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A simple 96-well microplate method for estimation of total polyphenol content in seaweeds

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Key words: seaweed, marine algae, total polyphenol, estimation, 96-well microplate

Abstract

Seaweed polyphenols are potent antioxidants and have also been shown to have α-glucosidase inhibiting activity. In our continuous efforts to develop new marine-based nutraceuticals and functional food ingredients, we have investigated many algal species collected on the Atlantic coast of Canada. A simple method for estimating the total polyphenol content in seaweeds and their extracts was developed based on the classic Folin-Ciocalteau colorimetric reaction. By using the 96-well microplate and a microplate reader, this new method saves experimental time, significantly reduces the amount of sample required, handles large number of samples in one experiment, and also improves the repeatability of the results.

A number of algal samples collected on the seashore of Nova Scotia, Canada, were analyzed for their levels of polyphenol content using this microplate-based method. The antioxidant activity of these samples was also assessed by using DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The results showed that there is a strong correlation between the total polyphenol content and the potency of antioxidant effect.

Introduction

Polyphenol has been emerging as one major category of natural products that is important to human health (Shahidi & Naczk, 2004; Frei, 1994). Increasing scientific evidence shows that polyphenols are good antioxidants, are effective in preventing cardiovascular and inflammatory diseases, and can also be used as chemopreventing agents for cancer. Some polyphenol products, such as the ones from tea and grape, are becoming popular in the market place. In addition to the polyphenols from terrestrial plants, seaweeds have been shown as another source of polyphenols with unique structural properties. Phlorotannin is one such special type of polyphenolic compound widely distributed in brown algae. It consists of 1,3,5-trihydroxybenzenoid, or phloroglucinol, as the structural unit, with a molecular size ranging from several thousands to tens of thousands for high molecular weight polyphloroglucinols. Its antioxidant activity is one of those most studied, and several species of brown algae such as *Sargassum kjellmanianum* (Yan et al., 1996; Yan et al., 1997; Wei & Xu, 2003), *Eisenia bicyclis* (Nakmura et al., 1996), Cystoseira sp. (Chkhikvishvili & Ramazanov, 2000), *Fucus sp.* (Jimenez-Escrig et al., 2001), and Ecklonia stolonifera (Kang et al., 2003a, 2004) have been reported with respect to free radical scavenging and the inhibition of total reactive oxygen species generation by phlorotannin compounds. More recently, polyphloroglucinols from brown algae were revealed to have bactericidal properties (Nagayama et al., 2002), hyaluronidase inhibition (Shibata et al., 2002), alpha-glucosidase inhibition (Kurihara et al., 2002), chemopreventive properties (Kang et al., 2003b), secretory phospholipase A2s, lipoxygenases, and cyclooxygenase inhibition (Shibata et al., 2003), and HIV-1 reverse transcriptase and protease inhibition effects (Ahn et al., 2004) as well as antioxidative, anti-inflammatory, alpha- and glucosidase inhibitory activities. Brown algal polyphenols have been investigated for their

potential functional applications in beverages and edible oils (Yan et al., 1998; Nagai & Yukimoto, 2003).

Among the several assays available to quantify total polyphenols, the Folin-Denis method (Folin & Denis, 1912) is one of the most commonly used. It is based on a color reaction between easily oxidized polyphenols or hydroxylated aromatic compounds and phosphotungsten-polymolybdic acid. It was later improved by Folin and Ciocalteu (1927) with an addition of lithium sulfate to the reagent to prevent precipitation in the reaction in order to increase the sensitivity. Now, Folin-Ciocalteu reagent is commercially available for polyphenol quantification and is found to be a preferred assay (Singleton et al., 1999). The two assays have been widely used in estimating the level of phlorotannin content in algal materials (Van Alstyne, 1995; Jimenez-Escrig et al., 2001).

Although the polyphenol quantification method is well established, there are still opportunities for improvements in terms of time for sample color intensity measurement, the amount of sample, regents and solvents required, and analysis and management of data. We present here a 96-well microplate Folin-Cioalteu assay for estimating polyphenol content in marine algae. This modified Folin-Cioalteu method incorporates the convenience of spectrometric measurement using 96-well microplate, so that it consumes much less reagents and solvents, and it runs more efficiently in a plate that handles more samples in small quantities.

Materials and methods

Chemicals and reagents: Folin-Ciocalteu reagent was purchased from Sigma (Catalogue # F-9252, 500 mL). Sodium carbonate anhydrous: Sigma, S-6139, 500 g, 99.9%. Phloroglucinol dihydrate was from Aldrich (P3, 800-5, 25 g, 97%).

Instrument: 96-Well microplate reader (Molecular Devices Spectra MAX 190, Sunnyvale, CA, U.S.A.).

Algal material: Marine macroalgae collected on the coast of Nova Scotia during 2000–2003 were analyzed. These included the brown algae *Alaria esculenta, Ascophyllum nodosum, Fucus distichus, F. evanescens, F. vesiculosus,* and *Laminaria saccharina*, the red alga *Polysiphonia stricta* and the green alga *Codium fragile.*

Standard solution: 10 mg phloroglucinol (calculated as anhydrous) was dissolved in 100 mL distilled water and used as a stock solution (100 μg mL^{-1}) to make serial dilutions and obtain the standard solution at the concentration of 100, 50, 25, 12.5, and 6.25 μg mL^{-1}.

Sample preparation: Sample solution was prepared from either dry algal powder or algal extract.

Algal powder: weigh out 0.5–1 g in a test tube, add 20 mL MeOH-water (1:1), adjust pH to 2 and shake for 1 h (150 rpm) at room temperature. Centrifuge at 12,000 $\times g$ for 10 min and recover the supernatant. The residue is then extracted with acetone-water (7:3) under the same conditions and centrifuged. The two liquid extracts are pooled together and mixed well. Take 100 μL of this solution to make a 1:10 dilution with water and use as the sample solution. If the absorbance of the final sample solution is not in the range of the standard curve, further dilution may be required.

Algal extract: Weigh out 1–5 mg of MeOH or water extracts of dry algae, add 1 mL MeOH or water, and agitated by vortex for 30 seconds or until the sample is thoroughly dissolved. The solution is centrifuged for 10 min at 12,000 $\times$ g. Take 100 μL of the supernatant and dilute with water (1:10, can be changed based on the absorbance of the final sample solution. If the absorbance is not in the range of the standard curve, make 2–10 times further dilution). Use this as the sample solution.

Measurement: Load 20 μL of each sample solution and the serial standard solution on a 96-well microplate (as shown in Figure 1). Add 100 μL Folin-Ciocalteu reagent, mix well and wait 5 min. Add 80 L of 7.5% sodium carbonate solution and mix well. Cover the plate and leave it in the dark at room temperature for 2 h. Measure absorbance at λ 750 nm with a spectrophotometric microplate reader (set auto mix for 60 s before reading).

Distilled water was used as a blank. A reagent blank was carried out using the same procedure and was also measured against the water blank. Each standard solution and sample solution was run in triplicate, and the latter was assayed against sample control (i.e., sample solution without Folin-Ciocalteu reagent and sodium carbonate).

Blank: 200 μL distilled water.

Standard: The concentrations of phloroglucinol (anhydrous) for serial dilutions were: Std01: 100 μg mL^{-1}; Std02: 50 μg mL^{-1}; Std03: 25 μg mL^{-1}; Std04: 12.5 μg mL^{-1}; Std05: 6.25 μg mL^{-1}; Std06: 0 μg mL^{-1}.

Sp1–16: Samples 1–16.

CSp1–16: Control of samples 1–16, i.e., samples without 80 μL 7.5% sodium carbonate and 100 μL Folin-Ciocalteu reagent but using 180 μL distilled water instead. This is used to measure the background absorbance caused by the sample solution.

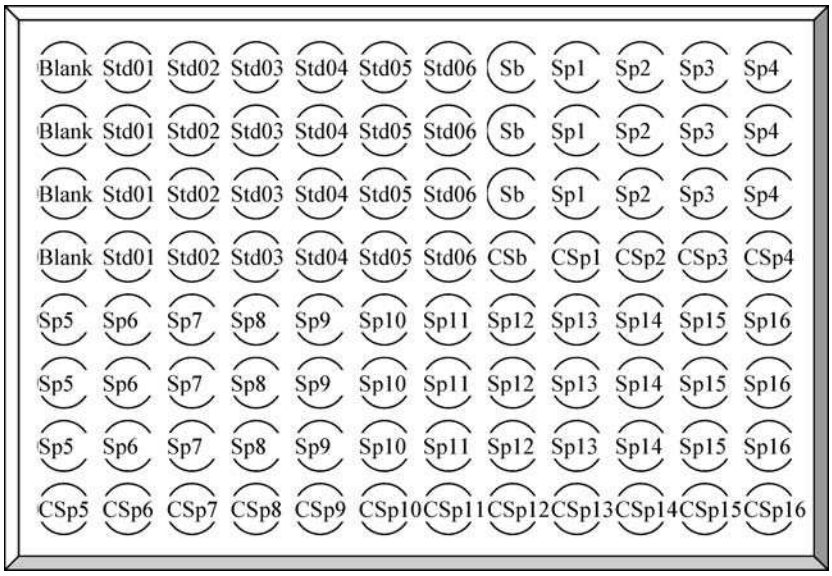

Figure 1. 96-well microplate template for standard phloroglucinol and seaweed sample solutions.

Sb: Sample blank or reagent blank. This is carried through the sample preparation process but without sample.

CSb: Control of sample blank. Sample blank without 80 μL 7.5% sodium carbonate and 100 μL Folin-Ciocalteu reagent while using 180 μL distilled water instead.

Calculation of total polyphenol content:

For algal powder: PGE% = {[(Norm × 40 × 10)÷ 1000] ÷ weight} × 100%

For algal extract: PGE% = {[(Norm × 1 × 10)÷ 1000] ÷ weight} × 100%

PGE: Phloroglucinol equivalents.

Norm: Mean result of sample-mean result of sample blank, where mean result of sample is the average of the triplicate results of each sample, and the result refers to the assay value obtained from the calibration curve (μg mL^{-1}).

Weight: Weight of algal powder or extract used (mg).

Please note that if additional dilution is made, Norm should be further multiplied by the dilution factor.

DPPH antioxidant assay: a 96-well microplate method (Lee et al., 1998; Fukumoto & Mazza, 2000) was used to measure the scavenging activity toward the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical.

Results and discussion

UV-Visible spectrum and standard curve: The absorbance in the range of 350–800 nm for Folin-Ciocalteu reagent and phloroglucinol standard solution, *A. nodosum* sample solution from combined MeOH-water (1:1, pH = 2) and acetone-water (7:3) extraction, and *A. nodosum* sample solution from a double MeOH-water (1:1, pH = 2) extraction were recorded and are shown in Figure 2. *A. nodosum* sample has a maximum absorbance at λ 750 nm, which is same as that of the phloroglucinol, so that it is appropriate to use the later as the standard for quantification of total polyphenol level. The UV-Visible spectrum of the combined acidic aqueous MeOH and aqueous acetone extract is basically the same as that of the extract obtained only with acidic aqueous MeOH, but the absorbance is higher. We therefore used the combined aqueous MeOH and acetone method to prepare sample solutions from algal samples.

The calibration curve of standard phloroglucinol solutions is shown in Figure 3. The curve is linear when the concentration of phloroglucinol is in the range of 0–100 μg mL^{-1} ($R \geq 0.998$).

Polyphenol content and antioxidant activity of some algal extracts from Atlantic Canada: The polyphenol content of *A. nodosum* powder and the MeOH, cold and hot water extracts of several other macroalgae from Atlantic Canada are shown in Table 1. Polyphenol content varies between extracts obtained by different solvents,

Table 1. Polyphenol content and antioxidant activity of some algae species from Atlantic Canada

Type of seaweed	Name	Powder or extract	Polyphenol content (PGE%)	Antioxidant potency* (EC_{50}, μg/mL)
Brown algae	*Alaria esculenta*	MeOH extract	9.58	ND
	(Linnaeus) Greville	Cold water extract	0.54	–
		Hot water extract	0.68	–
	Ascophyllum nodosum	Powder	5.26	ND
	(Linnaeus) Le Jolis	MeOH extract	38.95	9.96
		Cold water extract	14.80	33.90
		Hot water extract	12.36	29.97
	Fucus distichus	MeOH extract	30.40	10.32
	(Linnaeus)	Cold water extract	25.96	7.85
		Hot water extract	24.99	9.04
	Fucus evanescens	MeOH extract	23.85	19.43
	(C. Agardh)	Cold water extract	4.86	148.47
		Hot water extract	2.95	188.60
	Fucus vesiculosus	MeOH extract	23.21	ND
	(Linnaeus)	Cold water extract	10.84	37.47
		Hot water extract	12.51	32.97
	Laminaria saccharina	MeOH extract	2.17	–
	(Linnaeus)	Cold water extract	0.34	–
	J.V. Lamoroux	Hot water extract	0.44	–
Red algae	*Polysiphonia stricta*	MeOH extract	12.40	27.8
	(Dillwyn) Greville	Cold water extract	1.03	–
		Hot water extract	1.05	–
Green algae	*Codium fragile*	MeOH extract	1.15	–
	spp *tormentosoides*	Cold water extract	0.53	–
	(van Goor) P.C. Silva	Hot water extract	0.46	–

*BHA was used as a reference, EC_{50}=2.14–4.2 μg mL^{-1}; ND: not determined; "–": no activity.

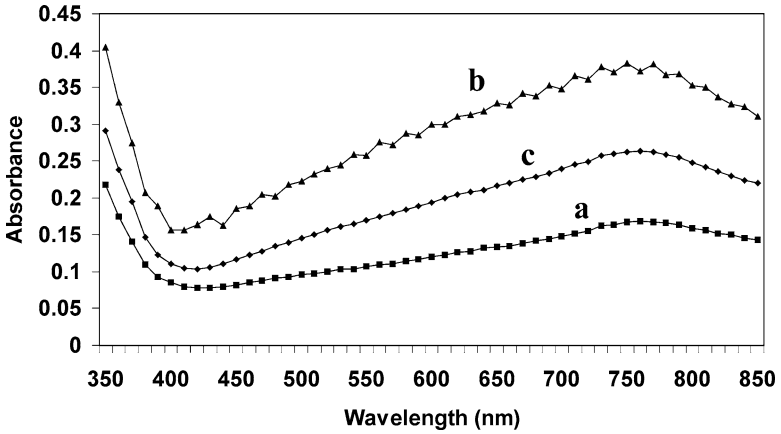

Figure 2. UV-visible spectra for phloroglucinol and *Ascophyllum nodosum* extract solutions. **a**: phloroglucinol; **b**: *A. nodosum* acidic MeOH-water (1:1, pH 2) and acetone-water (7:3) extract; **c**: *A. nodosum* acidic MeOh-water (1:1, pH 2).

such as MeOH, cold water and hot water. For most algal polyphenols, MeOH or aqueous alcohols are the preferred solvents for extraction. Solubility of polyphenols in water is fairly low, and more water-soluble polysaccharides or other components are extracted as well. If seaweed material, not its extract, is the target of analysis we would recommend acidic MeOH-water and acetone-water as the solvents for extraction

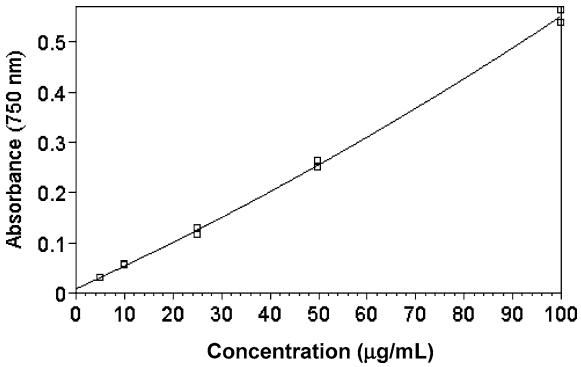

Figure 3. Phloroglucinol standard calibration curve (concentration within 0–100 μg mL^{-1}).

as described above; acidic aqueous MeOH and acetone are more powerful in recovering polyphenolics than the neutral solvents. The DPPH radical scavenging activities of those extracts indicate a good correlation between polyphenol content and antioxidant activity.

Absolute polyphenol content: Due to the complexity of the chemical nature of phenolic components in seaweeds, no method is regarded to be a perfect approach to determine the total level of phenolic compounds. However, some studies on brown algae species, such as *A. nodosum* and *F. vesiculosus*, indicate that by employing a conversion factor (or estimation factor, EF) obtained by using gravimetric methods, one can calculate the absolute polyphenol content by multiplying the EFs with the relative content value from Folin-Denis colorimetric determination (Ragan & Jensen, 1977 and 1978). This approach gives a relatively accurate and absolute value, and makes possible direct comparisons between different algal species. However, EF is sample dependent and varies to some degree according to species, collection time, and location. Determination of EFs is also tedious and time consuming. Using the phloroglucinol equivalents obtained from the Folin-Ciocalteu colorimetric method as a relative determination of polyphenol level appears to be practical for routine QC purpose and is reasonably reliable.

Bioassay directed fractionation and structure characterization of the bioactive polyphenols are in progress.

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