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**MACROMOLECULAR ANTIOXIDANTS AND DIETARY FIBER IN EDIBLE
SEAWEEDES**

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ABSTRACT

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

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

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

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

At the same time, MACAN are related to another well-known bioactive constituent of seaweeds: dietary fiber. For a long time, polyphenols and dietary fiber were considered to be independent constituents. However, research during last decade have showed that a fraction of polyphenols, in particular MACAN, is indeed as a constituent of dietary fiber (Le Bourvellec and Renard 2005; Bunzel and others 2006; Goñi and others 2009; Saura-Calixto 2011). Therefore, an additional role of dietary fiber would be the delivery of MACAN through the digestive tube (Saura-Calixto 2011). Although many studies have explored dietary fiber in seaweeds, the contribution of MACAN to this dietary constituent in these natural products has not been considered.

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

MATERIALS AND METHODS

Chemicals and reagents

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

Samples

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

Polyphenols and associated antioxidant capacity

Obtaining polyphenol fractions

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

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

For the aqueous–organic extractions, dried algal samples were subjected to successive extraction with methanol/water (50:50 v/v, pH 2) and with acetone/water (70:30, v/v), a procedure previously applied for the extraction of polyphenols in very different natural products (Pérez-Jiménez and others 2008). The extracts were combined and the supernatant that corresponded to extractable polyphenols. Independent residues from removing the extractable polyphenols were subjected to two different procedures in order to obtain the two different fractions of MACAN. To obtain hydrolyzable polyphenol concentrates, the residues were treated with methanol and concentrated sulfuric acid at 85°C for 20 h (Arranz and others 2009; Hartzfeld and others 2002), followed by pH adjustment to 5.5 and salt removal with an Oasis HLB cartridge (5400 mg, 3cc, ref. 30 µm) from Waters (Milford, MA, USA) (Pérez-Jiménez and Saura-Calixto 2015). To obtain non-extractable proanthocyanidin concentrates, the residues were treated with butanol/FeCl₃ (5:95, v/v) at 100°C for 1 h (Pérez-Jiménez and others 2009; Porter and others 1985).

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

Polyphenol analysis

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

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

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

Antioxidant capacity

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

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

Dietary fiber analysis and evaluation of functional properties

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

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

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

Statistical analysis

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

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

RESULTS AND DISCUSSION

Macromolecular antioxidants

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

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

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

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

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

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

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

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

Antioxidant capacity from macromolecular antioxidants

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

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

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

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

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

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

Dietary fiber content and associated functional properties

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

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

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

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

CONCLUSIONS

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

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AUTHORS' CONTRIBUTIONS

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

REFERENCES

Alves A, Sousa RA, Reis RL. 2013. A practical perspective on ulvan extracted from green algae. *J Appl Phycol* 25: 407-424.

Arnold TM, Targett NM. 2003. To grow and defend: lack of tradeoffs for brown algal phlorotannins. *Oikos*: 100:406-08.

Arranz S, Saura-Calixto F. 2009. Analysis of polyphenols in cereals may be improved performing acidic hydrolysis: A study in wheat flour and wheat bran and cereals of the diet. *J Cer Sci* 51: 313-18.

Arranz S, Saura-Calixto F, Shaha S, Kroon PA. 2009. High contents of nonextractable polyphenols in fruits suggest that polyphenol contents of plant foods have been underestimated. *J Agric Food Chem* 57: 7298-7303.

Benzie IFF, Strain JJ. 1998. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol* 299: 15-27.

Bunzel M, Ralph J, Brüning P, Steihart, H. 2006. Structural identification of dehydrotriiferulic acid and dehydrotetraferulic acid isolated from insoluble maize bran fiber. *J Agric Food Chem* 54: 6409-18.

435 Flodin C, Whitfield FB. 1999. 4-hydroxybenzoic acid: A likely precursor of 2,4,6-
 436 tribromophenol in *Ulva lactuca*. *Phytochem* 51: 249-255.

437 Francavilla M, Franchi M, Monteleone M, Caroppo C. 2013. The red seaweed
 438 *Gracilaria gracilis* as a multi products source. *Marine Drugs* 11: 3754-3776.

439 Goñi I, Díaz-Rubio ME, Pérez-Jiménez J, Saura-Calixto F. 2009. Towards an updated
 440 methodology for measurement of dietary fibre, including associated polyphenols, in
 441 food and beverages. *Food Res Intl* 42:840-46.

442 Guillon F, Champ M. 2000. Structural and physical properties of dietary fibres, and
 443 consequences of processing on human physiology. *Food Res Intl* 33: 233-245.

444 Hartzfeld PW, Forkner R, Hunter MD, Hagerman AE. 2002. Determination of
 445 hydrolyzable tannins (gallotannins and ellagitannins) after reaction with potassium
 446 iodate. *J Agric Food Chem* 50: 1785-1790.

447 Heffernan N, Smyth TJ, Fitzgerald RJ, Soler-Vila A, Brunton N. 2014. Antioxidant
 448 activity and phenolic content of pressurised liquid and solid-liquid extracts from four
 449 Irish origin macroalgae. *Intl J Food Sci Technol* 49:1765-1772.

450 Koivikko R, Lojonen J, Honkanen T, Jormalainen V. 2005. Contents of soluble, cell-
 451 wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with
 452 implications on their ecological functions. *J Chem Ecol* 31: 195-212.

453 Kuda T, Tsunekawa M, Goto H, Araki Y. 2005. Antioxidant properties of four edible
 454 algae harvested in the Noto Peninsula, Japan. *J Food Compos Anal* 18: 625-633.

455 Le Bourvellec C, Renard M. 2005. Non-covalent interaction between procyanidins and
 456 apple cell wall material. Part II: quantification and impact of cell wall drying.
 457 *Biochimica Biophysica Acta* 1725: 1-9.

458 Li YX, Kim SK. 2011. Utilization of seaweed derived ingredients as potential
 459 antioxidants and functional ingredients in the food industry: An overview. *Food Sci*
 460 *Biotechnol* 20: 1461-1466.

461 Li YX, Kim SK. 2001. Phlorotannins as bioactive agents from brown algae. *Process*
 462 *Biochem* 46: 2219-2224.

463 Liu M, Hansen PE, Lin X. 2011. Bromophenols in marine algae and their bioactivities.
 464 *Marine Drugs* 9: 1273-1292.

465 Mamatha BS, Namitha KK, Senthil A, Smitha J, Ravishankar G.A. 2007. Studies on use
 466 of Enteromorpha in snack food. *Food Chem* 101: 1707-1713.

467 Marlett JA. 1997. Sites and mechanisms for the hypocholesterolemic actions of soluble
 468 dietary fiber sources. *Advances Experimental Med Biol* 427: 109-121.

469 Pérez-Jiménez J, Arranz S, Tabernero M, Díaz-Rubio ME, Serrano J, Goñi I, Saura-
 470 Calixto F. Updated methodology to determine antioxidant capacity in plant foods, oils
 471 and beverages: extraction, measurement and expression of results. *Food Res Intl* 41:
 472 274-85.

473 Pérez-Jiménez J, Serrano J, Tabernero M, Arranz S, Díaz-Rubio ME, García-Diz L and
 474 others. 2008b. Effects of grape antioxidant dietary fiber in cardiovascular disease risk
 475 factors. *Nutr* 24: 646-653.

476 Pérez-Jiménez J, Arranz S, Saura-Calixto F. 2009. Proanthocyanidin content in foods is
 477 largely underestimated in the literature data: An approach to quantification of the
 478 missing proanthocyanidins. *Food Res Intl* 42: 1381-1388.

479 Pérez-Jiménez J, Torres JL. 2011. Analysis of non-extractable polyphenols in foods: the
 480 current state of the art. *J Agric Food Chem* 59: 12713-24

481 Pérez-Jiménez J, Díaz- Rubio ME, Saura-Calixto F. 2013. Non-extractable polyphenols,
 482 a major dietary antioxidant: occurrence, metabolic fate and health effects. *Nutr Res Rev*
 483 26: 118-129.

484 Pérez-Jiménez J, Díaz-Rubio ME, Mesías M, Morales FJ, Saura-Calixto F. 2014.
 485 Evidence for the formation of Maillardized insoluble dietary fiber in bread: a specific
 486 kind of dietary fiber in thermally processed foods. *Food Res Intl* 55: 391-96.

487 Pérez-Jiménez J, Saura-Calixto F. 2015. Macromolecular antioxidants or non-
 488 extractable polyphenols in fruit and vegetables: intake in four European countries. *Food*
 489 *Res Intl* 74: 315-23.

490 Plaza M, Amigo-Benavent M, del Castillo MD, Ibáñez E, Herrero M. 2010.
 491 Neoformation of antioxidants in glycation model systems treated under subcritical water
 492 extraction conditions. *Food Res Intl* 43: 1123-1129.

493 Porter LJ, Hrstich LN, Chan BG. 1985. The conversion of procyanidins and
 494 prodelphinidins to cyanidin and delphinidin. *Phytochem.* 25: 223-230.

495 Pulido R, Bravo L, Saura-Calixto F. 2000. Antioxidant activity of dietary polyphenols as
 496 determined by a modified ferric reducing/antioxidant power assay. *J Agric Food Chem*
 497 48: 3396-3402.

498 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999.
 499 Antioxidant activity applying an improved ABTS radical cation decolorization assay.
 500 Free Radicals Biol Med 26: 1231-1237.

501 Rupérez P, Saura-Calixto F. 2001. Dietary fibre and physicochemical properties of
 502 edible Spanish seaweeds. Eur Food Res Technol 212: 349-354.

503 Saura-Calixto F. 2011. Dietary fiber as a carrier of dietary antioxidants: an essential
 504 physiological function. J Agric Food Chem 12: 43-49.

505 Shalaby EA, Shanab SMM, El-Fayoumy EA. 2011. *Enteromorpha compressa* exhibits
 506 potent antioxidant activity. J Biomed Biotechnol 2011: 726405.

507 Singleton VL, Orthofer R, Lamuela-Raventós RM. 1998. Analysis of total phenols and
 508 other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent.
 509 Methods Enzymol 299: 152-178.

510 Tello-Ireland C, Lemus-Mondaca R, Vega-Gálvez A, López J, Di Scala K. 2011.
 511 Influence of hot-air temperature on drying kinetics, functional properties, colour,
 512 phycobiliproteins, antioxidant capacity, texture and agar yield of alga *Gracilaria*
 513 *chilensis*. LWT Food Sci Technol 44: 2112-2118.

514 Vergara-Salinas JR, Pérez-Jiménez J, Torres JL, Agosin E, Pérez-Correa JR. 2012.
 515 Effects of temperature and time on polyphenolic content and antioxidant activity in the
 516 pressurized hot water extraction of deodorized thyme (*Thymus vulgaris*). J Agric Food
 517 Chem 60: 10920-10929.

518 Vidal A, Silva De Andrade-Wartha ER, De Oliveira E, Silva AM, Pavan R, Lima A,
 519 Fallarero and others 2009. Antioxidant activity and polyphenols of seaweed species
 520 *Halimeda opuntia* and *Halimeda monile*. Ars Pharmaceutica 50: 24-31.

- 521 Wijesekara I, Pangestuti R, Kim SK. 2011. Biological activities and potential health
522 benefits of sulfated polysaccharides derived from marine algae. Carbohydrate Polymers,
523 84: 14-21.
- 524 Zeng LM, Wang CJ, Su JY, Li D, Owen NL, Lu Y and others. 2001. Flavonoids from
525 the red alga *Acanthophora spicifera*. Chinese J Chem 19: 1097-1100.
- 526 Zurita J, Díaz-Rubio ME, Saura-Calixto F. 2012. Improved procedure to determine non-
527 extractable polymeric proanthocyanidins in plant foods. Intl J Food Sci Nutr 63: 936-
528 939.

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

Sample	Extractable polyphenols	Macromolecular antioxidants ^a	Total polyphenols	Macromolecular antioxidants contribution to total content (%)
<i>Gracilaria</i>	216.4 ± 6.6 ^a	792.7 ± 63.5 ^a	1025.0 ± 57.7 ^a	21
<i>Callophyllis</i>	218.6 ± 20.7 ^a	841.3 ± 54.7 ^a	1056.0 ± 42.6 ^a	21
<i>Macrocystis</i>	343.2 ± 22.4 ^b	593.9 ± 22.2 ^b	943.7 ± 23.2 ^b	36
<i>Scytosiphon</i>	1297.4 ± 55.1 ^c	280.0 ± 19.8 ^c	1577.4 ± 68.4 ^c	82
<i>Ulva</i>	551.1 ± 17.5 ^d	677.8 ± 48.6 ^{b,d}	1228.9 ± 34.4 ^d	45
<i>Enteromorpha</i>	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 <i>U</i> tests (significance level, <i>P</i> < 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
<i>Gracilaria</i>	276.9 ± 30.4 ^a	60.3 ± 6.7 ^a	92.8 ± 12.2 ^a	430.1 ± 43.6 ^a
<i>Macrocystis</i>	154.3 ± 26.5 ^b	30.5 ± 0.5 ^b	70.0 ± 14.8 ^a	254.8 ± 37.7 ^b
<i>Ulva</i>	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 ($\mu\text{mol Trolox}/100\text{ g dw}$).

Treatment		Polyphenol content ($\text{mg}/100\text{g dw}$)	ABTS ($\mu\text{M Trolox}/100\text{g dw}$)
100°C, 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
150°C, 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
200°C, 30'		$1,016.6 \pm 52.9^g$	$23,116.1 \pm 1114.2^d$

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 ($\mu\text{mol Trolox}/100 \text{ g dw}$)

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