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Identification and efficient extraction method of phlorotannins from the brown seaweed *Macrocystis pyrifera* using an orthogonal experimental design

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ABSTRACT

The brown seaweed contains a type polyphenol compound characteristic of its species, the phlorotannins, which are produced from the polymerization of phloroglucinol units. They have been extensively studied due to their pharmacological and nutraceutical properties, but there is still a need for an optimized extraction protocol. In this study, the brown seaweed *Macrocystis pyrifera* was employed to determine the best conditions for extraction of phlorotannins. A set of different variables were evaluated such as the use of pre-treatment, type of solvent, drying temperature, particle size, temperature and extraction time as well as the solid/liquid ratio upon extraction. The optimal conditions for the extraction of phlorotannins were: pre-treatment with hexane, extraction with water, drying temperature 40 °C, particle size below 1.4 mm, at 55 °C for 4 h and a solid-to-liquid ratio of 1:15. Under these conditions, the concentration of phlorotannins achieved in the extract was 200.5 ± 5.6 mg gallic acid equivalent (GAE)/100 g dry seaweed (DS) and total antioxidant activity of the extract of 38.4 ± 2.9 mg trolox equivalent (TE)/100 g DS. Further, it was possible to identify two phlorotannins through HPLC-ESI-MS analyses: phloroeckol and a tetrameric phloroglucinol. These phlorotannins have been reported in the literature to have an antidiabetic effect and prevention of Alzheimer's disease for phloroeckol, and free radical scavenging ability and antiallergic effect for tetrameric phloroglucinol. Therefore, the extract of phlorotannins has potential as medicinal foods or therapeutics for human health applications.
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1. Introduction

In recent decades, the quest to identify new natural products with antioxidant potential has been increasing as several studies have recognized synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), as substrates with potential health hazards [1,2]. Consequently, natural antioxidants of marine origin have been the subject of intense research, and new substances with pharmacological and nutraceutical properties have been identified [3]. Interestingly, seaweed is one of the main sources of these compounds [4,5].

The macroalgae that occupy the littoral zone can be classified as red (*Rhodophyta*), brown (*Phaeophyta*) or green (*Chlorophyta*) types, depending on pigment present and chemical composition [6,7]. The brown algae contain a substantial amount of soluble carbohydrates and they completely lack lignin, which is important when aiming for an extraction process. Also, they contain unique compounds known as phlorotannins [8,9]. These compounds are polyphenols produced by seaweed as secondary metabolites and biosynthesized via the acetate malonate pathway [10]. Further, they are derived from the polymerization of phloroglucinol units, which constitute up to 15% of the dry weight of the seaweeds [11,12]. Depending on the types of interlinkage present, phlorotannins can be classified into various subclasses, i.e., phlorotannins with phenyl linkages, ether linkages or ether and phenyl linkages that are characteristics for different types of phlorotannins [13,14].

The phlorotannins are present in the algae in soluble form, stored in physodes, and insoluble form forming part of the structure of the cell walls of algae, forming complexes with proteins and alginic acid [15]. Concentration and molecular size of these compounds vary according to intrinsic factors (reproductive condition, age and size of the algae), as well as by extrinsic factors (environmental and ecological stimuli) [15–17]. The molecular size of phlorotannins reported varies between 126 Da (phloroglucinol) and 650 kDa (phlorofucoroeckol) [18] and has been extensively studied due to their pharmacological and nutraceutical properties exhibiting antioxidative, antiangiogenic, antiallergic, anti-inflammatory, and antidiabetic effects [15–23].

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The extraction of phlorotannins has been performed from different brown seaweeds (*Himanthalia elongata*, *Stypocaulon scoparium*, *Ascophyllum nodosum*, *Ecklonia stolonifera*, *Fucus vesiculosus* between others). Also, the influence of temperature, drying time and nature of the extractant as well as the quality of antioxidant compounds, phlorotannins, has been studied [16–18,24–27]. However, there is only limited information on the extraction of phlorotannins from *Macrocystis pyrifera* [28,29]. This brown seaweed, *M. pyrifera* is abundantly present in the Pacific Ocean [8,29,30] and is very abundant in the coastal areas of Chile. *M. pyrifera* is an alga with rapid growth and it is used as feed source in the abalone industry as well as a thickening agent in the cosmetics and food industries. Consequently, the development of extraction conditions that permit successful extraction of phlorotannins from *M. pyrifera* is of potential industrial interest.

Further, the identification of the different phlorotannins extracted is of considerable interest for the industry. In this respect, the main objectives of this study were to determine the effect of extraction conditions: (1) use of pre-treatment, (2) type of solvent, (3) drying temperature of algae and (4) extraction parameters (particle size, time and temperature of extraction and solid/liquid ratio) on total phlorotannin concentration (TPC) and total antioxidant activity (TAA) from *M. pyrifera* as well as the identification of the various phlorotannins extracted.

2. Materials and methods

2.1. Algal materials

M. pyrifera were collected by scuba diving 30 km southwest of Puerto Montt, Chile. The samples were harvested in November 2011 (M1), March 2013 (M2) and June 2013 (M3). The chemical analyses of *M. pyrifera* samples were carried out in order to determine its initial composition in terms of protein, lipids, carbohydrates and ash elements. The analyses were done at the Institute of Agroindustry, University of the Frontera, Chile. The moisture content as well as protein, lipid, ash and fiber contents were quantified following the official methods of the Association of Official Analytical Chemistry (AOAC): 930.04, 978.04, 991.36, 930.05 and 962.09, respectively [31]. The carbohydrate content was calculated from the difference in the initial mass and the sum of values reported for proteins, humidity, lipids and ash [32].

2.2. Effect of the extraction solvent on TPC and TAA

Prior to obtaining extracts, the dry seaweed (M1 dried at room temperature) was pretreated according to Koivikko, et al. [27]. The pretreatment consisted of washing the alga with hexane (solid/liquid ratio of 1:5 w/v) three (3) times to remove pigments and lipids compound. Additionally, 5.0 g of dry algae washed with hexane was dispersed separately in 50 ml of different solvents (methanol, ethanol, water, water/methanol 50:50, hexane/ethanol 88:12, ethanol/water 25:75 or 80:20, ethyl acetate/water 50:50, water/acetone 20:80 or 30:70 and methanol/chloroform 66:33% v/v) [27–30,33–35], and incubated in a platform at 200 rpm and 40 °C for 2 h. The mixture was centrifuged at 2000 rpm at 4 °C for 20 min and supernatant was stored at −20 °C before further analysis for the quantification of TPC and TAA values.

2.3. Effect of drying temperature on TPC and TAA

The best drying conditions were determined using the fresh algae (M2) dried at four temperatures 30, 40, 50 and 60 °C [25]. The experiments were performed in a horizontal dryer with hot air flow (Tray drier, Armfield UOP8) at an air flow speed of 1.3 m/s. Once the samples were dried, they were milled (1.4–2 mm) and stored at 4 °C prior extraction.

As the next step, 0.5 g of alga (dried and pretreated) was placed in test tube containing distilled water at a solid/liquid ratio of 1/10 and incubated in a platform shaker at 200 rpm and 40 °C for 2 h. The sample was centrifuged at 7000 rpm for 10 min at 4 °C and the supernatant was stored for further analyses. The best drying temperature was determined in terms of highest TPC and TAA obtained.

2.4. Effect of extraction parameters on TPC and TAA using Taguchi orthogonal array

The different extraction variables, namely extraction time, extraction temperature, solid/liquid (S/L) ratio and algae particle size were simultaneously evaluated using Taguchi orthogonal array L_9, 3^4 where 9 is the number of experiments, 4 is the number of factors tested and 3 is the number of work levels [36]. Seaweed M3 was previously dried at 40 °C, washed with hexane and water was used as extractant. Further, Table 2 demonstrates the different variables evaluated and describes the conditions chosen for the different experimental runs. The best extraction conditions were determined in terms of the highest TPC and TAA values measured after the extraction.

2.5. Determination of TPC

The amount of total phlorotannins compounds in extracts was determined according to the Folin–Ciocalteu assay [37] adapted to 96-well plates. Standards containing gallic acid with a concentration varying from 20 to 100 mg/l were prepared to measure the amount of phlorotannins in the extracts. Samples and standard (20 μl) were introduced separately into 96-well plates each containing 100 μl of Folin–Ciocalteu's reagent diluted with water (10 times) and 80 μl of sodium carbonate (7.5% w/v). The plates were mixed and incubated at 45 °C for 15 min. The absorbance was measured at 765 nm using a UV–visible spectrophotometer (Asys, UVM 340). The phlorotannin concentration was determined by the regression equation of the calibration curve expressed as gallic acid (GAE) mg equivalent/100 g dry seaweed (DS).

2.6. Determination of DPPH radical scavenging activity, total antioxidant activity (TAA)

The free radical scavenging activity was measured using the modified method of von Gadow et al., [38]. Consequently, 40 μl of 0.4 M 1,1-diphenyl-2-picryl-hydrazyl (DPPH) solution in ethanol was added to 50 μl of the sample solution, complemented with 110 μl of ethanol. The plates were mixed and allowed to stand for 30 min in the absence of UV light to avoid decomposition. The absorbance was measured at 520 nm against an ethanol blank. Calibration curves of Trolox (0–24 mg/l) were prepared and the results were expressed as the number of equivalents of Trolox (mg TE/100 g DS).

2.7. Characterization of phlorotannin extract

The composition of the phlorotannin extract obtained under the best extraction conditions was characterized and the type of phlorotannin present was identified as follows.

2.7.1. Fourier transform infrared spectroscopy — FTIR

The sample was pre-fractioned with methanol in order to induce precipitation of the co-extracted carbohydrates (methanol was chosen among different solvents tested, not shown), concentrated in rotary evaporator at 40 °C and pressure bellow 0.1 mbar and finally lyophilized at −50 °C under vacuum (below 0.1 mbar) overnight.

The IR spectra obtained for the sample and phloroglucinol were recorded on an ATI Mattson Infinity Series IR spectrometer at room temperature. The samples were freeze-dried before the FTIR analyzes and blended in a 3% w/w ratio with potassium bromide (KBr) powder,

Table 1
Chemical analysis of *Macrocystis pyrifera* harvested during different periods.

Proximal analysis	Unit	*M. pyrifera* M1 (Nov 2011)		*M. pyrifera* M2 (March 2013)		*M. pyrifera* M3 (Jun 2013)	
		Wet algae	Dry basis	Wet algae	Dry basis	Wet algae	Dry basis
Humidity	%	10.18	–	17.61	–	15.99	–
Protein	%	10.01	11.15	8.52	10.34	9.59	11.42
Ash	%	36.16	40.26	21.50	26.10	25.16	29.95
Lipids	%	1.03	1.15	1.17	1.42	5.42	6.45
Fiber	%	17.45	19.43	6.53	7.93	7.62	9.07
NNE[a]	%	25.16	28.01	44.67	54.22	36.22	43.11
Carbohydrates[b]	%	42.61	47.44	51.20	62.14	43.84	52.18

[a] NNE, no nitrogen elements which was calculated as 100−(Fiber + Lipids + Protein + ash).
[b] Carbohydrate amount was calculated as 100−(Humidity + Protein + Ash + Lipids).

followed by tableting (10 tons for 1 min) before measurement. A region of 4,000–400 cm^{-1} was used for scanning.

2.7.2. Carbohydrate analysis

Carbohydrates in the obtained phlorotannin extract were quantified with an acid methanolysis method followed by sample silylation and GC analysis as follows: a volume of 2 ml of methanolysis reagent containing 2 M of hydrochloric acid (HCl) in methanol was added to 10 mg of freeze dried algae samples and a calibration solution containing known amounts of carbohydrates. As the next step, the tubes were inserted into an oven operating at 100 °C for 3 h. Once the reaction was completed, 200 μl of pyridine was added to neutralize any excess of HCl, and 2 ml of each internal standard solution containing 0.1 mg/ml of sorbitol and resorcinol in methanol, respectively, was added to each sample. After mixing, methanol was evaporated at 50 °C under nitrogen stream and the sample was further dried under vacuum (Heraeus VTR 5022) at 42 °C below 50 mbar for 20 min prior silylation of the samples [39]. Once the samples were completely dry, silylation was commenced by adding 150 μl of pyridine, 150 μl of hexamethyldisilazane (HMDS) and 70 μl of chlorotrimethylsilane (TMCS), followed by a thorough mixing using a high-sheer vortex mixer. Further, the samples were kept in an oven at 70 °C for 45 min and the clear liquid phase was analyzed in order to determine the carbohydrate content of the samples by gas chromatography [39]. Consequently, about 1 μl of a silylated sample was injected via a split injector (260 °C, split ratio 1:15) into a 30 m/0.32 mm i.d. column coated with dimethyl polysiloxane (HP-1, Hewlett Packard) with the film thickness of 0.17 μm. The column temperature program was as follows: a temperature ramp from 100 to 175 °C (4 °C/min), followed by a ramp of 175 to 290 °C (12 °C/min). The detector (FID) temperature was 290 °C. Hydrogen was used as a carrier gas.

2.7.3. High Precision Liquid Chromatography Mass Spectrometry (HPLC-ESI-MS/MS) identification

A volume of 14 ml of extract phlorotannins obtained under the best extraction condition was concentrated at room temperature in a vacuum concentrator (SpeedVac, Savant Instruments Inc., NY-USA). Consequently, the concentrated extract was re-suspended in 4.5 ml of water and a volume of 20 μl was analyzed using a LC-ESI-MS/MS system which consisted of the HP1100 liquid chromatography (Algilent Technologies Inc., CA-USA) connected to the mass spectrophotometer (Esquire 4000 ESI-Ion Trap LC/MS(n) system, Bruker Daltonik GmbH, Germany). A Luna C18 150 × 4.6 mm, 5 μm and 100 Å analytical column (Phenomenex Inc., CA-USA) was used in the analysis; at the exit of the column a split divided the eluent for simultaneous UV and mass spectrometry detection. The mobile phase used was 1% v/v formic acid in water deionized (solvent A) and acetonitrile (solvent B), fed at a flow rate 1 ml/min according to the following elution gradient: 0–15 min, 5% B; 15–75 min, 5–100% B; 75–85 min, 100% B and 85–90 min, 100–5% B [40]. The detection wavelength was set to 280 nm. The mass spectral data were acquired in positive and negative modes; ionization was performed at 3000 V assisted by nitrogen as nebulising gas at 45 psi, drying gas at 345 °C and flow rate 10 l/min. All scans were performed in the range 20–2200 m/z. The trap parameters were set in ion charge control using manufacturer's default parameters. The collision induced dissociation (CID) was performed by collisions with the helium

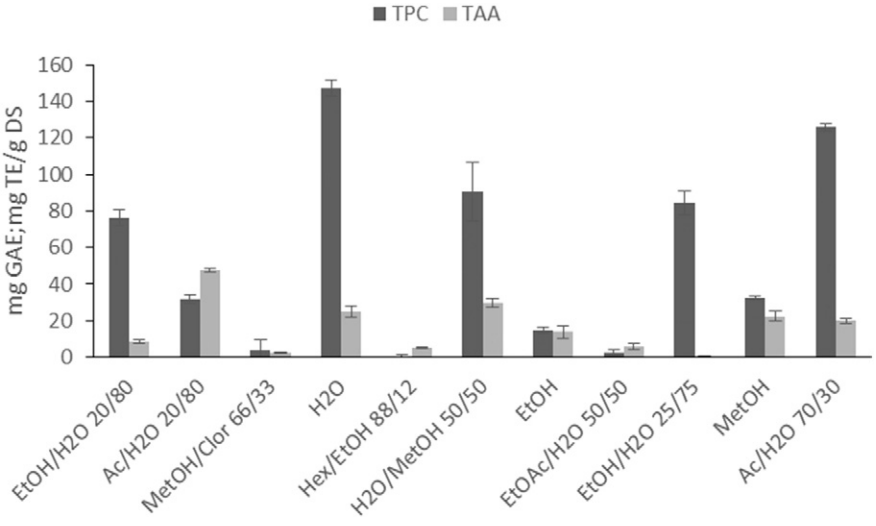

Fig 1. Evaluation of the best extractant for total polyphenols (TPC) and total antioxidant activity (TAA) in *Macrocystis pyrifera*. Ac, acetone.

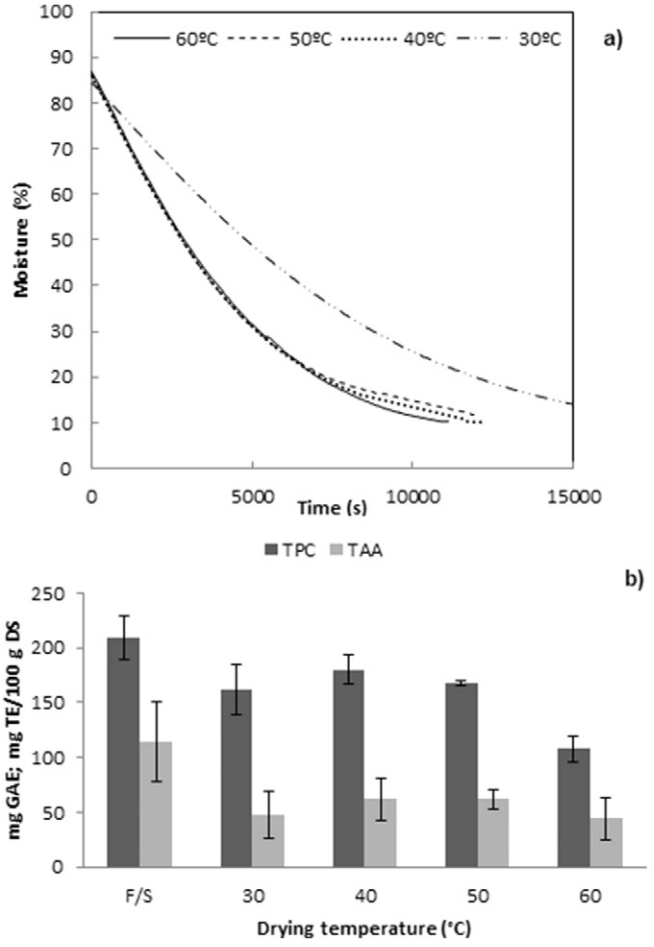

Fig 2. a) Drying curves for *Macrocystis pyrifera* at different drying temperatures, b) evaluation of the different drying temperatures for *Macrocystis pyrifera* in terms of total concentration polyphenols (TPC) and total antioxidant activity (TAA). F/S denotes fresh seaweed.

background gas present in the trap and automatically controlled trough SmartFrag option.

2.8. Statistical analysis

All extracts and fractions were analyzed in triplicate and the measurements were presented as average ± standard deviation. To evaluate the effect of the extraction solvent and drying temperature of phlorotannins from *M. pyrifera* were selected the best condition used as criteria higher-the-better for TPC and TAA.

Table 3
Variance analysis (ANOVA) for the total concentration of phlorotannins (TPC) and the total antioxidant activity (TAA) for *Macrocystis pyrifera*.

Response	Factors	DOF[a]	Sums of squares	Variance	F-ratio	Pure sum	%
TPC	Time	2	9636.9	4818.4	39.1	9390.3	21.7
	Temperature	2	6137.3	3068.7	24.9	5890.8	13.6
	S/L[b] ratio	2	12,960.9	6480.5	52.6	12,714.4	29.4
	Particle size	2	12,266.2	6133.1	49.8	12,019.6	27.8
	Other/error	18	2218.8	123.3	–	3204.9	7.4
	Total	26	43,220.1	–	–	43,220.1	100
TAA	Time	2	830.8	415.4	23.8	795.9	18.5
	Temperature	2	242.4	121.2	6.9	207.5	4.8
	S/L[b] ratio	2	2490.4	1245.2	71.4	2455.5	57.0
	Particle size	2	433.2	216.6	12.4	398.3	9.2
	Other/error	18	314.0	17.4	–	453.5	10.5
	Total	26	4310.8	–	–	4310.8	100

[a] DOF, degree of freedom.
[b] S/L ratio, solid/liquid ratio of extraction.

The significance and relative influence of each extraction parameter in TPC and TAA using the orthogonal Taguchi array were determined using the ANOVA variance analysis with a 5% confidence level.

3. Results and discussion

3.1. Raw material analysis

The chemical analysis of *M. pyrifera* indicated that the ash content was higher in the batch M1 (40.26% w/w), whereas the lipid content was higher in the batch M3 (6.45% w/w) and the carbohydrate content was highest in the batch M2 (62.14% w/w, Table 1). According to the literature [41], alginate–phenol linkages play an essential role in the brown algal cell wall structure and it is expected that this correlates with high phlorotannin concentration in the brown alga. On the other hand, the differences observed in the lipid and carbohydrate content, for the alga batches harvested in November and March, could be attributed to environmental factors associated with the season, such as, water temperature, 12.5 and 13.5 °C on average, respectively [42], and the level of UV radiation, UV-B 0.5 and 4 W/m^2 on average, respectively [43].

3.2. Selection of operational conditions: effect of the extractant type and drying temperature

The TPC and TAA levels obtained using different solvents are depicted in Fig. 1. The two highest concentrations of TPC were attained using water and 70% v/v acetone as extraction solvents, giving 147 ± 2.9 and 125 ± 1.5 mg GAE/100 g DS, respectively. On the other hand, the highest TAA values were attained for 80% v/v acetone and water, 25 ± 2.0 and 20.2 ± 4.2 mg TE/100 g DS, respectively. The better yields of TAA in the case of aqueous acetone may be due to the fact that

Table 2
The influence of extraction parameters determined by Taguchi orthogonal array design L_9, 3^4 on TPC and TAA from the extraction of *Macrocystis pyrifera*.

Runs	Time [h]	Temperature [°C]	S/L[a] ratio	Particle size [mm]	TPC [mg GAE/100 g DS]	TAA [mg TE/100 g DS]
1	2	25	1/10	<1.4	120.8 ± 11.3	42.7 ± 0.4
2	2	40	1/15	2–1.4	58.5 ± 4.0	29.1 ± 8.4
3	2	55	1/20	>2	39.5 ± 3.1	14.4 ± 4.2
4	3	25	1/15	>2	31.4 ± 18.1	27.3 ± 4.3
5	3	40	1/20	<1.4	47.2 ± 5.9	42.0 ± 3.2
6	3	55	1/10	2–1.4	136.9 ± 20.8	55.6 ± 1.9
7	4	25	1/20	2–1.4	100.0 ± 6.3	20.8 ± 2.0
8	4	40	1/10	>2	89.1 ± 9.3	46.1 ± 4.8
9	**4**	**55**	**1/15**	**<1.4**	**200.5 ± 5.6**	**38.4 ± 2.9**

Bold values in this table highlights the best conditions of TPC and TAA extraction.
[a] S/L, solid to liquid ratio.

Table 4
Condition giving the maximal total phlorotannins concentration (TPC) and total antioxidant activity (TAA) from *Macrocystis pyrifera*.

Factors	TPC		TAA	
	Optimum level	Contribution	Optimum level	Contribution
Time	4 h	25.0	3 h	6.5
Temperature	55 °C	20.8	40 °C	3.9
S/L[a] ratio	1:10	30.7	1:10	13.0
Particle size	<1.4	17.9	<1.4	5.9
Total contribution from all factors		94.4		29.2
Expected result at optimum ± CI[b] condition		218.9 ± 15.0		64.4 ± 2.9

[a] S/L, solid/liquid ratio of extraction.
[b] CI, confidence interval.

Table 5
Average concentration (±standard deviation) of carbohydrates present in alga *Macrocystis pyrifera* and in its phlorotannin extract.

Carbohydrate	Phlorotannins extract	*M. pyrifera* (M3)
	mg/g DS	mg/g DS
Alginate	23.4 ± 6.6	191.7 ± 3.4
Arabinose	1.0 ± 0.6	1.7 ± 0.5
Fucose	39.7 ± 5.7	73.5 ± 0.1
Fructose	1.4 ± 0.1	14.7 ± 0.1
Galactose	5.7 ± 1.3	31.7 ± 2.7
Galacturonic acid	0.5 ± 0.7	6.8 ± 0.1
Glucose	2.0 ± 0.6	11.1 ± 0.1
Glucuronic acid	0.8 ± 0.1	4.9 ± 0.1
Mannitol	38.1 ± 2.3	38.2 ± 0.4
Mannose	2.0 ± 0.9	11.5 ± 1.1
Rhamnose	2.7 ± 0.4	2.0 ± 0.1
Xylose	1.2 ± 0.4	9.0 ± 1.3
Total	118.5	396.9

acetone is a less polar solvent. Consequently, acetone has a higher affinity for apolar compounds such as lipid compounds and pigments, which may have produced an increment in the TAA measured in the sample. The presence of apolar compounds present in the extract suggests that the pretreatment conditions used are not optimized [44,45]. Therefore, these results suggested that water was the best solvent for the extraction of phlorotannins. Similar results were obtained by Kindleysides, et al. [28]. Moreover, when studying the extraction strategies of phlorotannins for other brown seaweed species (*Scytosiphon lomentaria*, *Papenfussiella kuromo* and *Nemacystus decipiens*), water was considered as the best extraction solvent [46].

The influence of temperature and drying time on the extraction and quality of phlorotannins extracted from *M. pyrifera* was evaluated. The drying curves are presented in Fig. 2a. As can be observed, the drying profiles for the temperatures 40, 50 and 60 °C are relatively similar. On the contrary, at 30 °C, a much slower release of moisture was observed. In order to evaluate the best drying temperature, the extraction of phlorotannins was carried out using water as an extractant. Notwithstanding, the phlorotannin compounds are easily decomposed, as also reported earlier [27]. Consequently, the addition of 0.3% ascorbic acid as a preservative during extraction was evaluated. The results of the total phlorotannins content (TPC) and TAA concentration obtained are presented in Fig. 2b. The optimal drying temperature was 40 °C, giving TPC and TAA values in the extract amounting to 180 ± 13 mg GAE/100 g DS and 62 ± 19 mg TE/100 g DS, respectively. On the other hand, for fresh seaweed (85% average moisture content; fresh seaweed, F/S) gave 210 ± 20 mg GAE/100 g DS of TPC and 115 ± 36 mg TE/100 g DS of TAA. Consequently, the TPC and TAA values obtained for non-dried alga gave better results, 14 and 46% higher than those values of dry seaweed at 40 °C, respectively, presumably due to the degradation of some phlorotannins during the drying process. Admittedly, the aim of the drying process was to reduce the moisture content in order to minimize the deterioration of the algae due to microbial growth, thus increasing the storage time of the seaweed prior any treatments. Further, the drying process also reduces the weight and volume of the seaweed, thus minimizing the packaging, storage and transportation costs [47].

The difference observed in the concentration of TPC and TAA for the extract obtained previously, 147 ± 2.9 mg GAE/100 g DS and 20.2 ± 4.2 mg TE/100 g DS for the effect of extractant type and, 180 ± 13 mg GAE/100 g DS and 62 ± 19 mg TE/100 g DS for effect of drying temperature, can be explained as due to the effect of different drying conditions (temperature and time) and by the fact that algae samples correspond to different harvest periods [48], Table 1. Nevertheless, it is possible to minimize the differences observed by controlling drying conditions and via standardization of biomass through changes in the form of culture [49].

3.3. Optimized extraction of phlorotannins: effect of extraction parameters

The TPC and TAA values measured upon different conditions of water extraction are recorded in Table 2. The best extraction conditions correspond to the entry N°9, whereupon the extraction performed for a sample particle size below 1.4 mm, and extraction with water at 55 °C for 4 h (S/L ratio of 1/15). Consequently, TPC of 200.5 ± 5.6 mg GAE/100 g DS and TAA of 38.4 ± 2.9 mg TE/100 g DS were recorded, resulting in 10% higher TPC value compared with previous conditions, 180 ± 13 mg GAE/100 g DS. The reported value is higher than the corresponding values reported for other types of brown algae. Indeed, Chandini,

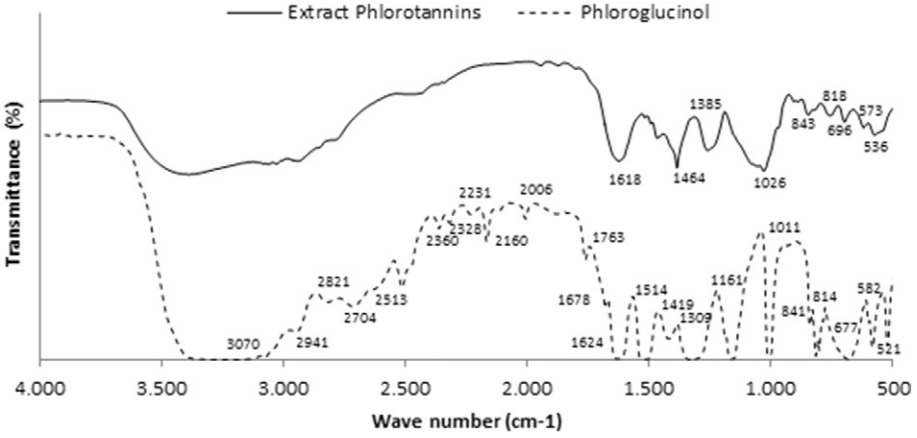

Fig 3. FTIR spectra of standard phloroglucinol and water phlorotannin extracts from *Macrocystis pyrifera*.

et al. [50] reported a TPC value of 0.29 mg GAE/100 g DS upon the water extraction of *Sargassum maginatum*, whereas in case of *E. stolonifera*, 8.21 ± 0.01 mg GAE/100 g DS was reported [51]. Further, in an additional study of *Turbinaria conoides* and using diethyl ether extraction, 119 ± 0.1 mg GAE/100 g DS of TPC was obtained [52]. Thus, it is evident that *M. pyrifera* has potential since much higher TPC values were attained for this worldwide relatively abundant seaweed.

Finally, all factors had significant effects ($p < 0.05$) on the TPC and TAA (Table 3), the highest influence was obtained with the S/L ratio, particle size and extraction time of 29.4, 27.8 and 21.7%, respectively, the sum of these three factors accounted for 78.9% of the TPC. For the TAA the highest influence, 57%, was that of factor S/L ratio. The statistical methodology applied predicted a maximum TPC of 218.9 ± 15.0 mg GAE/100 g DS and TAA of 64.4 ± 2.9 mg TE/100 g DS under the optimal extraction conditions (Table 4).

3.4. Characterization of phlorotannin extract

3.4.1. Fourier transform infrared (FTIR) spectroscopy

As can be seen, the chemical composition of phlorotannin extract obtained under the optimal condition, and of standard phloroglucinol was similar, see Fig. 3. The phloroglucinol standard gave rise to twenty

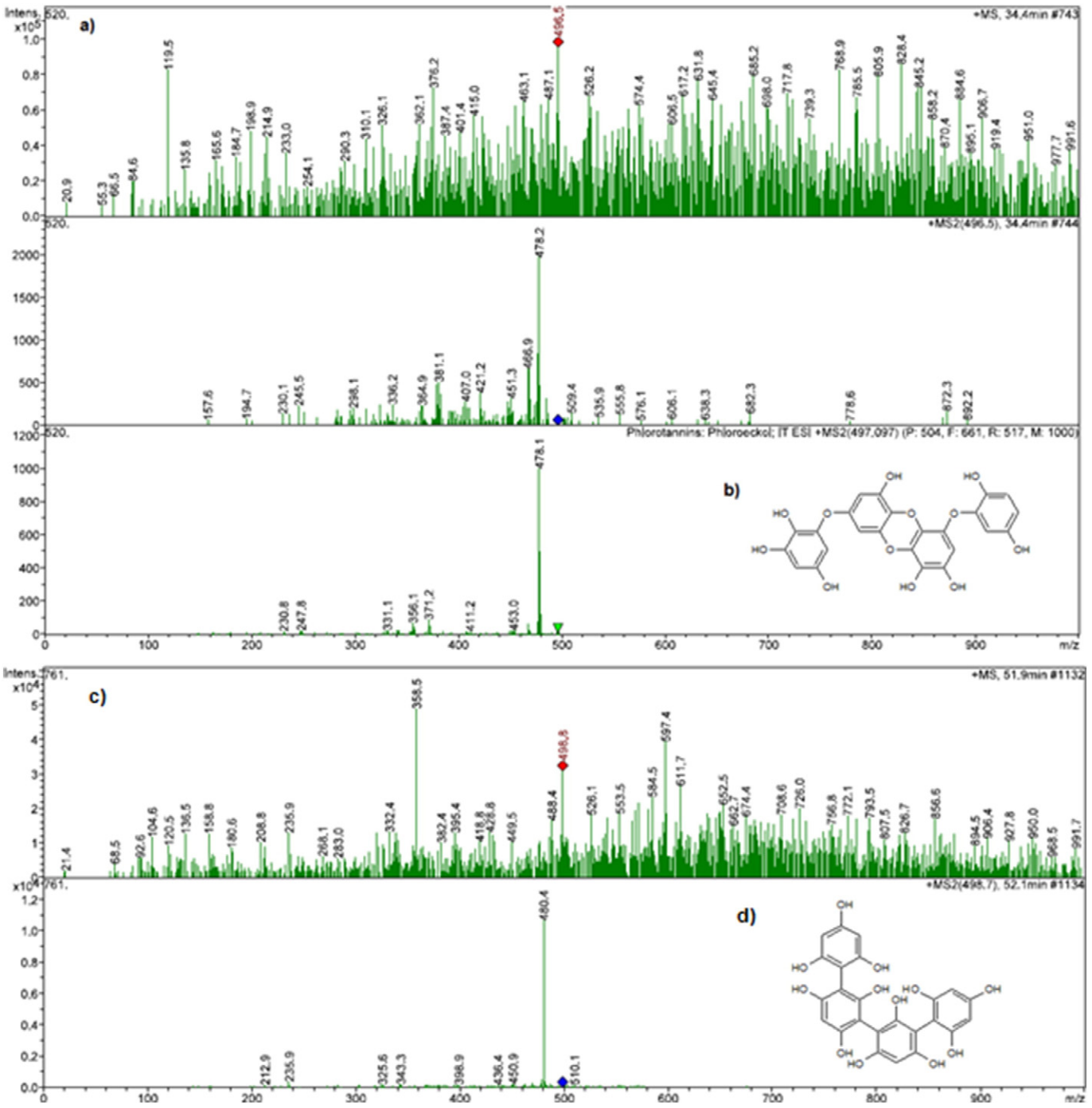

Fig. 4. Identification of phlorotannins detected in *Macrocystis pyrifera*. A, mass spectrum (MS) for peak 1 (superior) and fragmentation spectrum (MS2) for m/z 497 (inferior); B, chemical structure and library fragmentation spectrum for phloroeckol. C, mass spectrum for peak 2 (superior) and fragmentation spectrum for m/z 499; B, chemical structure for phloroglucinol tetramer.

three major peaks within the range between 3070 and 521 cm^{-1}, meanwhile similar peaks were observed for phlorotannins extract between 1618 and 536 cm^{-1}. Indeed, in phlorotannin extract, the stretching bands around 1470–1450 cm^{-1} correspond to the aromatic nuclei. The characteristic stretch band of carboxyl groups, the peak at 536 cm^{-1}, was presumably caused by the stretching vibration of O—H with relatively high strength in the axial position. In turn, the bands at 1026 cm^{-1} correspond to the glycosidic linkage vibrations of C—OC and C—O—H, indicating the presence of some carbohydrates in the sample.

Nonetheless, the stretch bands identified for some carbohydrate compounds may reveal the presence of some phlorotannin compounds in association with some carbohydrates. The signal at 843 cm^{-1} corresponds to characteristic absorption of mannuronic acid (a monomeric unit of alginate, one of the main constituents of brown algae) [53].

3.4.2. Analysis of the carbohydrate present in the phlorotannins extract

Various carbohydrates could be identified in the water extract of phlorotannins (Table 5), such as polysaccharide alginate, different types of monosaccharides (arabinose, fucose, fructose, galactose, glucose, mannose, rhamnose and xylose) sugar acid, (galacturonic and glucuronic acid) and a polyol (mannitol).

3.4.3. HPLC-ESI-MS/MS identification

The chromatograms obtained were compared to the reported ones for phlorotannins [54–57]. The identification was carried out using all m/z signals for each corresponding separated fragment. Additionally, an extracted ion chromatogram (EIC) of the expected precursor signal was performed. Two peaks were identified upon the analysis and compared with earlier data [54–56]. The peak 1 (t_R 34.4 min) was detected with a signal m/z 497, (the fragmentation gave m/z values of 478.2 ($[M\text{-}H_2O + H]^+$) and m/z 245.5 ($[M\text{-}2xPhloroglucinol + H]^+$)), likely corresponding to a derivative of phloroglucinol named phloroeckol (Fig. 4a and b). The peak 2 (t_R 52.1 min) was detected with a signal m/z of 499, (the fragmentation gave signals with m/z of 480.4 ($[M\text{-}H_2O + H]^+$), m/z 245.8 ($[M\text{-}2xPhloroglucinol + H]^+$) and m/z 235.9 ($[Phloroglucinol\text{-}H_2O + H]^+$)), likely originating from a tetramer of phloroglucinol isomers: difucophloroethol, fucodiphloroethol, tetrafucol or tetraphloroethol (Fig. 4c and d) [54].

These phlorotannins have interesting properties. Fucodiphloroethol exhibits free radical scavenging ability [58] and antiallergic effect [59], whereas phloroeckol exhibits antidiabetic effect [60] and prevents Alzheimer's disease [61]. Finally, we propose that phlorotannin extracts could find use as medicinal foods or therapeutics substrates in human health products.

4. Conclusions

The orthogonal design set of experiments allowed identification of the S/L ratio, particle size and time of extraction as the most important variables to obtain the highest concentrations of phlorotannins in the extract. The best extraction conditions upon extraction of phlorotannins from brown algae *M. pyrifera*, were determined: pre-treatment with hexane, extraction with water, drying temperature 40 °C, particle size below 1.4 mm, at 55 °C for 4 h and solid-to-liquid ratio of 1:15. At optimal conditions, the TPC value obtained was 200.5 ± 5.6 mg GAE/100 g DS and the TAA of 38.4 ± 2.9 mg TE/100 g DS. Finally, two types of phlorotannins were identified in the water extract, corresponding to phloroeckol and a tetramer of phloroglucinol.

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