and 1.2–1.7 for dilutions of 1:50. This resulted in an overestimation of the phenolic content of at least 20% when using the F-C method instead of the FBBB.

Ratios of FC/FBBB higher than one indicates that the phenolic quantification using the Folin-Ciocalteu method is accounting not only for the phenols but also for other reducing agents. This ratio seemed to increase when higher dilutions were performed as the effect caused by the existing interferences as well as the background readings were magnified. Seaweeds are widely reported to be a rich source of antioxidants (Gómez-Mascaraque et al., 2021; Ismail, Gheda, Abo-Shady, & Abdel-Karim, 2020), and although this bioactivity is mainly attributed to the phenolic content (Baldrick et al., 2018; Chandini, Kumar, & Bhaskar, 2008; Guendouze-Bouchefa et al., 2015), brown seaweeds are rich in fucoidan which have also been reported to display antioxidant properties (Abu et al., 2013; Imbs, Skriptsova, & Zvyagintseva, 2014). Fucoidans are polysaccharides that mainly consist of a linear or branched backbone of α-(1–3) or alternating α-(1–3) with α-(1–4)-linked L-fucopyranoside subunits that can be mono- or di- sulphated or acetylated, and can also contain mannose, xylose, galactose, glucose, and uronic acids (Ale & Meyer, 2013). Seaweeds are rich in reducing sugars as these are naturally present free in the seaweed matrix and some of them are attached to major polysaccharide units. These two components are expected to contribute to higher Folin-Ciocalteu values. Ratios above 1 indicate the presence of a higher concentration of non-phenolic reducing constituents, as these are also detected by the Folin-Ciocalteu method and contribute to the overestimation.

3.3. Analysis of ethanolic extracts from Ascophyllum nodosum

The ethanolic seaweed extracts were analysed directly after extraction and centrifugation. The polyphenols extracted and retained in the ethanolic supernatant were directly quantified using both the Fast Blue BB (C) and Folin-Ciocalteu Methods. The conditions under which the ethanolic seaweed extracts were produced are summarized in Table 1.

The results from the phenolic quantification by both the FBBB and F-

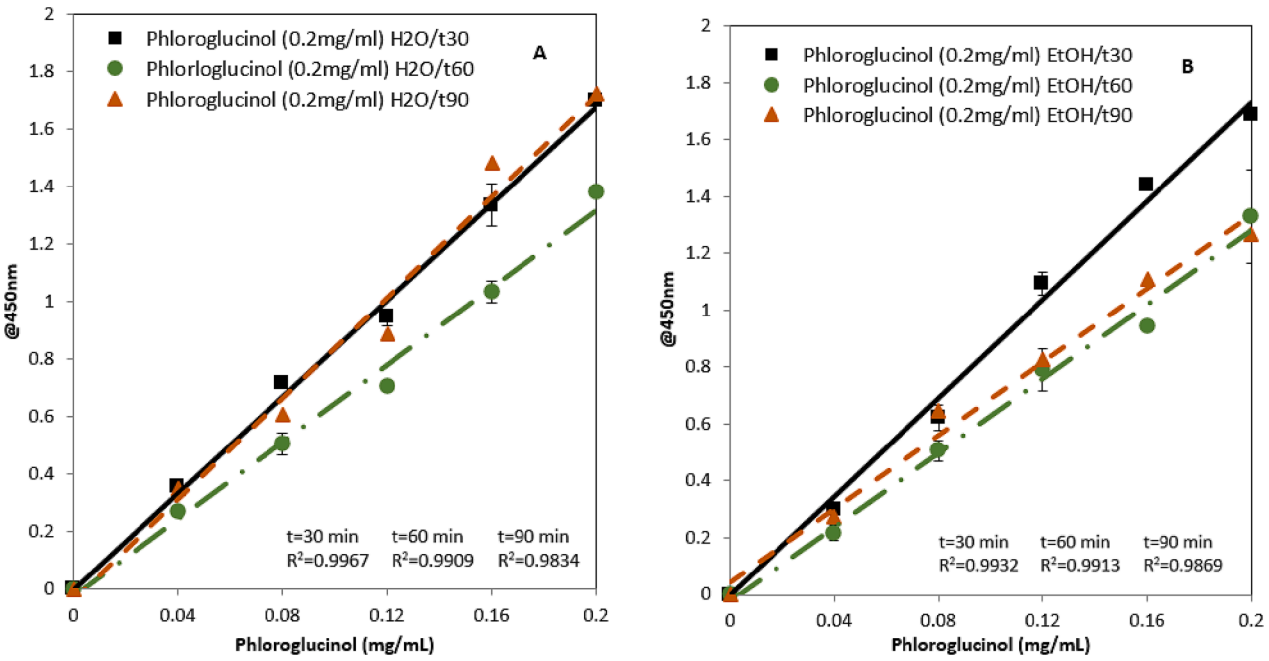

Fig. 3. Absorbance of the Fast Blue BB interaction with 0, 40, 80, 120, 160 and 200 μg/mL Phloroglucinol using the Fast Blue BB Method C in deionized water **(A)** and 70%(v/v) ethanol **(B)** at 450 nm after 30, 60 and 90 min of reaction time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Total Polyphenolic Content of water extracts from *Ascophyllum nodosum* measured by Fast Blue BB (FBBB) and Folin-Ciocalteu (F-C) Methods [a]

Extract from Ascophyllum nodosum in distilled water	Dilution	FBBB method (g of PGE/ 100 g dry extract)	±SD	F-C method (g of PGE/ 100 g dry extract)	±SD	Ratio FC/ FBBB
Sample H1	1:25	18.48	1.04	22.86	0.44	1.2
	1:50	15.93	1.50	25.21	2.10	1.6
Sample H2	1:25	13.24	1.18	16.62	0.54	1.3
	1:50	13.78	1.18	18.95	1.22	1.4
Sample H3	1:25	15.86	1.33	21.42	0.34	1.4
	1:50	14.39	0.50	24.34	0.46	1.7
Sample H4	1:25	13.23	1.10	17.99	0.43	1.4
	1:50	12.12	0.23	18.88	0.76	1.6
Sample H5	1:25	20.94	0.89	21.39	0.48	1.0
	1:50	18.79	0.45	21.84	0.94	1.2
Sample H6	1:25	14.45	1.39	14.54	0.74	1.0
	1:50	14.44	2.12	21.71	0.37	1.5
Sample H7	1:25	18.77	0.94	24.69	0.86	1.3
	1:50	16.20	0.89	26.88	0.61	1.7
Sample H8	1:25	19.89	0.74	19.70	0.57	1.0
	1:50	16.16	0.29	20.63	0.33	1.3
Sample H9	1:25	17.14	0.65	21.45	0.43	1.3
	1:50	17.19	2.38	23.03	1.42	1.3

[a] Polyphenols measured as PGE per 100 g of dry extract. Each value was analysed at least in triplicate (n = 3).

C methods of the ethanolic extracts from *Ascophyllum nodosum* are presented in Table 3. The results obtained by the FBBB method (dilution 1:5) showed that the shorter extraction time (1 h) at both low (25 °C) and high (70 °C) temperatures yielded a higher phenolic content for the extractions that used the highest ratio of extractant (70% (v/v) EtOH) to seaweed. Among the hot extracts, those that yielded the highest content in polyphenols were E4 (590 (mg of PGE/100 g dry seaweed) and E8 (554 (mg of PGE/100 g dry seaweed). This, again, demonstrated that a higher E:S (extractant to seaweed) ratio seemed to have a stronger relevance than the extraction duration in the phenolic extraction yield maximization. When compared, the phenolic content of the ethanolic extractions using cold temperature for 6 h (E5 and E6) was similar; E5 yielded 503 and E6 produced 511(mg of PGE/100 g dry seaweed, respectively. The phenolics extracted for just 1 h at a cold temperature (25 °C) with a ratio extractant to seaweed of 2:1 (E1) was the one that yielded a smaller phenolic content (297 mg of PGE/100 g dry seaweed).

Nevertheless, results obtained by the F-C method (dilution 1:5) showed that the extractions E2 (992 (mg of PGE/100 g dry seaweed), E4 (1060 (mg of PGE/100 g dry seaweed), E6 (980 (mg of PGE/100 g dry seaweed) and E8 (879 (mg of PGE/100 g dry seaweed) yielded the highest phenolic content meaning that the major phenolic extraction was obtained for ratios E:S of 5:1. Similarly to the results obtained using the FBBB method, the extraction that yielded the smaller phenolic content was E1 (667 (mg of PGE/100 g dry seaweed).

The ratios of Folin-Ciocalteu to Fast Blue BB were 1.3–1.5 for dilutions of 1:2, 1.5–2.3 for dilutions of 1:5 and 2.6–3.9 for dilutions of 1:10. This resulted in a minimum overestimation of the phenolic content that oscillated between 30% and 50% when using the F-C method instead of the FBBB. Ratios of FC/FBBB higher than one indicated that the phenolic quantification using the Folin-Ciocalteu method accounted not only for the phenols but also for other reducing agents. In this regard, high ratios may indicate that the concentration of the interference was probably similar to that of the polyphenols. The ratio seemed to increase when higher dilutions were performed as the effect caused by the existing interferences as well as the background readings were magnified after the application of the dilution factor.

Results reported in literature indicated that the total phenolic

Table 3
Total Polyphenolic Content of extracts from *Ascophyllum nodosum* produced using 70% (v/v) ethanol as extractant measured by Fast Blue BB (FBBB) and Folin-Ciocalteu (F-C) Methods [a]

Extract from *Ascophyllum nodosum* in 70% (v/v) EtOH	Dilution	FBBB method (mg of PGE/100 g dry seaweed)	±SD	F-C method (mg of PGE/100 g dry seaweed)	±SD	Ratio FC/ FBBB
Sample E1	1:2	327	17	447	30	1.4
	1:5	297	12	667	7	2.2
	1:10	263	51	724	85	2.8
Sample E2	1:2	502	96	808	183	1.6
	1:5	470	90	992	143	2.1
	1:10	305	104	1118	5	3.7
Sample E3	1:2	389	35	507	40	1.3
	1:5	357	85	798	134	2.3
	1:10	356	32	918	20	2.6
Sample E4	1:2	629	3	825	23	1.3
	1:5	590	13	1060	18	1.8
	1:10	315	13	1244	88	3.9
Sample E5	1:2	426	6	569	45	1.3
	1:5	503	27	877	10	1.7
	1:10	385	24	1037	53	2.7
Sample E6	1:2	530	16	766	7	1.4
	1:5	511	21	980	71	1.9
	1:10	293	1	1115	82	3.8
Sample E7	1:2	373	18	466	16	1.3
	1:5	379	4	718	7	1.9
	1:10	268	34	880	115	3.3
Sample E8	1:2	555	11	715	69	1.3
	1:5	554	35	879	52	1.5
	1:10	318	18	1088	181	3.4
Sample E9	1:2	501	2	734	10	1.5
	1:5	408	17	875	72	2.1
	1:10	294	13	925	159	3.1

[a] Polyphenols measured as PGE per 100 g of dry seaweed. Each value was analysed at least in triplicate (n = 3).

content and the antioxidant properties of brown seaweed extracts produced using mixtures of ethanol in water as extraction solvent, were higher if the concentration of ethanol used was increased and the extraction time and temperature were augmented (Boi et al. 2016). The ratio of solvent to seaweed seemed to be less significant when the extraction solvent aqueous ethanol. Some TPC results reported for extracts from *A.nodosum* obtained using aqueous ethanol are (80% (v/v) EtOH) 0.101 mg PGE/g (Tierney et al., 2013), (60% (v/v) EtOh) 0.3–1.0 mg PGE/100 mg (Parys et al., 2009). These data are in line with the results reported for the ethanolic algal extracts tested.

4. Conclusions

This study proposes an alternative to the commonly used Folin-Ciocateu method for the direct quantification of total phenolics in seaweeds that relies on the coupling property of the diazonium salt to form azo compounds with hydroxyl of phenols. Moreover, the interaction between phloroglucinol and the Fast Blue BB diazonium salt at alkaline pH to form tri-azo complex has been reported for the first time. The concentration of the Fast Blue BB, acid (HCl), alkali (NaOH) and reaction time were optimized to develop a three-step procedure, that mainly consists of transferring the samples to individual microplate wells, acidifying the samples, adding the Fast Blue BB diazonium compound, incorporating the alkali (NaOH) and measuring the absorbance at 450 nm after 30 min of incubation at 25 °C in darkness. An assay of 46 tests and a positive and negative control (4n) may be completed in less than 1 h at an estimated cost of 0.24€ per test (4n). Linear regression correlation values (R2) were ≥0.99 with phloroglucinol as standard. Additionally, direct quantification of TPCs (phloroglucinol equivalents, PGEs) in crude aqueous and ethanolic extracts from *A. nodosum* demonstrated that the new FBBB assay is not subject to side-redox interference and provides a more accurate estimate of TPC (1.2–3.9-fold lower than with the FC assay) in a relatively rapid and inexpensive microplate format. The proposed FBBB method will ensure a more consistent and accurate direct report of TPC in seaweed research and potentially in studies of other biomasses with relevant content of reducing interferences. The phenolic content and biochemical composition in seaweeds highly fluctuate depending on species, environmental conditions, harvesting season as well as on extraction and storage procedures. Therefore, the use of a direct method that allows a rapid, consistent, and affordable quantification of polyphenols is required to unify the results reported in seaweed research minimizing fluctuations associated to the method of quantification. The results of the present study shall pave the way to achieve this. Future studies may focus on the investigation of the optical properties of the Fast Blue BB interaction with different phenolics of interest and the identification of the phenolic-azo reaction products to better understand the possibilities of this technique.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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