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Macromolecular Antioxidants and Dietary Fiber in Edible Seaweeds.

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4

5 # MACROMOLECULAR ANTIOXIDANTS AND DIETARY FIBER IN EDIBLE
6 # SEAWEEDS

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23 **ABSTRACT**

24 Seaweeds are rich in different bioactive compounds with potential uses in drugs,
25 cosmetics and the food industry. The objective of this study was to analyze
26 macromolecular antioxidants or non-extractable polyphenols, in several edible seaweed
27 species collected in Chile (*Gracilaria chilensis*, *Callophyllis concepcionensis*,
28 *Macrocystis pyrifera*, *Scytosyphon lomentaria*, *Ulva* sp. and *Enteromorpha compressa*),
29 including their first HPLC characterization. Macromolecular antioxidants are commonly
30 ignored in studies of bioactive compounds. They are associated with insoluble dietary
31 fiber and exhibit significant biological activity, with specific features that are different
32 from those of both dietary fiber and extractable polyphenols. We also evaluated
33 extractable polyphenols and dietary fiber, given their relationship with macromolecular
34 antioxidants. Our results show that macromolecular antioxidants are a major polyphenol
35 fraction (averaging 42% to total polyphenol content), with hydroxycinnamic acids,
36 hydroxybenzoic acids and flavonols being the main constituents. This fraction also
37 showed remarkable antioxidant capacity, as determined by two complementary assays.
38 The dietary fiber content was over 50% of dry weight, with some samples exhibiting the
39 target proportionality between soluble and insoluble dietary fiber for adequate nutrition.
40 Overall, our data show that seaweed could be an important source of commonly ignored
41 macromolecular antioxidants.

42

43 **Keywords**: edible seaweeds; polyphenols; macromolecular antioxidants, dietary fiber

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47 **PRACTICAL APPLICATION**

48 In this study, the composition of several edible seaweeds from Chile, in terms of
49 macromolecular antioxidants and dietary fiber, was evaluated. All the seaweeds showed
50 relevant content of these constituents. Given the nutritional interest of these compounds,
51 the consumption of these seaweeds might be promoted within the frame of a healthy
52 diet and they can also be used as sources of macromolecular antioxidants and dietary
53 fiber for the production of new ingredients.

INTRODUCTION

Seaweeds, or macroalgae, are very rich in a range of compounds that exhibit significant biological activities, in particular, polyphenols and dietary fiber (Rupérez and Saura-Calixto 2001). Thus, they are promising candidates for use in the design and production of drugs, foods, dietary supplements and cosmetics, and this potential is increasingly being exploited (Li and Kim 2011). However, in order to move forward with this, it is necessary to have a complete characterization of the bioactive compounds included in these natural products.

With regards to polyphenols, brown seaweeds (Phaeophyta) are known to contain phlorotannins (Li and Kim 2001), a family of structures derived from the polymerization of phloroglucinol (1,3,5-trihydroxybenzene). In contrast, the most characteristic phenolic compounds in red seaweeds (Rhodophyta) and green seaweeds (Chlorophyta) are bromophenols, which are polyhydroxylated and polybromated structures derived from the transformation by bromoperoxidases of polyphenols (Flodin and Whitfield 1999). Both phlorotannins and bromophenols are specific to seaweeds and are not found in any other natural product. They have been reported to have different biological effects, such as antioxidant and anti-inflammatory activities (Li and others 2011; Liu and others 2011).

Most studies on polyphenols in seaweeds only consider a fraction of them, the so-called extractable polyphenols, i.e., those present in the supernatants of the aqueous-organic extractions that are commonly analyzed. However, an important fraction of polyphenols remains in the residues of these extractions, specifically the macromolecular antioxidants (MACAN) or non-extractable polyphenols. They are either macromolecular phenolic compounds or small polyphenols and carotenoids associated

78 with macromolecules of the food matrix (Pérez-Jiménez and Saura-Calixto 2015). Thus,
79 there is a gap in most of the literature on polyphenols from different origins, including
80 those from seaweeds, since MACAN are not included. This gap was recently shown, for
81 instance, in common fruits and vegetables (Pérez-Jiménez and Saura-Calixto 2015).
82 Therefore, in order to properly evaluate total polyphenol content in a sample, analyses
83 of both extractable polyphenols and MACAN should be carried out. Moreover,
84 including MACAN in studies of food antioxidants is especially relevant for their
85 quantitative contribution to total antioxidants, and also for the emerging evidence of
86 their physiological effects (Pérez-Jiménez and others 2013).

87 At the same time, MACAN are related to another well-known bioactive constituent of
88 seaweeds: dietary fiber. For a long time, polyphenols and dietary fiber were considered
89 to be independent constituents. However, research during last decade have showed that
90 a fraction of polyphenols, in particular MACAN, is indeed as a constituent of dietary
91 fiber (Le Bourvellec and Renard 2005; Bunzel and others2006; Goñi and others 2009;
92 Saura-Calixto 2011). Therefore, an additional role of dietary fiber would be the delivery
93 of MACAN through the digestive tube (Saura-Calixto 2011). Although many studies
94 have explored dietary fiber in seaweeds, the contribution of MACAN to this dietary
95 constituent in these natural products has not been considered.

96 Therefore, this study aimed to characterize MACAN in a selection of edible seaweeds,
97 also analyzing their closely related constituents: extractable polyphenols and dietary
98 fiber.

99 **MATERIALS AND METHODS**

100 **Chemicals and reagents**

101 Pepsin (2000 FIP-U/g) and glucose were obtained from Merck (Darmstadt, Germany).
102 Amyloglucosidase (14 IU/mg) was from Roche (Manheim, Germany). Pancreatin, α-
103 amylase (17.5 IU/mg), ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid),
104 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), *p*-coumaric acid,
105 gallic acid and quercetin were all obtained from Sigma-Aldrich (St. Louis, MO, USA).
106 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was from Fluka Chemicals (Madrid, Spain).
107 Dinitrosalicylic acid and iron III-chlorure-6-hydrate were from Panreac (Castellar del
108 Vallés, Barcelona, Spain).

109 **Samples**

110 In October 2013, the following macroalgae were collected at the Huinay Scientific Field
111 Station (42°22´ S, 72°24´ W), located in Comau Fjord: the red seaweeds *Gracilaria*
112 *chilensis* and *Callophyllis concepcionensis*; the brown seaweeds *Macrocystis pyrifera*
113 and *Scytosyphon lomentaria*; and the green seaweeds *Ulva* sp. and *Enteromorpha*
114 *compressa*. This area was highly representative of the fjords of Southern Chile.
115 *Callophyllis* was collected in the subtidal zone by scuba diving, while the other samples
116 were collected in the intertidal zone. Once collected, they were properly identified
117 according to morphological characteristics and they were dried at 50ºC for 3 h, milled to
118 a particle size of 0.5 mm in a ZM 2000 centrifuge mill (Retsch, Haan, Germany) and
119 stored under vacuum until analyzed.

120 **Polyphenols and associated antioxidant capacity**

121 **Obtaining polyphenol fractions**

122 Two different procedures were used to obtain polyphenol fractions: a) aqueous–organic
123 extraction followed by different hydrolysis procedures in order to release non-
124 extractable polyphenols or MACAN present in the residues of the extraction; b)

125 extractions with pressurized hot water as an alternative procedure for a direct release of
126 MACAN from the original matter.

127 For the aqueous–organic extractions, dried algal samples were subjected to successive
128 extraction with methanol/water (50:50 v/v, pH 2) and with acetone/water (70:30, v/v), a
129 procedure previously applied for the extraction of polyphenols in very different natural
130 products (Pérez-Jiménez and others 2008). The extracts were combined and the
131 supernatant that corresponded to extractable polyphenols. Independent residues from
132 removing the extractable polyphenols were subjected to two different procedures in
133 order to obtain the two different fractions of MACAN. To obtain hydrolyzable
134 polyphenol concentrates, the residues were treated with methanol and concentrated
135 sulfuric acid at 85ºC for 20 h (Arranz and others 2009; Hartzfeld and others 2002),
136 followed by pH adjustment to 5.5 and salt removal with an Oasis HLB cartridge (5400
137 mg, 3cc, ref. 30 μm) from Waters (Milford, MA, USA) (Pérez-Jiménez and Saura-
138 Calixto 2015). To obtain non-extractable proanthocyanidin concentrates, the residues
139 were treated with butanol/$FeCl_3$ (5:95, v/v) at 100ºC for 1 h (Pérez-Jiménez and others
140 2009; Porter and others 1985).

141 Regarding the extraction with pressurized hot water, a *Gracilaria* sample was mixed
142 with neutral quartz sand and placed in a stainless steel extraction cell in order to carry
143 out the successive extractions at different temperatures with MilliQ water in Accelerated
144 Solvent Extraction equipment (ASE 150, Dionex, Sunnyvale, CA, USA) (Vergara-
145 Salinas and others 2012). Successive extractions were carried out at 100ºC for 5 minutes
146 (3 extraction cycles), at 150ºC for 5 minutes (3 extraction cycles), and at 200ºC for 30
147 minutes (1 extraction cycle).

148 **Polyphenol analysis**

149 The polyphenol contents of the extractable polyphenol concentrates (obtained by
150 aqueous–organic extraction and by pressurized hot water extraction) and the
151 hydrolyzable polyphenol concentrates were evaluated using the spectrophotometric
152 Folin-Ciocalteau assay (Singleton and others 1998). The non-extractable
153 proanthocyanidin content was determined in the concentrates by measuring the sum of
154 absorbance at 450 and 555 nm; the results are expressed as mg non-extractable
155 proanthocyanidins/100 g dw, by using a standard curve from a polymeric
156 proanthocyanidin concentrate (Zurita and others 2012). All these measurements were
157 carried out in a Lambda 12 spectrophotometer (Perkin-Elmer, Waltham, MA, USA).

158 Additionally, the profiles of the polyphenol classes in the hydrolyzable polyphenol
159 concentrates were determined by high- performance liquid chromatography with diode
160 array detection (HPLC-DAD) in Agilent 1200 series equipment (Agilent Technologies,
161 Waldbroon, Germany), according to a procedure previously validated (Arranz and
162 others 2009; Pérez-Jiménez and Saura-Calixto 2015). A 20 µL sample was separated in
163 a Luna C18 (50 x 2.1 mm i.d.) 3.5-µm particle size column with a Phenomenex
164 Securityguard C18 (4 x 3 mm i.d.) column (Torrance, CA, USA). Gradient elution was
165 performed with a binary system consisting of [A] 0.1% aqueous formic acid and [B]
166 0.1% formic acid in acetonitrile. The following increasing linear gradient (v/v) of [B]
167 was used: 0 min 6% B, 10 min, 23% B; 15 min, 50% B; 20 min, 50% B; 23 min, 100%
168 B; 25 min, 100% B; 27 min, 6% B and 30 min, 6% B. The flow was set at 0.4 mL/min.
169 Detection was carried out at several wavelengths corresponding to the different
170 polyphenol classes: 280 nm (hydroxybenzoic acids), 320 nm (hydroxycinnamic acids)
171 and 365 nm (flavonols). Polyphenols belonging to the different classes were quantified
172 using a corresponding standard: gallic acid for hydroxybenzoic acids (y= 85.594x –
173 3.6489, R^2= 0.9983), *p*-coumaric acid for hydroxycinnamic acids (y= 152.52 x + 18.922

174 , R^2= 1), and quercetin for flavonols (y= 40.756x + 69.24, R^2= 0.9914). Nevertheless,
175 the selected standard may still show a different response from that of the actual
176 compound in the sample, so this method cannot be considered to provide proper
177 quantification and should therefore be used mainly for comparative purposes.

178 **Antioxidant capacity**

179 Antioxidant capacity was determined by ferric reducing/antioxidant power (FRAP)
180 and 2, 2′-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) assays in both
181 extractable polyphenols and hydrolyzable polyphenols obtained from the six edible
182 seaweeds included in the study as described above. Additionally, the ABTS assay was
183 carried out in the extracts of *Gracilaria chilensis* obtained with pressurized hot water.

184 FRAP reagent, freshly prepared and warmed to 37 °C, was mixed with distilled water
185 and the test sample, standard or appropriate blank reagent. Readings at 595 nm in a
186 Lambda 12 spectrophotometer after 30 min were selected to calculate the FRAP values
187 (Benzie and Strain, 1996; Pulido and others 2000). For ABTS assays, after the addition
188 of the sample or Trolox standard to the $ABTS^{\bullet+}$ solution, absorbance readings were
189 taken at 595 nm every 20 s for 6 min by a DU-640 spectrophotometer (Beckman
190 Instruments Inc., Fullerton, CA, USA). The percentage inhibition of absorbance was
191 plotted against time and the area under the curve (0-6 min) was calculated (Re and
192 others 1999).

193 **Dietary fiber analysis and evaluation of functional properties**

194 Dietary fiber was determined using the indigestible fraction method (Goñi and others
195 2009), in which the six dried edible seaweeds were subjected to several enzymatic
196 treatments (pepsin, pancreatin, α-amylase and amyloglucosidase) and to dialysis in
197 order to remove the digestible components of the sample and to separate soluble dietary

198 fiber from insoluble dietary fiber. In the soluble dietary fiber, non-starch
199 polysaccharides were hydrolyzed with sulfuric acid and spectrophotometrically
200 quantitated in a Lambda 12 spectrophotometer after alkalinization and reaction with
201 dinitrosalicylic acid (Englyst and Cummings, 1988). Insoluble dietary fiber was
202 determined by gravimetry and the content of resistant protein (determined by using an
203 automated nitrogen analyzer FP-2000, Dumas Leco Corp., Waltham, MA, USA) was
204 substracted. Total dietary fiber was determined as the sum of the soluble and the
205 insoluble dietary fiber.

206 Procedures previously reported (Rupérez and others 2001) were used to evaluate dietary
207 fiber’s functional properties: water retention capacity, where a distilled water–algal
208 sample mixture was centrifuged and the residue was weighed, dried and weighed again;
209 oil retention capacity, determined by the same procedure but starting with a mixture of
210 commercial extra virgin olive oil instead of water; and swelling capacity, calculated as
211 the amount of distilled water added to a known initial volume of sample, minus the final
212 volume after 18 h.

213 **Statistical analysis**

214 Three parallel extractions were carried out on each sample. The determinations were
215 performed in duplicate for each extract and are reported on a dry matter basis. The
216 results are expressed as the mean value $\pm$ s.d. Levene’s test and the Kolmogorov-
217 Smirnov test were applied to assess variance equality and normal distribution,
218 respectively. One-way analysis of variance, followed by Tukey's post-hoc significance
219 test, was used when the assumptions of normality and equal variance were met.
220 Otherwise, non-parametric tests (Kruskal–Wallis and Mann–Whitney *U* rank-sum) were

221 used to assess significance. Differences were considered to be statistically significant
222 for $P < 0.05$. The SPSS IBM22 for Windows was used throughout.

223 **RESULTS AND DISCUSSION**

224 **Macromolecular antioxidants**

225 Six edible seaweeds were included in this study. Some of them are already consumed in
226 many countries, such as *Ulva* sp. (commonly known as sea lettuce) or *Scytosyphon*
227 *lomentaria* (known in Japan as kayamo-nori), while others are not yet commonly
228 consumed. However, although some previous studies (Kuda and others 2005; Shalaby
229 and others 2011; Tello-Ireland and others 2011) reported the antioxidant capacity or the
230 extractable polyphenols contents of some of these species, no systematic study of their
231 MACAN content had been previously carried out.

232 The total polyphenol contents of the samples, including extractable polyphenols and
233 MACAN, are shown in **Table 1**. For extractable polyphenols, the values were in the
234 same range as those previously described, for instance for another seaweed of the genus
235 *Gracilaria* (Tello-Ireland and others 2011). But the most remarkable fact is the
236 MACAN content that these samples exhibited, in particular in the fraction of
237 hydrolyzable polyphenols. All the samples exhibited significant hydrolyzable
238 polyphenol content: between 200 and 800 mg/100 g dry weight. Indeed, all the
239 seaweeds except *Scytosiphon* contained more hydrolyzable polyphenols than extractable
240 polyphenols, which indicates the importance of this fraction. Overall, the mean
241 contribution of hydrolyzable polyphenols to total polyphenol content was 41%. Despite
242 this, only a few studies have previously evaluated the hydrolyzable polyphenol content
243 of some seaweeds of other genera (Koivikko and others 2005; Vidal and others 2009).

244 Furthermore, a preliminary HPLC analysis –per classes- of hydrolyzable polyphenols
245 was carried out on a selected sample of each phylum- brown, red and green seaweeds
246 (**Table 2**). This method was recently validated in a wide selection of common fruit and
247 vegetables (Pérez Jiménez and Saura-Calixto, 2015). To the best of our knowledge, this
248 is the first time that a chromatography method has been applied for the characterization
249 of hydrolyzable polyphenols in seaweeds. Hydroxybenzoic acids, hydroxycinnamic
250 acids and flavonols were detected in the three samples selected. Considering the sum of
251 the different classes of hydrolyzable polyphenols, the ranking of total content was *Ulva*
252 > *Gracilaria* > *Macrocystis*. Although phlorotannins and bromophenols are the most
253 well-known polyphenol classes in seaweeds, some previous studies reported the
254 presence in them of polyphenols belonging to the classes we identified here.:
255 hydroxybenzoic acids in other species of *Ulva* and *Enteromorpha*, (Flodin and others
256 1999; Mamatha and others 2007), hydroxycinnamic acids in *Enteromorpha* (Mamatha
257 and others 2007) and flavonols, present as glycosides with fucose, a characteristic
258 seaweed sugar, in red seaweeds (Zeng and others 2001). Nevertheless, in those cases
259 they were present in the fraction of extractable polyphenols. Therefore, we show here
260 for the first time that these polyphenol classes are also present as constituents of
261 MACAN in seaweeds.

262 The analytical conditions used in this assay, specifically acid hydrolysis instead of
263 alkaline hydrolysis for the release of these compounds, have been previously validated
264 in several previous studies, including samples of different nature (Arranz and others
265 2009; Pérez-Jiménez and Saura-Calixto, 2015); similarly, the drastic conditions used
266 have been proven to be needed in order to release hydrolysable polyphenols from their
267 strong associations with macromolecules in the food matrix (Pérez-Jiménez and Torres
268 2011). Nevertheless, we cannot rule out that these conditions degrade some of the

269 original phenolic structures; this resulted in some of the signals not corresponding in
270 fact to the direct release of small phenolic compounds from the food matrix, but rather
271 to the release followed by partial degradation. This has been previously described for
272 some flavonoids present in the fraction of hydrolysable polyphenols in common fruits
273 (Pérez-Jiménez and Saura-Calixto 2015) and it could have been the case in the instance
274 of the fraction of phlorotannins in brown seaweeds that is associated with alginic acid as
275 a cell wall constituent (Arnold and Targett 2003). The results obtained show the
276 relevance of the commonly ignored hydrolysable polyphenols in these natural products,
277 which should be therefore included in future studies of bioactive constituents in
278 seaweeds. At the same time, the relevance of these compounds emphasizes the need for
279 developing methodologies that allow their direct determination in the original sample,
280 i.e., as associated with macromolecules, instead of small phenolic compounds released
281 after hydrolysis; thus, it is remarkable that the analysis of MACAN is still much less
282 developed than the analysis of extractable polyphenols (Pérez-Jiménez and Torres,
283 2011).

284 Non-extractable proanthocyanidins, the other class of macromolecular antioxidants that
285 may be found in extraction residues, were also searched for in the samples according to
286 the procedure described above. This is a spectrophotometric method, where the
287 cleavage of proanthocyanidins releases colored cations with specific absorbance
288 maximum. These compounds were not detected in any of the samples, which provided
289 colorless hydrolyzates after treatment with butanol/HCl with no absorbance at the
290 measured wavelengths.

291 Additionally, the efficiency of pressurized hot water as an alternative procedure for the
292 direct release of MACAN from the original matter was evaluated, since this technique
293 would reduce the use of solvents and the analytical steps. For this purpose, *Gracilaria*

294 was used as a case-study. First, common conditions for extractions with pressurized hot
295 water – 3 cycles for 5 min at 100ºC and 150ºC- were tested. Comparing the results
296 obtained by this technique (**Table 3**) with those obtained after aqueous–organic
297 extraction from *Gracilaria* (**Table 1**), the extractions with pressurized hot water did not
298 result in a significant increase in total polyphenol content, as previously reported for
299 other seaweeds when using this procedure at 90ºC (Heffernan and others 2014).
300 Therefore, these conditions did not allow the release of a part of MACAN from the
301 original matter. Additionally, an extraction was carried out with pressurized hot water at
302 200ºC for 30 min. These drastic conditions tried to emulate those needed in the
303 chemical hydrolysis described above to release hydrolysable polyphenols (sulfuric acid,
304 85ºC, 20 h). The extract obtained from this process contained more polyphenols than
305 that obtained by aqueous–organic extraction, what was probably due to the release of a
306 fraction of hydrolyzable polyphenols under the drastic conditions used. Nevertheless,
307 the generation of new antioxidants compounds with these conditions, as reported by
308 other authors (Plaza and others 2010; Vergara-Salinas and others 2012), should not be
309 discarded when applying this procedure as an alternative technique for the release of
310 MACAN.

311 **Antioxidant capacity from macromolecular antioxidants**

312 The antioxidant capacity derived from both extractable and hydrolyzable polyphenols
313 was evaluated in these seaweeds by using two complementary methods: ABTS assay,
314 based on radical scavenging capacity, and the FRAP test, which evaluates the metal
315 reducing power of a sample.

316 Regarding the ABTS assay (**Table 4**), the results for extractable polyphenols were in
317 agreement with previous data for some other species of the genera studied here

318 (Francavilla and others 2013). *Enteromorpha* showed the highest value, despite not
319 being the sample with the highest polyphenol content. However, it was recently reported
320 that this seaweed is a potent antioxidant due to its content of a non-phenolic compound,
321 ethyl [2-(benzylsulfunyl)-4-(4-nitrophenyl)-1H-imidazol-1-yl] acetate (Shalaby and
322 others 2011). The presence of this compound in the analyzed sample might therefore
323 explain this apparent discrepancy between polyphenol content and antioxidant capacity.
324 Be that as it may, considering all the samples, significant correlations were found
325 between extractable and hydrolyzable polyphenol contents as determined by the Folin-
326 Ciocalteau assay and their associated antioxidant capacity determined by ABTS assay
327 ($P < 0.001$ and $P < 0.01$, respectively).

328 Antioxidant capacity values again show the relevance of MACAN as key bioactive
329 compounds in seaweeds. In particular, *Gracilaria* and *Callophyllis*, that provided an
330 ABTS value below the limit of quantification for extractable polyphenols, provided
331 remarkable ABTS values for hydrolyzable polyphenols, but the opposite happened with
332 *Scytosiphon*. Therefore, both fractions should be systematically considered in order to
333 have a closer approach to the antioxidant capacity of seaweeds. Regarding the use of
334 pressurized hot water in *Gracilaria* as an alternative procedure for the release of
335 MACAN and their associated antioxidant capacity (**Table 3**), it yielded similar
336 tendencies for that ABTS assay as those obtained for the polyphenol content
337 determination by the Folin-Ciocalteau assay in those extracts, as described above- see
338 "Macromolecular antioxidants".

339 The same tendencies observed for the antioxidant capacity in the six edible seaweeds
340 when applying the ABTS assay were observed by the FRAP assay (**Table 4**), where
341 significant correlations were found again between these values and polyphenol content
342 data ($P < 0.05$ for extractable polyphenols and $P < 0.001$ for hydrolyzable polyphenols).

343 *Gracilaria*, *Callophyllis* and *Enteromorpha* showed the highest antioxidant capacity
344 associated with hydrolyzable polyphenols. In fact, when using this assay, this fraction of
345 polyphenols provided at least a 40% of the total antioxidant capacity in all the samples.

346 **Dietary fiber content and associated functional properties**

347 The dietary fiber contents were evaluated in the samples previously selected for HPLC
348 analysis of hydrolyzable polyphenols, i.e., one of each phylum- brown, red and green
349 seaweeds. These samples showed a remarkable total dietary fiber content (**Table 5**):
350 above 50% of dry weight, which is higher than that commonly found in plant foods, as
351 previously reported (Rupérez and others 2001). Previous studies with other species of
352 the genera *Ulva* and *Gracilaria* obtained similar values to those found here (Wijsekara
353 and others 2011). Both *Ulva* and *Gracilaria* showed a high proportion of soluble dietary
354 fiber (33% and 40%, respectively), a fraction of dietary fiber for which specific health
355 effects have been reported (Marlett 1997). Although soluble dietary fiber from brown
356 and red seaweeds have commonly received more attention, green seaweeds such as
357 *Ulva* also have a specific class of soluble dietary fiber, the ulvans, whose biological
358 activities have yet to be elucidated (Alves and others 2013).

359 Some functional properties associated with dietary fiber, related to its *in vivo*
360 physiological activities as well as to its technological potential (Guillon and Champ
361 2000), were also evaluated (**Table 6**). *Ulva* showed the highest values of all of them,
362 especially in the case of oil retention capacity. This makes this green seaweed especially
363 recommendable for stabilizing food emulsions with a high percentage of fat. Moreover,
364 from a nutritional point of view, it may show an enhanced capacity to reduce fat
365 absorption, which should be confirmed in *in vivo* studies.

366 Dietary fiber has been traditionally considered as a food component independent of
367 polyphenols. However, in recent years it has been emphasized that a fraction of dietary
368 polyphenols are indeed constituents of dietary fiber in different types of foods of
369 different nature, e.g., fruits, nuts or foods subjected to the Maillard reaction (Goñi and
370 others 2009; Pérez-Jiménez and others 2014). In the case of seaweeds, some authors
371 have described that a fraction of polyphenols appears as constituents of the cell wall
372 (Arnold and Targett 2003; Koivikko and others 2005), but polyphenols and dietary fiber
373 are still commonly considered independently. Here, we evaluated dietary fiber by the
374 indigestible fraction method and insoluble dietary fiber was quantified gravimetrically.
375 Based on previous studies (Goñi and others 2009; Pérez-Jiménez and others 2014) it is
376 to be expected that in fact this gravimetric residue contains not only non–digestible
377 polysaccharides, but also a fraction of MACAN. This interaction between these two
378 constituents should be taken into account either when carrying out analysis of dietary
379 fiber (since in fact it will include MACAN) or of polyphenols (since an important
380 fraction of them will be ignored, if MACAN associated with the food matrix are not
381 released and also analyzed). The contribution of MACAN to the functional properties of
382 dietary fiber should also be further studied. Moreover, the fact of a single matrix
383 including significant contents of dietary fiber and polyphenols -a fraction of both
384 constituents forming a single complex- may create synergistic bioactivities (Pérez-
385 Jiménez and others 2008), which makes these products especially relevant as sources of
386 bioactive compounds. It should be remarked that MACAN present physiological
387 activities with specific features as compared to both extractable polyphenols
388 (stimulation of colonic fermentation due to their association with dietary fiber, sustained
389 circulation of bioactive metabolites due to a prolonged fermentation) (Pérez-Jiménez
390 and others 2013) and dietary fiber (production of colonic metabolites different to those

391 derived from carbohydrates fermentation, antioxidant capacity and other biological
392 effects) (Saura-Calixto 2011).

393

394 **CONCLUSIONS**

395 MACAN, commonly ignored in studies on bioactive compounds in seaweeds, were
396 analyzed in six edible seaweeds. They were present as hydrolyzable polyphenols in all
397 the samples, with a mean contribution to total polyphenols content of 41%.
398 Additionally, the first HPLC data on their presence in seaweed were provided, detecting
399 hydroxybenzoic acids, hydroxycinnamic acids and flavonols. MACAN also provided
400 relevant antioxidant capacity by FRAP and ABTS assays. This data shows the relevance
401 of MACAN in seaweeds, as major bioactive constituents with specific biological
402 activity.

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413 **AUTHORS' CONTRIBUTIONS**

414 J.P.-J. and F.S.-C. designed the research and collected the samples. N.S.-P., J.P.-J. and
415 J.R.V.-S. carried out the experimental work. J.R.P.-C. designed and supervised the
416 extraction with pressurized hot water. All the authors revised the manuscript.

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Table 1. Polyphenol content in Chilean seaweeds, as determined by Folin-Ciocalteau assay (mg gallic acid equivalents/100 g dw)

Sample	Extractable polyphenols	Macromolecular antioxidants[a]	Total polyphenols	Macromolecular antioxidants contribution to total content (%)
Gracilaria	216.4 ± 6.6[a]	792.7 ± 63.5[a]	1025.0 ± 57.7[a]	21
Callophyllis	218.6 ± 20.7[a]	841.3 ± 54.7[a]	1056.0 ± 42.6[a]	21
Macrocystis	343.2 ± 22.4[b]	593.9 ± 22.2[b]	943.7 ± 23.2[b]	36
Scytosiphon	1297.4 ± 55.1[c]	280.0 ± 19.8[c]	1577.4 ± 68.4[c]	82
Ulva	551.1 ± 17.5[d]	677.8 ± 48.6[b,d]	1228.9 ± 34.4[d]	45
Enteromorpha	498.9 ± 42.2[d]	704.5 ± 54.7[b,d]	1215.6 ± 94.8[d]	41

Comparisons were performed using Kruskal-Wallis and Mann-Whitney *U* tests (significance level, $P < 0.05$)

[a] Corresponding exclusively to the class of hydrolysable polyphenols, since non-extractable proanthocyanidins were not detected in the samples.

Table 2. Hydrolyzable polyphenols content in Chilean seaweeds, as determined by HPLC-DAD (mg/100 g dw)

Sample	Hydroxybenzoic acids	Hydroxycinnamic acids	Flavonols	Total
Gracilaria	276.9 ± 30.4[a]	60.3 ± 6.7[a]	92.8 ± 12.2[a]	430.1 ± 43.6[a]
Macrocystis	154.3 ± 26.5[b]	30.5 ± 0.5[b]	70.0 ± 14.8[a]	254.8 ± 37.7[b]
Ulva	386.3 ± 9.2[c]	63.0 ± 5.2[a]	124.2 ± 8.2[b]	573.6 ± 7.8[c]

Comparisons were made using one-way ANOVA and Tukey's *post hoc* significance tests (significance level, $P < 0.05$)

Table 3. Polyphenol content and associated antioxidant capacity (ABTS assay) in extracts of *Gracilaria chilensis*, obtained with pressurized hot water (µmol Trolox/100 g dw).

Treatment		Polyphenol content (mg/100g dw)	ABTS (µM Trolox/100g dw)
100ºC, 5'	Extraction 1	195.0 ± 2.6[a]	1434.9 ± 142.7[a]
	Extraction 2	8.7 ± 0.1[b]	22.1 ± 2.9[b]
	Extraction 3	4.8 ± 0.6[c]	< LOQ[c]
150ºC, 5'	Extraction 1	44.4 ± 1.6[d]	< LOQ[c]
	Extraction 2	22.9 ± 3.5[e]	< LOQ[c]
	Extraction 3	10.7 ± 0.9[f]	17.1 ± 4.3[b]
200ºC, 30'		1,016.6 ± 52.9[g]	23,116.1 ± 1114.2[d]

ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid); LOQ, limit of quantification

Comparisons were performed using Kruskal-Wallis and Mann-Whitney *U* tests (significance level, $P < 0.05$)

Table 4. Antioxidant capacity in Chilean seaweeds, as determined by ABTS and FRAP assays (µmol Trolox/100 g dw)

	ABTS assay			FRAP assay		
Sample	Extractable polyphenols	Hydrolyzable polyphenols	Total polyphenols[1]	Extractable polyphenols	Hydrolyzable polyphenols	Total polyphenols
Gracilaria	< LOQ[a]	2065.4 ± 267.6[a]	2065.4 ± 267.6[a]	511.7 ± 29.6[a]	2527.7 ± 218.8[a]	2928.0 ± 138.0[a]
Callophyllis	< LOQ[a]	453.8 ± 50.6[b]	453.8 ± 50.6[b]	1267.0 ± 95.6[b]	2830.7 ± 152.4[a]	4097.7 ± 246.0[b]
Macrocystis	2359.6 ± 576.1[b]	562.1 ± 90.0[b]	2921.7 ± 511.8[c]	1335.5 ± 96.3[b]	2260.7 ± 95.6[b]	3729.5 ± 144.1[c]
Scytosiphon	4529.1 ± 531.8[c]	< LOQ[c]	4529.1 ± 531.8[d]	1912.7 ± 57.0[c]	1478.9 ± 32.9[c]	3408.1 ± 36.9[c]
Ulva	4206.2 ± 376.0[c]	136.4 ± 11.0[d]	4354.0 ± 430.0[d]	1956.3 ± 162.3[c]	2202.3 ± 103.7[b]	4135.9 ± 175.1[b]
Enteromorpha	6530.4 ± 527.8[d]	306.9 ± 80.3[e]	6753.6 ± 574.4[e]	3059.9 ± 287.1[d]	2851.8 ± 212.6[a]	6155.5 ± 320.3[d]

[1] Calculated as sum of the antioxidant capacity of extractable polyphenols and the antioxidant capacity of hydrolysable polyphenols.

ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid); FRAP, ferric/reducing antioxidant power; LOQ, limit of quantification.

Comparisons were performed using Kruskal-Wallis and Mann-Whitney *U* tests (significance level, $P < 0.05$) within the same method.

1 **Table 5**. Dietary fiber content and associated functional properties in Chilean seaweeds

Sample	Soluble dietary fiber (g/100 g dw)	Insoluble dietary fiber (g/100 g dw)	Total dietary fiber (g/100 g dw)	Water retention capacity (g/g dw)	Oil retention capacity (mL/g dw)	Swelling capacity (mL/g dw)
Gracilaria	23.8 ± 1.9[a]	36.0 ± 3.7[a]	59.8 ± 4.2[a]	13.9 ± 0.3[a]	1.1 ± 0.1[a]	6.4 ± 0.01[a]
Macrocystis	6.4 ± 0.3[b]	43.6 ± 1.2[b]	50.0 ± 1.2[b]	20.5 ± 0.5[b]	1.6 ± 0.05[b]	6.7 ± 0.08[b]
Ulva	18.2 ± 0.8[c]	36.0 ± 1.2[a]	54.2 ± 1.4[c]	23.9 ± 0.5[c]	3.2 ± 0.04[c]	6.5 ± 0.006[c]

2 Comparisons were performed using Kruskal-Wallis and Mann-Whitney *U* tests (significance level, $P < 0.05$)

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