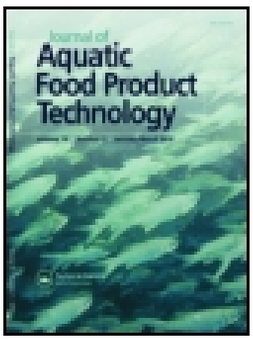

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Identification of Polyphenols from Chilean Brown Seaweeds Extracts by LC-DAD-ESI-MS/MS

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ABSTRACT

Polyphenol profiles were characterized in extracts of three Chilean brown seaweeds, *Durvillaea antarctica* (Chamisso) Hariot, *Lessonia spicata* (Suhr) Santelices, and *Macrocystis integrifolia* (Bory) by liquid chromatography with mass spectrometry detection (LC-MS/MS). Phlorotannins with different degrees of polymerization were identified in *D. antarctica* (trimers to octamers) and *L. spicata* (trimers to tetramers). No signals related to phlorotannins compounds were detected in *M. integrifolia*. *L. spicata* and *M. integrifolia* showed a great variety of flavonoid compounds in comparison with *D. antarctica*, mainly identified as glycoside forms in all the extracts. The antioxidant activity of brown seaweed extracts measured by ferric reducing power (FRAP) and oxygen radical absorbance capacity (ORAC) was significantly higher in *D. antarctica*, followed by *M. integrifolia* and *L. spicata*, in line with the total phenolic (TP) content. However, *D. antarctica* and *M. integrifolia* showed similar activity for free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in spite of the differences found in TP content. *D. antarctica* as well as *L. spicata* would represent a potential source of phlorotannins, whereas *M. integrifolia* could be considered as an alternative source of flavonoids. The identification of polyphenols in extracts of Chilean brown seaweeds opens innovative opportunities for their use in the food and pharmaceutical industries.

KEYWORDS

Phlorotannins; brown seaweeds; LC-MS/MS

Introduction

Chile, a country with over 9,000 km of coastline, has a great richness of seaweeds (González et al., 2012). *Durvillaea antarctica* (Chamisso) Hariot (Fucales, Phaeophyceae) is a brown seaweed commonly known as "Cochayuyo" (in Quechua language: "turnip of sea") that is distributed from Coquimbo (29°S) to the Strait of Magellan (55°S) and in New Zealand, Argentina, and Australia (Collantes et al., 2002). *Lessonia spicata* (Suhr) Santelices (Laminariales, Phaeophyceae), commonly known as "Huiro negro," is distributed from Coquimbo (29°S) to Chiloé Island (41°S) (González et al., 2012). *Macrocystis integrifolia* Bory (Laminariales, Phaeophyceae), known as "Huiro flotador," is distributed from Arica (18°S) to Concepción (36°S) (Santelices, 1989), and it is restricted to temperate regions of the Oriental Pacific as well as North and South America. The economic value of Chilean kelp beds is mainly associated with the alginate extraction industry. In addition, *M. integrifolia* has been used in the intense mariculture diet of *Haliotis* spp. (abalone) (Vásquez et al., 2012).

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Over the last two decades, the extraction and characterization of compounds with biological activity for the design of healthy or functional foods have become important areas of research in food science. In this context, brown seaweeds have been reported to be a rich source of bioactive compounds associated with several biological activities in both *in vitro* and *in vivo* models. Among the bioactive compounds in brown algae, polyphenols have been found to be responsible for antioxidant, antidiabetic, anticancer, antihypertensive, and anti-inflammatory activities (Holdt and Kraan, 2011). The study of phenolic compounds in brown seaweeds has been mainly focused on the identification of phlorotannins as a class of tannins exclusively synthesized in these seaweeds. Phlorotannins are phloroglucinol-based compounds biosynthesized by the acetate-malonate or polyketide pathway, are highly hydrophilic, and have a wide range of molecular sizes (Ferreres et al., 2012). Phlorotannins have been grouped according to interphloroglucinol linkages into four primary types as follows: fucols (with only phenyl linkages), phlorethols (with only aryl ether bonds), fucophlorethols (with phenyl and aryl ether linkages), and eckols (with dibenzodioxin linkages) (Isaza Martínez and Torres Castañeda, 2013). Phlorotannin compounds such as fucophloroethol, fucodiphloroethol, fucotriphloroethol, 7-phloroeckol, phlorofucofuroeckol, and bieckol/dieckol have been reported in the brown seaweeds *Pelvetia canaliculata, Fucus spiralis, Fucus vesiculosus, Ascophyllum nodosum, Saccharina longicruris, Cystoseira nodicaulis, Cystoseira tamariscifolia*, and *Cystoseira usneoides* (Ferreres et al., 2012; Steevensz et al., 2012). Different polymerization degrees of phlorotannins have been described in *Fucus vesiculosus* and *Sargassum muticum* (Montero et al., 2016; Steevensz et al., 2012). However, studies dealing with the extraction and characterization of polyphenols in Chilean seaweeds are scarce (Leyton et al., 2016; Tala et al., 2013) but essential to give added value to these marine natural products and to explore new potential uses in the food and pharmaceutical industries.

The objective of this study was to determine the content of total phenolic compounds and antioxidant activity of extracts of three Chilean brown seaweeds species (*D. antarctica, L. spicata*, and *M. integrifolia*), as well as to identify their polyphenol profiles by high performance liquid chromatography with mass spectrometry detection (HPLC-MS/MS).

Materials and methods

Sample collection

Durvillaea antarctica, Lessonia spicata, and *Macrocystis integrifolia* were collected in January 2013 from intertidal rocks on "El Tabo" beach (33° 27′ 31" S, 71° 39′ 43′ W), which is located in the Valparaiso region (Chile). Fresh seaweeds were washed with cold water spurts to remove sand, stored at room temperature for 24 h, and then dried at 40°C for 7 days in a forced-air oven (WTE, Germany). The dried seaweeds (10% moisture) were stored in sealed plastic bags at −20°C until extract preparation.

Preparation of seaweed extracts

Dry seaweeds (40 g) were ground in a windmill (Fuchs-Müllen, Masch. Kom. N° 18791, Kriens, Switzerland), macerated in ethanol:water (1:1 v/v) (100 mL) for 24 h at room temperature and then filtered (Whatman N°1). This procedure was carried out twice with 100 mL of ethanol:water (1:1 v/v). The extracts were combined, and the volume was reduced in a rotary evaporator (Buchi R-3, Vacuum Pump v-700, Switzerland) at 40°C to a final volume of 150 mL. Finally, the seaweed extract was centrifuged (Z206A, Hermle, Germany) at 3500 rpm for 10 min. The resulting seaweed extracts were frozen at −20°C.

Characterization of seaweed extracts

Total phenolic (TP) content was determined using the Folin–Ciocalteau method (Singleton and Rossi et al., 1965) and expressed as a phloroglucinol equivalent (PGE) (Koivikko et al., 2005), according to a calibration curve (0.101–0.811 mg·mL^{-1}, R^2 = 0.997).

Antioxidant activity (AA) was measured using the ferric-reducing antioxidant power (FRAP) method (Benzie and Strain, 1996), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Brand-Williams et al., 1995), and the oxygen radical absorbance capacity (ORAC) method (Cao et al., 1993). The DPPH and FRAP measurements were conducted using a UNICAM UV3 UV/Vis spectrophotometer (Rochester, NY, USA) at 517 and 593 nm, respectively. The DPPH bleaching activity was expressed as EC_{50} (mg of sample that bleached 50% of the DPPH ethanolic solution). For the FRAP experiments, the results were expressed as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents (TE). The ORAC was determined using a Fluorimeter Biotek FLx800 (BioTek Instruments, Inc., Winooski, VT, USA), and the results were expressed as TE. All the experiments were performed in triplicate.

Identification of polyphenols by liquid chromatography with mass spectrometry

Phenolic compounds were obtained from *D. antarctica, L. spicata*, and *M. integrifolia* extracts using liquid-liquid extraction according to Peña-Neira et al. (2007). Briefly, 25 mL of the extract was treated with ethyl ether (3 x 10 mL for each sample) and with ethyl acetate (3 x 10 mL for each sample). The organic fractions were combined and then evaporated in a rotary evaporator (Buchi R-3, Vacuum Pump v-700) at 35°C. The residue was dissolved in 2 mL ethanol:water (1:1 v/v), filtered (0.45 µm filter, Millipore), and injected into the LC-ESI-MS/MS system, which consisted of an HPLC (HP1100, Agilent Technologies Inc., Santa Clara, CA, USA) connected with a mass spectrometer (Esquire 4000 ESI-Ion Trap LC/MS(n) system, Bruker Daltoniks GmbH, Germany). A C18 column (5 µm, 4.6 mm i.d. x 25 cm, Waters) was used in the analysis. The mobile phase was formic acid in deionized water (1% v/v, solvent A) and acetonitrile (solvent B), at a flow rate of 1 mL/min, with the following elution gradient: 0–5 min, 5% B; 5–60 min, 5–30% B (linear); 60–70 min, 30–60% B (linear); 70–80 min, 60% B; and 80–90 min, 60–5% B (linear). Phenolic compounds were detected at 280 nm. The mass spectral data were acquired in positive and negative modes. Ionization was performed at 3000 V with nitrogen as nebulizing gas at 40 psi, drying gas at 365°C, and at a flow rate of 10 L/min. All the scans were performed in the range of 20–2200 m/z. The collision-induced dissociation (CID) was performed by collisions with the helium background gas present in the trap and automatically controlled through SmartFrag option. The analysis of chromatograms and mass spectra was performed using DataAnalysis 3.2 (Bruker Daltonik GmbH). Identification of phenolic compounds was performed by m/z values of the molecular ions, their fragmentation pattern, and comparison with published data.

Statistical analyses

All the statistical analyses were calculated using Statgraphics Centurion XVI (Manugistics Inc., Statistical Graphics Corp., The Plains, VA, USA).

Results and discussion

Total phenolic compounds and antioxidant activity of D. antarctica, L. spicata, and M. integrifolia extracts

Table 1 shows the content of total phenolic compounds (TPC) and antioxidant activity of the *D. antarctica, L. spicata*, and *M. integrifolia* extracts. The TPC content of the extract from *D. antarctica* was significantly higher than those of *M. integrifolia* and *L. spicata*. Higher TP contents have been reported in other brown seaweeds (*Fucus vesiculosus, Laminaria ochroleuca, Undaria*

Table 1. Total phenolic compounds (TP) and antioxidant activity for *D. antarctica, L. spicata*, and *M. integrifolia* extracts using three different methods (DPPH, FRAP, and ORAC).

Analysis	*D. antarctica*	*L. spicata*	*M. integrifolia*
TP content (g PGE/kg DW)	5.0 ± 0.05 [a]	1.21 ± 0.11 [c]	3.70 ± 0.14 [b]
DPPH (EC_{50} mg/mL)	0.97 ± 0.11 [b]	1.24 ± 0.35 [a]	1.07 ± 0.18 [b]
FRAP (mmoL TE/kg DW)	6.20 ± 0.5 [a]	2.95 ± 0.04 [c]	3.91 ± 0.18 [b]
ORAC (μmoles TE/g DW)	25.9 ± 1.8 [a]	4.75 ± 0.27 [c]	9.35 ± 0.41 [b]

PGE: phloroglucinol equivalent, DW: dry weight, TE: trolox equivalent. Values are means ± standard deviation ($n = 3$). Different letters in columns indicate significant differences ($p \leq 0.05$).

pinnatifida, Dictyota dichotoma, and *Sargassum polycistum*), ranging from 6.6 to 109 g PGE·kg-1 DW (Jiménez-Escrig et al., 2001; Koivikko et al., 2005; Matanjun et al., 2008).

Generally, various methods have been employed to measure the antioxidant activity of extracts, which include different mechanisms and/or action modes. FRAP and DPPH are based on single electron transfer, whereas ORAC is based on hydrogen atom transfer (Shahidi and Zhong, 2015). The antioxidant activity of brown seaweed extracts measured by FRAP and ORAC (Table 1) was significantly higher in *D. antarctica*, followed by *M. integrifolia* and *L. spicata*, in accordance with the TP content. However, no significant differences were found between *D. antarctica* and *M. integrifolia* in the antioxidant activity measured by DPPH, in spite of the lower TPC content of the latter, and the extract with the lowest TPC content showed the lowest DPPH activity. Similarly, several studies have reported a correlation between the content of total phenolic compounds in algae and their antioxidant activity, and the higher the TPC content, the higher the antioxidant activity (López et al., 2011; Rajauria et al., 2016). However, a lack of correlation between TP content and antioxidant activity have also been reported, suggesting that other components, such as chlorophyll and carotenoids, together with differences in the polyphenols profiles may affect the antioxidant activity (Belda et al., 2016). Furthermore, the predominant mode of antioxidant activity of each polyphenol should be taken into account also, since different antioxidant mechanisms are involved in the different methods used to evaluate the antioxidant activity. The comparison of these results with the previously reported values for both TPC content and antioxidant activity of brown seaweed is a difficult task, since these parameters may be affected by several factors such as the geographical location (Montero et al., 2016), seasonal variation (Conan et al., 2004; Maréchal et al., 2004), reproductive stage (Pansch et al., 2008), genus and species (Shibata et al., 2004). Furthermore, the extracting solvent used may play a key role in both TPC content and antioxidant activity of seaweed extracts (Sabeena Farvin and Jacobsen, 2013). In this study, a mixture of ethanol:water (50:50) was used as extracting solvent, since ethanol is a permitted solvent in the food industry, and these extracts are intended to be applied as food ingredients in the design of healthy or functional foods. In previous studies, ethanol was more efficient than water for phenolic extraction in the case of several brown seaweeds (Sabeena Farvin and Jacobsen, 2013). Mixtures of acetone:water have also showed high efficiency for the extraction of TPC and antioxidant activity in *D. antarctica* (70:30) (Tala et al., 2013) and *H. elongate* (60:40) (Belda et al., 2016). However, acetone is not permitted as extracting solvent for food applications.

Identification of polyphenols in brown seaweed extracts by HPLC MS/MS

Figure 1(a–c) shows the UV chromatograms at 280 nm for *D. antarctica, L. spicata*, and *M. integrifolia* extracts, respectively. The identification of the marked peaks is detailed in Tables 2–4, which contain the positive and negative polarity precursor m/z signals for each peak, as well as their corresponding fragmentations. The fragmentations are arranged in order of decreasing intensity from left (base peak) to the right.

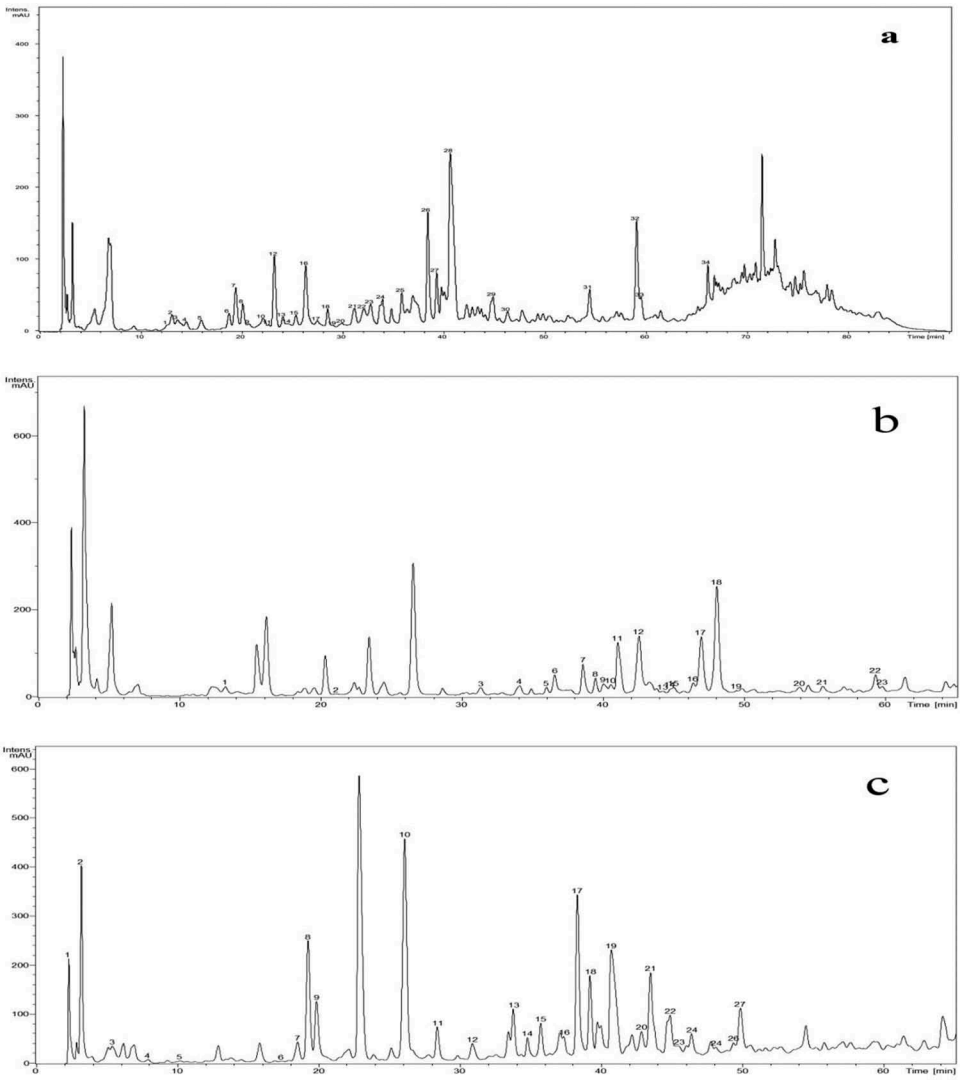

Figure 1. UV chromatograms for *D. antarctica* (a), *L. spicata* (b), and *M. integrifolia* (c) extracts. The identifications of numbered peaks are detailed in Tables 2–4.

Phlorotannins profile

Usually, the mass spectral data are acquired in negative or positive ionization mode in most of the studies dealing with the identification of phlorotannins by HPLC/MS–MS in brown seaweeds (Rajauria et al., 2016; Steevensz et al., 2012; Wang et al., 2012). However, both negative and positive ionization modes were considered in this study. [M-H]- ions corresponding to trimers (m/z 373) were detected in both *D. antarctica* (peaks 1, 2, and 3; Table 2) and *L. spicata* (peak 1, Table 3) extracts. Their fragmentation pattern showed successive loss of phloroglucinol ([M-126-H]-, m/z 247 and [M-126-H]-, m/z 124) and phloroglucinol and methyl ([M-14–126-H]-, m/z 233). Furthermore, a fragment resulting from the loss of phloroglucinol and one molecule of water ([M-18–126-H]-, m/z 229) was detected only in the *D. antarctica* extract. Protonated molecular ions ([M + H]+) were detected at m/z 375 in the case of the *D. antarctica* extract (Table 2, peaks 1 and 3). The fragmentation pattern showed losses of one molecule of water (m/z 357, [M-18 + H]+), phloroglucinol ([M-126 + H]+, m/z 249) and one molecule of water and phloroglucinol

Table 2. Identification of phenolic compounds of *D. antarctica* extract by HPLC MS/MS. Identifications based on ion positive MS^2 fragments are marked with P, while those based on ion negative MS^2 fragments are marked with N.

Peak	R_T (min)	$[M + H]^+$ (m/z)	Ion positive MS^2 fragments (m/z)						$[M-H]^-$ (m/z)	Ion negative MS^2 fragments (m/z)						Identification	References
1	12.7	375	357	249	231	165	234		373	233	247	229	124	189		Trimer (triphloroethol/ fucophloroethol)	Ferreres et al., 2012; Wang et al., 2012[P]
2	13.2	375							373	233	247	229	124	189		Trimer (triphloroethol/ fucophloroethol)	
3	13.8	375	356	231	249	221	339	165	373	233	247	229	124	189		Trimer (triphloroethol)	Ferreres et al., 2012; Wang et al., 2012[P]
4	14.6	623	605	497	355	479	373		621	373	603	495	371	479	355	Pentamer (pentaphloroethol/ trifucophloroethol)	Wang et al., 2012[P]
5	16.1	623	605	479	497	247			621	603	373	339	357	495	477	Pentamer (pentaphloroethol/ trifucophloroethol)	Wang et al., 2012[P]
6	18.8	747	621	481	603	357	729	497	745							Hexamer (hexaphloroethol/ bis-trifucophloroethol)	Wang et al., 2012[P]
7	19.5	747							745	497	479	513.0	619	495	727	Hexamer (hexaphloroethol/ bis-trifucophloroethol)	
8	20.2	747	621	729	479	357	603	497	745	497	479	727	495	481	513	Hexamer (hexaphloroethol/ bis-trifucophloroethol)	Liu et al., 2012; Wang et al., 2012[P]
									577	425	451	407	289	541	519	Proanthocyanidin dimer	
9	20.8	499	373	479	248	355	233	247	497	233	371	263	189	245	480	Tetramer (tetraphloroethol)	Ferreres et al., 2012; Wang et al., 2012[P]
10	22.2								745	371	727	355	511	603	481	Hexamer	
		497	478	371	356	467	453									Phloroeckol	Ferreres et al., 2012[P]
									289	245	205	178	231	271		(Epi)catechin	
11	22.7	747	481	603	621	373	355	495								Hexamer (hexaphloroethol/ bis-trifucophloroethol)	Wang et al., 2012[P]
12	23.3	623	357	497	604	481			621	495	233	479	355	245		Pentamer (pentaphloroethol/ trifucophloroethol)	Wang et al., 2012[P]
13	24.2	871	481	745	605	727	604	499	869	727	635	605	495	369	477	Heptamer	Wang et al., 2012[P]
14	24.7								869	635	621	603	727	495	369	Heptamer	
		623	604	357	481	497	371	462	621	495	479	355	477	247	233	Pentamer (pentaphloroethol/ trifucophloroethol)	Wang et al., 2012[P]
15	25.5	871	481	605	745	727	357	586	869	621	603	727	851	477	635	Heptamer	Ferreres et al., 2012; Wang et al., 2012[P]
16	26.4	871	605	499	479	586	728	357	869	635	621	477	603	495	479	Heptamer	Wang et al., 2012[P]
17	27.6								577	451	425	407	289	559		Proanthocyanidin dimer	
									315	161	285	297				Methoxy-kaempherol	(Alvarez-Suarez et al., 2010 [N]

(*Continued*)

Table 2. (Continued).

Peak	R_T (min)	$[M + H]^+$ (m/z)	Ion positive MS^2 fragments (m/z)						$[M-H]^-$ (m/z)	Ion negative MS^2 fragments (m/z)						Identification	References
18	28.5								635	465						Trigalloyl-glucose	
									289	245	205	231	178	160		(Epi)catechin	
19	29.1	995	605	481	729	623	851	462								Octamer	Liu and Gu, 2012 [P]
20	30.0	995	605	481	623	729	462	710								Octamer	Liu and Gu, 2012 [P]
21	31.2	747	728	481	603	497.0	619	373	745	511	619	495	481	387	605	Hexamer	Wang et al., 2012[P]
									631	479	317					Galloyl-myricetin-hexoside	
22	32.1	995	605	357	623	462	339	481	993	601	729	759	619	603	355	Octamer	Liu and Gu, 2012[P]
23	32.9	622	604	357	479	495	463	231	620	611	548	527	530			Pentamer	Wang et al., 2012[P]
24	34.0	481	319	463					479	316						Myricetin-O-hexoside	
25	35.9	617	315	303	599	345	297	447	615	463	301					Galloyl-hexose-quercetin	
26	38.5	611							609	301						Quercetin-O-rutinoside	FIO00597[N]
		465							463	316	178					Myricetin-O-rhamnoside	FIO00203; PR040144[N]
27	39.3								463	301						Quercetin-O-glucoside	FIO00168[N]
28	40.6	449	287						447	285						Luteolin-O-hexoside	
29	44.9								447	285	327	255	301			Kaempherol-O-hexoside	
30	46.3								609	301						Hesperitin-O-rutinoside	FIO00492; TY000206[N]
		449	287													Luteolin-O-hexoside	
31	54.4								517	323	341	179	160	355		Not identified	
		314	177	297					312	177	134	297	147			Not identified	
32	59.1								593	285	367					Kaempherol-O-rutinoside	
33	59.6	303	257	285	229	165	247	153	301	178	150	257	273	193		Quercetin	
34	66.1	271							269	225	148	182	201			Apigenin	

Table 3. Identification of phenolic compounds of *L. spicata* extract by HPLC MS/MS. Identifications based on ion positive MS^2 fragments are marked with P, while those based on ion negative MS^2 fragments are marked with N.

Peak	R_T (min)	$[M + H]^+$ (m/z)	Ion positive MS^2 fragments (m/z)						$[M-H]^-$ (m/z)	Ion negative MS^2 fragments (m/z)					Identification	References
1	13.3								373	233	247	305	124		Trimer (fucophloroethol/ triphloroethol)	Ferreres et al., 2012; Wang et al., 2012[P]
2	21.4								497	233	371	263	189		Tetramer (tetraphloroethol)	
3	31.3	655 (M+ Na)	337	637	341	413			631	479	317	299			Myricetin-O-galloylhexoside	Simirgiotis et al., 2013[N]
4	34.0	503 (M+ Na)	341	185					479	316					Myricetin-O-hexoside	Gordon et al., 2011[N]
5	35.9	655 (M + K)	606	525	756				615	463	301				Quercetin-O-galloylhexoside	Hevesi Tóth et al., 2009[N]
		639 (M+ Na)	337	485	338	611	621	325								
6	36.5								609	285	429	255	309		Kaempferol-O-diglucoside	Olsen et al., 2009[N]
7	38.5	649 (M + K)	631	463	485	560	495		609	301	271				Quercetin-O-rutinoside	Biesaga and Pyrzynska, 2009; FIO00597[N]
		633 (M+ Na)	331	487	485	476	560	480								
									463	316	178				Myricetin-O-deoxyhexoside	Gordon et al., 2011[N]
8	39.4	487 (M+ Na)	185	325	445	459	375		463	301					Quercetin-O-hexoside	Rattmann et al., 2011[P] Gordon et al., 2011[N]
9	39.9								463	301					Quercetin-O-hexoside	Rattmann et al., 2011[P] Gordon et al., 2011[N]
10	40.5								447	285					Luteolin-O-glucoside	Bouaziz et al., 2010; FIO00168[N]
11	41.0								449	285	303	431	150		Taxifolin-O-rhamnoside	Tatsis et al., 2007[N]
12	42.4								593	285					Kaempferol-O-rutinoside	Vallverdú-Queralt et al., 2010; Vallverdú-Queralt et al., 2014[N]
		457 (M+ Na)	325	507	155				433	301					Quercetin-O-pentoside	Gordon et al., 2011[N]
13	44.2								433	301					Quercetin-O-pentoside	Gordon et al., 2011[N]
14	44.7	471 (M+ Na)	309	185					447	284	327	301	255	227	Quercetin-O-rhamnoside	Rattmann et al., 2011[P]
15	44.9	471 (M+ Na)	325	309					447	301					Quercetin-O-rhamnoside	Boulekbache-Makhlouf et al., 2013[N]
16	46.4	633 (M+ Na)	483	331	487	337	615	325	609	301					Hesperetin-O-rutinoside	Vallverdú-Queralt et al., 2014; FIO00492; TY000206[N]
									431	269					Apigenin-O-glucoside	Vallverdú-Queralt et al., 2014[N]
17	46.9	449	287												Luteolin-O-hexoside	Termentzi et al., 2008[P]
									461	446	300	284			Kaempferol-methyl ether-O-hexoside	Falcão et al., 2013[N]
18	47.9								515	353	203				Dicaffeoylquinic acid	Gouveia and Castilho, 2011[N]
									503	341	281	251	179		Caffeic acid dihexoside	Vallverdú-Queralt et al., 2011[N]

(*Continued*)

Table 3. (Continued).

Peak	R_T (min)	$[M + H]^+$ (m/z)	Ion positive MS^2 fragments (m/z)		$[M-H]^-$ (m/z)	Ion negative MS^2 fragments (m/z)					Identification	References
19	49.4				615	317	463				Myricetin-O-galloyldeoxyhexoside	Saldanha *et al.*, 2013[N]
20	53.9				547	353	179	367	371	160	Dicaffeoylquinic acid derivative	Gouveia and Castillo, 2011[N]
21	55.5	663 (M+ Na)	361	645	639	463	477	301	315		Isorhamnetin-O-dihexoside	Gancel et al., 2008[N]
					609	463	301				Quercetin-O-rhamnosyl/ coumaroylhexoside	Gouveia and Castillo, 2011[N]
22	59.1				593	285	367	447			Luteolin-O-rutinoside	Jemai et al., 2009[N]
23	59.6				301	178	150	273	257		Quercetin	Tatsis et al., 2007[N]

Table 4. Identification of phenolic compounds of *M. integrifolia* extract by HPLC MS/MS. Identifications based on ion positive MS^2 fragments are marked with P, while those based on ion negative MS^2 fragments are marked with N.

Peak	R_T (min)	$[M + H]^+$ (m/z)	Ion positive MS^2 fragments (m/z)						$[M-H]^-$ (m/z)	Ion negative MS^2 fragments (m/z)					Identification	References
1	2.4	365 (M+ Na)	203	185					341	178	142	112			Caffeic acid hexoside	Vallverdú-Queralt et al., 2014[N]
2	3.3								633	301	275	249	231		HHDP-galloylglucose	Boulekbache-Makhlouf et al., 2013[N]
									483	331	313	168	457		Digalloylglucose	Boulekbache-Makhlouf et al., 2013;
3	5.5	657 (M+ Na)	337	487	645				633	301	275	249			HHDP-galloylglucose	Boulekbache-Makhlouf et al., 2013[N]
4	8.0								633	301	249	275			HHDP-galloylglucose	Boulekbache-Makhlouf et al., 2013[N]
5	10.3								483	331	168	313			Digalloylglucose	Boulekbache-Makhlouf et al., 2013; Regueiro *et al*., 2014[N]
6	17.5								341	178	160				Caffeic acid hexoside	Rivera-Pastrana et al., 2010[N]
7	18.6	601 (M+ Na)	449	431	463	311	475	355	577	425	451	407	559	289	Procyanidin dimer B	Mane *et al*., 2011; Simirgiotis, 2013[N]
8	19.3	579	427	409	291	453	439	247	577	425	451	407	559	289	Procyanidin dimer B	Aaby et al., 2007[P] Simirgiotis, 2013[N]
9	19.9								270	149	133				Apigenin	Eyles et al., 2007[N]
10	26.2								785	483	301	633	275	419	HHDP-digalloylglucose	Sanz et al., 2010; Gordon et al., 2011[N]
11	28.4	659 (M+ Na)	489						635	465					Trigalloylglucose	Sanz et al., 2010[N]
									289	245	205	179			Catechin/epicatechin	Sun et al., 2007[N]
12	30.9	671 (M + K)	625	541	653	412			631	479					Myricetin-O-galloylhexoside	Simirgiotis et al., 2013[N]
		655 (M+ Na)	337	637	341	543										
13	33.8	503 (M+ Na)	341	419	185	319	394									
		481	319						479	316					Myricetin-O-hexoside	Määttä et al., 2003[P]
14	34.7								593	300	285	463	271		Kaempferol-methyl ether-O-pentoside-hexoside	Liu et al., 2009[N]
15	35.7	639 (M+ Na)	485	337	479	476	325		615	463	301				Quercetin-O-galloylgalactoside	Sannomiya et al., 2005[N]
16	37.3	473 (M+ Na)	341	394	413	155										
		451	319	153	237				449	316					Myricetin-O-pentoside	Lin and Harnly, 2007[P]
17	38.3	649 (M + K)	631	463	485				609	301	343				Quercetin-O-rutinoside	Biesaga and Pyrzynska, 2009; FIO00597[N]
		633 (M+ Na)	331	487	485	560	476	325								

(*Continued*)

Table 4. (Continued).

Peak	R_T (min)	$[M + H]^+$ (m/z)	Ion positive MS^2 fragments (m/z)						$[M-H]^-$ (m/z)	Ion negative MS^2 fragments (m/z)						Identification	References
									463	316						Myricetin-O-deoxyhexoside	Gordon et al., 2011[N]
18	39.1	487 (M+ Na)	185	325	403	445	459		463	301						Quercetin-O-hexoside	Rattmann et al., 2011[P] Gordon et al., 2011; Boulekbache-Makhlouf et al., 2013[N]
19	40.6								449	285	303	287	150	431		Taxifolin-O-rhamnoside	Tatsis et al., 2007[N]
20	42.7	633 (M + K)	615	444	469	541	347	489	593	285	257					Luteolin-O-rutinoside	Siciliano et al., 2004[N]
		617 (M+ Na)	331	471	460	542	599	589									
		471 (M+ Na)	185	309	453				447	284	327	286	255	150		Kaempferol-O-glucoside	Rattmann et al., 2011[P] Vallverdú-Queralt et al., 2014[N]
21	43.4	663 (M + K)	645	479	499	576	517		623	315	300	271	255			Isorhamnetin-O-rutinoside	Ding et al., 2008[N]
		647 (M+ Na)	331	501	490	486	499										
22	44.7	539 (M+ Na)	377	359	521				515	353						Dicaffeoylquinic acid	Vallverdú-Queralt et al., 2014[N]
		471 (M+ Na)	309	185	443				447	284	301	327	286	255		Kaempferol-O-glucoside	Rattmann et al., 2011[P] Vallverdú-Queralt et al., 2014[N]
23	45.3								477	314	357	271	285	299	316	Isorhamnetin-O-glucoside	Simirgiotis and Schmeda-Hirschmann, 2010[N]
24	46.3	633 (M+ Na)	483	331	487	337	615	325	609	301						Hesperetin-O-rutinoside	Vallverdú-Queralt et al., 2014; FIO00492; TY000206[N]
25	48.1	539 (M+ Na)	377	521	359	519	493	523	515	353	203	299				Dicaffeoylquinic acid	Gouveia and Castilho, 2011[N]
26	49.2	639 (M+ Na)	321	303	487	341			615	317	463					Myricetin-O-galloyldeoxyhexoside	Saldanha *et al.*, 2013[N]
27	49.7								371	209						Sinapyl alcohol-O-glucoside	Eyles et al., 2007[N]

([M-18–126 + H]+, m/z 231). According to MS data and fragmentation pattern, the trimers identified would be triphloroethol y/o fucophloroethol (Ferreres et al., 2012; Wang et al., 2012). The fragmentation patterns for trimers, together with their proposed chemical structures, are shown as supplementary material (supplementary material 1 and 2).

Isomers with [M-H]- at m/z 497 were also detected in both *D. antarctica* (Table 2) and *L. spicata* (Table 3), which were considered to be phlorotannins composed by four phloroglucinol (phlorotannin tetramers). The product ions detected may be due to the loss of bifuhalol (m/z 233), one phloroglucinol unit (m/z 371), two phloroglucinol units (m/z 245), and successive loss of water (m/z 189) (Wang et al., 2012). Tetramers were also detected in the positive ionization mode for the *D. antarctica* extract, with protonated molecular ions ([M + H]+) at m/z 499 (peak 9, Table 2), whose fragmentation pattern also showed the combined loss of phloroglucinol and water (m/z 355), bifuhalol (m/z 233), and successive losses of phloroglucinol (m/z 373, m/z 247/248). Loss of phloroglucinol units suggest the breaking of C-O-C bonds, which would indicate that most of the detected phlorotannins corresponded to phloroethols. Similar phenolic compounds have been reported in other brown algae such as *F. spiralis, C. usneoides*, or *F. vesiculosus*, where these tetramers were tentatively identified as tetrafucol and/or fucodiphoroethol (Ferreres et al., 2012; Wang et al., 2012). In the *D. antarctica* extract, other compounds with m/z in the range 621–993 in the negative ion mode and m/z 623–995 in the positive ion mode were considered polymers with several phloroglucinol units. Thus, m/z of 621/623 were tentatively identified as pentamers (pentaphoroethol/trifucophloroethol) (Ferreres et al., 2012). The fragmentation patterns for pentamers, together with their proposed chemical structures, are shown as supplementary material (supplementary material 3 and 4). Molecular ions detected at m/z 745/747, 869/871, and 993/995 may be phenolic compounds composed by six, seven, or eight phloroglucinol units as described in other brown algae such as *F. vesiculosus* and *F. spiralis* (Ferreres et al., 2012; Wang et al., 2012). The product ions detected from these polymers showed the successive loss of phloroglucinol units. The phlorotannins profile of *D. antarctica* extract showed a higher degree of polymerization (trimers to octamers) than *L. spicata* extract, where only trimers and tetramers were identified. Geographic location, in addition to genus and species, has been reported to affect the degree of polymerization of phlorotannins in brown seaweeds (Steevensz et al., 2012). In contrast, signals related to phlorotannin compounds were not detected in *M. integrifolia* extract at positive or negative polarity (Figure 1c, Table 4).

Other polyphenols in brown seaweeds

Although phlorotannins are the major polyphenols found in brown algae (Ferreres et al., 2012; Isaza Martínez and Torres Castañeda, 2013; Rajauria et al., 2018; Steevensz et al., 2012; Wang et al., 2012), other polyphenols such as several flavonoid derivatives and phenolic acids have been reported also (Belda et al., 2016; López et al., 2011; Rajauria et al., 2016; Sabeena Farvin and Jacobsen, 2013). Most of the phenolic compounds were detected in negative polarity mode, and for some peaks, sodium ([M+ Na]+) or potassium ([M + K]+) adducts were detected in positive polarity mode. In a few cases, the identification required further analysis of the fragmentation, as was the case for the differentiation between luteolin and kaempferol derivatives (data not shown). Here, characteristic fragments, such as m/z 199 and m/z 175, were found in the negative fragmentation of luteolin (Sánchez-Rabaneda et al., 2003, 2004) and m/z 165 and m/z 121 were found in the positive fragmentation of kaempferol (Cuyckens and Claeys, 2004; Justino et al., 2009). Analyses of the fragmentation patterns were also pivotal for distinguishing between quercetin and hesperetin (data not shown); quercetin shows characteristic fragments in negative polarity mode (m/z 179 and m/z 151) (Gates and Lopes, 2012; Yang et al., 2012), and hesperetin shows a characteristic fragment of m/z 286 in negative polarity mode (Gates and Lopes, 2012; Zhao et al., 2013).

Flavonoid derivatives such as flavonols, flavones, flavanones, flavanonols, and flavanols were detected in the extracts of the three brown algae. In the case of flavonols, aglycone or glycoside forms of myricetin, quercetin, and kaempferol were identified (Figure 1a–c; Tables 2–4), as

concluded from the fragmentation pattern where losses corresponding to one or two units of glucose or galactose ([M-162-H]- or [M-324-H]-), one unit of rhamnose ([M-146-H]-), one unit of pentose ([M- 132-H]-), or one unit of rhamnoglucose ([M-308-H]-) were detected (Parejo et al., 2004). Furthermore, isorhamnetin-*O*-dihexoside as well as isorhamnetin-*O*-glucoside and isorhamnetin-*O*-rutinoside were tentatively identified in the case of *L. spicata* and *M. integrifolia* extracts, respectively. Two types of flavones were tentatively identified in the three algae extracts: aglycone or glycoside forms of apigenin and glycoside forms of luteolin. Two luteolin derivatives were proposed in the negative and positive ionization modes, luteolin-*O*-hexoside and luteolin-*O*-rutinoside, which showed losses corresponding to one unit of glucose or galactose ([M-162-H]-) and one unit of rhamnoglucose ([M-308-H]-) (Parejo et al., 2004). The flavonone heperitin was found as heperitin-*O*-rutinoside in the three extracts, with the loss of one unit of rhamnoglucose ([M-308-H]-) (Parejo et al., 2004). Furthermore, catechin/epicatechin was tentatively identified in both *D. antarctica* and *M. integrifolia* in the negative ionization mode. The peak at m/z 285 was the result of the molecular ion fragmentation. According to literature, fragmented ions at m/z 245 (which results from losing a CO_2 group) and 205 (probably due to the loss of the A-ring) were obtained after further fragmentation (Savic et al., 2014). The flavononol taxifolin was found in both *L. spicata* and *M. integrifolia* extracts as taxifolin-*O*-rhamnoside, and fragmented ions m/z 303 and m/z 285 were obtained, probably due to the successive loss of one unit of rhamnose ([M-146-H]-) and water ([M-18-H]-). Other phenolic compounds such as dimeric procyanidins were tentatively identified in both *D. antarctica* and *M. integrifolia*. Furthermore, in the case of *M. integrifolia*, other tannins such as gallotannins (digallloylglucose and trigalloylglucose) and the ellagitannins HHDP-galloylglucose were also proposed. Phenolic acid derivatives of dicaffeoyl-quinic acid and caffeic acid were also detected in the case of *L. spicata* and *M. integrifolia*.

Conclusions

D. antartica is a source of phlorotannins with higher degree of polymerization (trimer to octamer) than *L. spicata* (trimer to tetramer), whereas phlorotannins were not detected in *M. integrifolia*. A greater diversity of flavonoid compounds was identified in *M. integrifolia*, mainly as glycoside forms in all the extracts. The identification of polyphenols profile in both negative and positive ionization mode, together with the quantification of total phenolic compounds as well as the antioxidant activity of these Chilean marine resources opens new opportunities for their use in the food and pharmaceutical industries.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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