

Nutritional and Functional Bioactivity Value of Selected Azorean Macroalgae: *Ulva compressa*, *Ulva rigida*, *Gelidium microdon*, and *Pterocladia capillacea*

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Abstract: This study presents information on the biochemical composition (dry weight basis), nutritional aspects, and angiotensin I-converting enzyme (ACE) inhibitory properties of selected macroalgae (*Ulva compressa*, *Ulva rigida*, *Gelidium microdon*, and *Pterocladia capillacea*) from Azores. Moisture content was very high (83.2% to 90.0% of fresh weight). Total dietary fiber (33.7% to 41.0%) that presented a good balance of both soluble and insoluble fibers (15.5% to 19.2% and 18.2% to 21.8%, respectively) was the most abundant component in these macroalgae. Protein and ash (ranged from 15.7% to 23.4% and 10.7% to 20.7%, respectively) were the 2nd most abundant components in red and green macroalgae, respectively. Moderate soluble carbohydrate contents (14.5% to 19.8%) were found in all species. Lipid contents were low (1.0% to 4.3%), particularly in *Ulva* species (1.0% to 1.7%), but contained higher unsaturated fatty acids (FAs) (7.5% to 32.9% and 29.6% to 69.2% of total FA for monounsaturated fatty acid and polyunsaturated fatty acid groups, respectively) than saturated fatty acid (23.3% to 46.8% of total FA) contents. All the macroalgal proteins had high digestibility *in vitro* (82.2% to 89.4%, relatively to sodium caseinate), contained high quantity of essential amino acids (45.3% to 58.1% of total amino acids), but in different proportions, and were rich in aspartic and glutamic acids that together account for 17.2% to 36.2% of the total amino acids. These results suggested that regular consumption of the selected macroalgae may improve human health and revealed that they can be used for producing food supplements for human and animal nutrition and/or pharmaceuticals with potential effect on the regional economy. Furthermore, the ACE-inhibitory IC₅₀ values of 0.095 to 0.695 mg/mL for the <1 kDa protein hydrolysate fraction revealed a potential impact on hypertension disorder.

Keywords: antihypertensive protein hydrolysates, functional foods, nutraceuticals, nutrient composition, pharmaceuticals

Introduction

Many species of marine macroalgae have been consumed in Asian countries since ancient times, especially China, Japan, and Korea, due to their high nutritional and low caloric values. They have also found use in traditional Eastern medicine because of their perceived health benefits (for example, Dharmaranda 2002; Kolanjinathan and others 2014). In the Western countries, macroalgae have mainly been exploited as a source of techno-functional polysaccharides (for example, the phycocolloids agar, carrageenans, and alginates) for the food, cosmetic, and pharmaceutical industries, among others (Bocanegra and others 2009; Faggio and others 2016), and as fertilizers and feed ingredients for animal nutrition (Pereira 2011; Makkar and others 2016). However, nowadays, edible macroalgae are increasingly consumed in

Europe, and in some countries are considered novel functional foods (Bocanegra and others 2009; Rupérez and others 2014).

Marine macroalgae, or seaweeds or sea vegetables as they are commonly known, are a large and diverse group of marine organisms that have developed complex biochemical pathways to survive in highly competitive and aggressive surroundings, which are very different in many aspects from terrestrial environment. Such situations demand the production of quite specific and potent bioactive compounds which may lead to the development of novel drugs and functional foods or nutraceuticals that may be a natural alternative therapy to commercial synthetic drugs against certain chronic diseases. Indeed, seaweeds are increasingly being recognized either as a rich natural resource of health-promoting ingredients (including high-quality proteins, dietary fiber, polysaccharides, polyunsaturated fatty acid (PUFA), minerals, vitamins, pigments, and phytochemicals such as polyphenols), or a source of potential bioactive protein hydrolysates and peptides, which may have protective effects against allergy, cancers, degenerative disorders, diabetes, digestive disorders, heart diseases, hypertension, inflammation, lipidemia, obesity, and oxidative stress (Fleurence 1999; Ortiz and others 2006; Bocanegra and others 2009; Lordan and others 2011; Mohamed and others 2012; Harnedy and FitzGerald 2013; Kolanjinathan and others 2014). The nutraceutical value of these seaweed compounds has recently been reviewed (for example, Holdt and Kraan 2011; Pereira 2011; Pal and others 2014; Michalak and Chojnacka 2015). However, as is well established, the chemical composition of seaweeds, and consequently

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their nutritional and medicinal value, depends on many factors, including species and its developmental stage, geographical origin or area of cultivation, habitat, season, climate, environmental conditions, time of harvest, and processing methods, like different sampling methodologies and drying methods (for example, Chan and others 1997; Fleurence 1999).

Today, the global utilization of products obtained from macroalgae is a multibillion dollar industry; however, macroalgae species are often regarded as an underexploited bioresource (Holdt and Kraan 2011; Milledge and others 2014; Rupérez and others 2014). Taking into account that in recent decades, consumer awareness has been increased regarding the influence of diet on health and well-being and that worldwide demand for macroalgae as a natural source of functional ingredients is growing, several reports have appeared on the nutritional composition and biological activities of edible macroalgae from some coastal areas. Seaweeds are abundant and structuring organisms on the coastal areas of the Azores Islands (Portugal), known as environmentally healthy habitats (Neto and others 2009) and located in the middle of the Atlantic Ocean (37°40' N and 25°31' W). Some species are available throughout the year, whereas others have a markedly seasonal pattern (Neto and others 2005). Traditionally, the Azorean population has gathered seaweeds either as food (for example, *Fucus spiralis*, *Porphyra*, *Laurencia*, *Osmundea*, and *Ulva*) or for chemicals extraction, such as the red algae *Gelidium microdon* and *Pterocladia capillacea* that are exported for agar production (Neto and others 2005). However, a complete report on the nutritional and/or functional metabolites content of edible Azorean macroalgae is still scarce (Paiva and others 2014, 2016a, b, c). As a result, the aim of the present study was to investigate the nutritional aspects and health-promoting attributes of the Azorean and locally abundant *Ulva compressa*, *Ulva rigida*, *G. microdon*, and *P. capillacea* in terms of moisture, lipids, soluble carbohydrates, protein, dietary fiber, ash, fatty acid (FA) groups (saturated fatty acid [SFA], monounsaturated fatty acid [MUFA], and PUFA), amino acid (AA) profiles, *in vitro* protein digestibility, and angiotensin I-converting enzyme (ACE) inhibition, in order to evaluate their use as functional foods and/or for producing health-care products for the pharmaceutical, medical, and food industries. This, in turn, can provide an incentive for the biodiversity conservation and for the maintenance of a clean environment in the Azores while also having a potential impact on the regional economy and public health.

Materials and Methods

Chemicals and reagents

Acetonitrile, dichloromethane, chloroform, methanol, hexane, and HPLC grade were purchased from Fluka Chemika (Steinheim, Switzerland). Ethanol, acetone, sodium chloride, sodium sulphate anhydrous, ammonium acetate, phenol, triethylamine (TEA), sulphuric acid, hydrochloric acid, trichloroacetic acid (TCA), boric acid, Kjeldahl catalyst, and standard glucose were obtained from E. Merck (Darmstadt, Germany). Derivatization reagent (14% boron trifluoride in methanol) was purchased from Alltech Associates (Deerfield, Ill., U.S.A.). Total Dietary Fiber Assay Kit (TDF-100A Kit) that contains α -amylase (A3306), amyloglucosidase (A9913), protease (P3910), and celite, fatty acids methyl esters standards "FAME Mix C4-C24 (18919 Supelco)" and "PUFA No. 1 Marine Source (47033 Supelco)," sodium carbonate anhydrous, disodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide, sodium caseinate, 2-mercaptoethanol, trizma base, zinc chloride (ZnCl_2), hippuric acid (HA), hippuryl-L-histidyl-L-leucine (HHL), and

the commercial enzymes ACE from porcine kidney, bromelain (B4882), chymotrypsin (C9381), pepsin (P77161), peptidase (P7500), and trypsin (T8003) were obtained from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Amino acids standard mixture, sequential grade 6 N HCl, and phenyl isothiocyanate (PITC) were purchased from Pierce Chemicals (Rockford, Ill., U.S.A.). All other reagents used in this study were reagent grade chemicals. Deionized water was obtained from a Milli-Q water purification and filtration system with 18 M Ω /cm resistivity (Millipore, Bedford, Mass., U.S.A.). All reagents, eluents, and buffers were filtered through 0.2 μm membranes.

Macroalgae collection

All samples used in this study were collected from the littoral of São Miguel Island of Azores Archipelago, Portugal, and voucher specimens were prepared and deposited in the Herbarium AZB – Ruy Telles Palhinha of the Dept. of Biology at the Univ. of Azores. The Rhodophyta *Gelidium microdon* Kützting (SMG-13-03) and *Pterocladia capillacea* (S.G. Gmelin) Santelices & Hommers and (SMG-13-05) were collected in January 2013, and the Chlorophyta *Ulva compressa* Linnaeus (SMG-13-15) and *Ulva rigida* C. Agardh (SMG-13-12) in April 2013.

Macroalgae sample preparation

Within 24 h of collection, the algal samples were 1st washed in seawater to remove encrusting material and epiphytes, carefully rinsed with distilled water to remove salts, partially dried with a paper towel, and then air-dried and stored in an air-tight container in a freezer (−80 °C) for not more than 6 mo until further analysis. Prior to the analytical procedures, the samples were defrosted and dried at 40 to 45 °C for 48 h (avoiding overheating that could lead to oxidation). Dried triplicate samples were grounded into a fine powder of 0.5 mm particle size, redried at 40 °C, and stored in the dark under N₂ in desiccators at a refrigerated temperature of 4 to 5 °C.

Nutrient analysis

Dry weight and moisture content determination. The dry weight (DW) of fresh matter was estimated after drying the algal samples at 70 °C in a hot air oven until a constant weight was obtained. The moisture content of the fresh material was calculated by subtracting the DW of the sample from the wet weight.

Crude protein determination. The organic nitrogen content was quantified using the modified Kjeldahl procedure (AOAC 1990) in a Velp Scientifica UDK 132 apparatus. Each of the algal samples (1 g of homogenized dry powder algal material) was digested with 12 mL of sulphuric acid 96%, then distilled with sodium hydroxide (35%) and acid boric solution (2%), and finally titrated with 0.1 M HCl. Estimation of the crude protein content was calculated multiplying the organic nitrogen content by a conversion factor of 6.25 (% Protein = % N \times 6.25).

***In vitro* algae protein digestibility evaluation.** To provide a good estimate of the true protein digestibility of the studied algae, a multiproteolytic enzyme system was used in order to reproduce the actual digestion environment *in vivo*. The *in vitro* digestibility was determined in the algal protein concentrate suspension, using a multienzyme freshly prepared solution (trypsin, chymotrypsin, and peptidase), according to Hsu and others (1977) method. Fifty milliliters of aqueous protein suspension (6.25 mg protein/mL) in glass distilled water were adjusted to pH 8.0, while stirring in a 37 °C water bath. The multienzyme solution (1.6 mg/mL trypsin [10000 BAEE units/mg protein], 3.1 mg/mL chymotrypsin

[80 units/mg solid], and 1.3 mg peptidase/mL [50 to 100 units/g solid]) was maintained in an ice bath and adjusted to pH 8.0. Five milliliters of the multienzyme solution were then added to the protein suspension and the pH change in the mixture, caused by the enzymatic digestion, was measured after exactly 10 min. Sodium caseinate was used as control and the *in vitro* protein digestibility of the algae was expressed as a relative percentage to that of the sodium caseinate normalized at 100% (FAO/WHO 1991).

Amino acids composition. The proteins were precipitated with cold (4 °C) 25% TCA (2.5:1, TCA:homogenate, v/v), according to Barbarino and Lourenço (2005). The tubes containing TCA and homogenate were kept in an ice bath for 30 min and then centrifuged (15000 × g) for 20 min at 4 °C. Supernatants were discarded and pellets were washed with cold (4 °C) 10% TCA and centrifuged again. Pellets formed after the 2nd centrifugation were suspended in 5% TCA at room temperature (21 °C), in a proportion of 5:1 (TCA:precipitate, v/v) and centrifuged (15000 × g) for 20 min. Supernatants were discarded and the protein pellets were recovered. The protein pellet was hydrolyzed using 6 N HCl and derivatized with PITC, as previously described (Sánchez-Machado and others 2003; Paiva and others 2014). The AA profile was performed on an Agilent Technologies (Palo Alto, Calif., U.S.A.) HPLC system series 1200 on a HP Aminoquant reverse-phase column (20 cm × 2.1 mm i.d., 5 μm particle size) using a linear gradient of phase (A) 0.14 M ammonium acetate with 0.05% TEA (pH 6.4) and phase (B) acetonitrile:water (60:40, v/v): $t = 0$ min – 5% B, $t = 13$ min – 5% B, $t = 20$ min – 40% B, $t = 22$ min – 45% B, $t = 25$ min – 50% B, $t = 26$ min – 80% B, $t = 27$ min – 100 B, $t = 31$ min – 5% B at a flow rate of 0.35 mL/min under UV detection at 254 nm (Paiva and others 2014). The AA content was expressed as mg/g of protein.

Crude lipid extraction and total lipid and fatty acid groups determination. The crude lipid content was determined gravimetrically after soxhlet extraction (AOAC 1995) with chloroform:methanol 2:1 (v/v) during 4 h of reflux, in order to obtain high-yield, following the Folch and others (1957) methodology. For FA groups determination, a cold extraction with chloroform:methanol (2:1, v/v) in the absence of light to minimize lipids oxidation was adopted (Folch and others 1957) and then the sample was transmethylated using 0.5 N potassium hydroxide methanol solution and derivatized with 14% boron trifluoride in methanol according to the modified protocol of Leite and others (2007). Fatty acids methyl esters (FAME) were analyzed on a Bruker model 450 GC equipped with split/splitless injector and a flame ionization detector, using a fused silica CP-Wax 58 (FFAP) CB column (25 m × 0.25 mm i.d., 0.20 μm film thickness) from Varian. The temperature was held at 150 °C for 2 min, programmed at a rate of 4 °C/min to 250 °C, and then held at this temperature for 10 min. Helium was the carrier gas at a flow rate of 1.3 mL/min. The injector and detector temperatures were held constants at 260 and 280 °C, respectively. FAME identifications were carried out by comparison of their retention times with those of the FAME standard and for the more difficult assignments were also confirmed by GC/MS experiments carried out with a Varian 3800 GC (Palo Alto, Calif., U.S.A.) interfaced with a 4000 Varian MS, using the same analytical conditions. The mass spectrometric conditions were: electron impact ionization at 70 eV, source temperature 220 °C, 100 μA trap current, and the sweep time was 1.5 s/decade scan, with a mass range of 50 to 450 *m/z*. FAME peaks were quantified by area normalization using the workstation software from Bruker (Paiva and others 2014).

Soluble carbohydrate content determination. The soluble carbohydrates were extracted from samples with 2.5 N HCl and the concentrations determined by the phenol-sulphuric acid colorimetric method described by Dubois and others (1956). Percent soluble carbohydrate was calculated based on absorptions at 490 nm in a Shimadzu 160-A UV/VIS spectrophotometer model 1800 (Shimadzu Co., Kyoto, Japan). The results were calculated from a multiple level glucose calibration curve at 5 different concentrations, constructed from peak-area versus glucose concentration (Paiva and others 2014).

Total, soluble, and insoluble dietary fiber determination. The total dietary fiber (TDF) and insoluble dietary fiber (IDF) were determined according to the AOAC enzymatic gravimetric method (AOAC 2006), following the Sigma-Aldrich protocol provided in the TDF-100A Kit, with slight modifications. Briefly, 1 g of dried fat-free macroalgae sample was suspended in 50 mL phosphate buffer (0.08 M, pH 6.0) in a beaker and 0.10 mL of α-amylase was added and mixed well. Each beaker was covered with aluminum foil and incubated in a boiling water bath for 15 min. After this time, the pH was adjusted to 7.5 (0.275 N NaOH) and 0.1 mL of freshly prepared protease solution (50 mg/mL in phosphate buffer, 0.08 M, pH 6.0) was added to each beaker that was covered again with an aluminum foil and incubated in 60 °C water bath (with continuous agitation) for 30 min. Then, 0.1 mL of amyloglucosidase was added after adjusting the pH to 4.0 to 4.6 with 0.325 M HCl. The beakers were covered again with an aluminum foil and incubated in 60 °C water bath (with continuous agitation) for 30 min. The undigested fraction was then precipitated with ethanol and filtered. The residue was washed with ethanol and acetone, dried overnight in a 105 °C air oven or 70 °C vacuum oven, cooled, and then weighed and corrected for protein, ash, and blank (B) to give the macroalgae fiber material or TDF using the equation: % TDF = $[(R_{\text{sample}} - P_{\text{sample}} - A_{\text{sample}} - B) / S] \times 100$, where *R*, *P*, *A*, and *S* is the average weight (in mg) for the residue, protein, ash, and sample, respectively, and $B = R_{\text{blank}} - P_{\text{blank}} - A_{\text{blank}}$. For IDF determination, another sample was digested as referred before. The enzyme mixture was filtered, and the insoluble residue was washed with distilled water, ethanol, and acetone, dried overnight in a 105 °C air oven or 70 °C vacuum oven, cooled, and then weighed and corrected for protein and ash contents and blank. The soluble dietary fiber (SDF) was calculated by difference as TDF – IDF.

Ash determination. The total inorganic material (ash) in insoluble indigestible fractions (1 g) was determined gravimetrically after incineration of dried algal material at 550 °C for 2 to 3 h using an electric muffle furnace (AOAC 1995). The ash content was determined using the equation: % Ash = weight of ash / weight of sample × 100.

Biological activity assay

Algae enzymatic hydrolysates preparation and fractionation. Twenty grams of dried algal samples were suspended in 300 mL of distilled water to induce cell lysis by osmotic shock that facilitated subsequent protein extraction. Then, the suspension of ice-cold distilled water (in order to prevent the heating of the sample) was homogenized with an Ultra-turrax T50 at 5635 × g for 10 min and then hydrolyzed by pepsin (substrate/enzyme ratio to 100:1, w/w) for 20 h to maximize the hydrolysis at the conditions recommended by the enzyme manufacturer (37 °C and pH 2.0, adjusted using 2 N HCl). After 20 h, the digestion solution was boiled for 10 min in order to inactivate the enzyme, adjusted to pH 7.0 using 5 M NaOH and immediately

concentrated until an approximate volume of 150 mL. The concentrated digestion solution (substrate) was subsequently hydrolyzed with bromelain (substrate/enzyme ratio to 100:1, w/w) at 37 °C for 20 h. The digest was boiled for 10 min and immediately transferred to a 12 kDa molecular weight cutoff (MWCO) dialyzing tube. The outer solutions (permeate) from 5 changes (approximately 3 L) were combined and concentrated for further fractionation through 3 different ultrafiltration (UF) membranes in cell dialyzer system having a range of MWCO of 10, 3, and 1 kDa. The obtained fractions were lyophilized and kept at -80 °C for further ACE-inhibitory activity determination.

ACE-inhibitory activity determination. The determination of ACE-inhibitory activity was performed *in vitro* by RP-HPLC adapted from the spectrophotometric method described by Cushman and Cheung (1971) with slight modifications. This method is based on the liberation of HA from HHL catalyzed by ACE. For the assay, 42.5 µL of each algae enzymatic hydrolysate solution (2 mg/mL) was preincubated at 37 °C for 5 min with 10 µL ACE (0.6 mU/mL) enzyme. The mixture was subsequently incubated at the same temperature for 60 min with 20 µL of the substrate (5 mM HHL in 10 µM zinc chloride containing 100 mM sodium trizma base and 300 mM NaCl at pH 8.3). The reaction was terminated by adding 12.5 µL of 5 M HCl. The ACE-inhibition (IC₅₀) was determined by HPLC analysis. An aliquot of 20 µL from the reaction mixture was analyzed on a reverse-phase Ultrasphere ODS analytical column (25 m × 4.6 mm i.d., 5 µm particle size) from Beckman Coulter (Miami, Fla., U.S.A.), using an isocratic elution of MeOH:ACN:0.1% HCl (25:25:50, v/v/v) at a constant flow-rate of 0.6 mL/min, and HA and HHL were detected by UV at 228 nm. The average value from 3 determinations at each concentration was used to calculate the ACE-inhibition rate as follows: % ACE-inhibition = $[B - A / B - C] \times 100$, where *A* is the absorbance of HA generated in the presence of ACE-inhibitor, *B* is the absorbance of HA generated without ACE-inhibitor, and *C* is the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay). The IC₅₀ value (mg/mL) was defined as the concentration of the inhibitor required to reduce the HA peak by 50% (corresponding to 50% inhibition of ACE activity). The captopril was used as a positive control for ACE-inhibition.

Statistical analysis

All determinations were performed at least in triplicate and the results were expressed as means ± standard deviations (SD). One-way analysis of variance test (ANOVA) was carried out to assess for any significant differences between the means. Differences between means at the 5% (*P* < 0.05) level were considered significant.

Results and Discussion

Nutrient analysis

Moisture. The moisture content of fresh algal samples (Table 1) is very high, varying from 83.2% to 90.0%. These results are within the range (80.0% to 90.0%) referred to several marine seaweeds (Bocanegra and others 2009).

Protein. Protein content of macroalgae varies between 3.0% and 47.0% of DW, depending to a large extent on the phylum, species, geographical origin, and time of harvest (for example, Fleurence 1999). According to this author, generally, the green and red algae contain higher protein levels (10.0% to 47.0%) than brown algae (3.0% to 15.0%).

The protein content of the studied algae (Table 1) was low in *U. compressa* and *U. rigida* which presented similar values (15.7% and 15.8%, respectively), but within the range reported for the genus *Ulva* (10.0% to 26.0%) (Fleurence 1999). In the Rhodophyta, values were higher, respectively, 23.4% for *G. microdon* and 20.2% for *P. capillacea*. These results are also within the range reported for Rhodophyta algae in general and comparable to those of common edible algae, such as *Porphyra* sp. (24.1%) (Sánchez-Machado and others 2004), as well to those of edible land vegetables (around 20.0%) (Wong and others 2004).

Different protein levels were, however, reported by other authors for the same species (for example, 7.3% for *U. rigida* from Tunisia) (Frikha and others 2011). This fact may be related with differences in the analytical methods employed, different geographical location, and time of harvest, among other factors (for example, Barbarino and Lourenço 2005).

Amino acids composition. Table 2 shows the AA profiles of the studied algae, which were compared to those of some high-protein sources to obtain a 1st estimate of the nutritional value of the algae proteins. Concerning the total AA content, *U. compressa* showed a value of 228.1 mg/g protein which is significantly higher than those found in the other studied algae (59.7 to 130.0 mg/g protein) and presented significant differences (*P* < 0.05).

The studied algae proteins contain, in general, all the essential amino acids (EAAs), except for tryptophan that was destroyed during acid hydrolysis, but in different proportions. The EAA percentage of the total AAs was 45.3%, 48.4%, 48.6%, and 58.1% for *P. capillacea*, *U. rigida*, *U. compressa*, and *G. microdon*, respectively. These values were higher than those reported for the Rhodophyta *Palmaria* sp. (37.0%) and *Porphyra* sp. (39.5%) (Sánchez-Machado and others 2003), and are comparable to that of *U. rigida* (43.5%) from Brittany coast (Fleurence and others 1995). On the other hand, all the studied algae presented similar or higher EAA percentage than casein (45.7%), but lower than leguminous proteins (77.6%), and *G. microdon* presented higher values than ovalbumin (57.0%). The EAA profile of *U. compressa* showed superior levels for all EAA, except for His and Ile, than the remaining studied algae. On the other hand, the percentage of Leu found in *U. compressa* proteins is higher than that reported for the protein standards (casein, ovalbumin, and leguminous proteins), and the Leu/Ile ratio value (1.6) is similar to that from casein (1.9) and leguminous plants (2.0). The other *Ulva* species, *U. rigida*, had a similar EAA profile to that found in *U. compressa*, showing, however, much lower levels for all EAA and absence of His and revealed a significant difference between the 2 algae in the total EAA (*P* < 0.05). Compared with the red algae, *U. rigida* also showed inferior levels for almost all EAAs. Similar results were reported in some other studies, which show that *Ulva* species are a rich source of Leu and present a balanced Leu/Ile ratio (for example, 2.0 for *Ulva pertusa* Kjellman) (Bocanegra and others 2009). The EAA profile of *P. capillacea* showed higher levels for Ile than the remaining studied algae. The EAA profile of the other red algae, *G. microdon*, showed that His, which is an EAA for children, is present at a higher level than that in the remaining studied algae and protein standards.

All the studied algae had similar profiles of non-EAA, the higher values being recorded in *U. compressa*. Aspartic and glutamic acids constitute together the main AAs representing 36.2%, 28.4%, 22.2%, and 17.2% of the total AAs in *P. capillacea*, *U. compressa*, *U. rigida*, and *G. microdon*, respectively. *Ulva* species values are similar to that of casein (28.8%), and *G. microdon* ones similar to those of leguminous plants (18.5%) and ovalbumin (18.4%). Similar results were also reported by Fleurence and others (1995).

Table 1—Composition (moisture, protein, lipids, soluble carbohydrates, ash, and total, soluble, and insoluble dietary fiber) of the studied macroalgae.^a

Composition (%)	Macroalgae species			
	<i>Ulva compressa</i>	<i>Ulva rigida</i>	<i>Gelidium microdon</i>	<i>Pterocladia capillacea</i>
Moisture ^b	90.00 ± 0.91 ^a	84.50 ± 0.70 ^{bc}	83.20 ± 0.88 ^c	86.00 ± 0.65 ^b
Protein ^c	15.66 ± 0.09 ^c	15.78 ± 0.10 ^c	23.40 ± 0.65 ^a	20.16 ± 0.15 ^b
Lipids ^c	1.67 ± 0.16 ^c	1.02 ± 0.09 ^d	2.44 ± 0.09 ^b	4.32 ± 0.33 ^a
Carbohydrates ^c	14.45 ± 0.43 ^d	16.74 ± 0.17 ^c	17.62 ± 0.27 ^b	19.76 ± 0.23 ^a
Ash ^c	18.03 ± 0.23 ^b	20.60 ± 0.27 ^a	20.69 ± 0.24 ^a	10.69 ± 0.17 ^c
TDF ^c	33.67 ± 0.35 ^b	34.67 ± 0.55 ^b	39.77 ± 0.95 ^a	41.00 ± 0.80 ^a
SDF ^c	15.50 ± 0.56 ^b	15.57 ± 0.51 ^b	18.70 ± 0.46 ^a	19.17 ± 0.45 ^a
IDF ^c	18.17 ± 0.78 ^b	19.10 ± 0.96 ^b	21.07 ± 0.51 ^a	21.83 ± 0.35 ^a

^aValues are mean ± SD ($n = 3$). Means with different superscripts (a to d) in the same row differ significantly ($P < 0.05$).

TDF, total dietary fiber; SDF, soluble dietary fiber; IDF, insoluble dietary fiber. The SDF was calculated by difference as TDF – IDF.

^bPercentage of fresh weight.

^cPercentage of dry weight.

Table 2—Amino acid profiles of the studied macroalgae (mg amino acids/g protein)^a compared to some traditional high-protein foods and protein standards (%).

Amino acid (AA)	Macroalgae species				Animal and vegetable protein sources		
	<i>Ulva compressa</i>	<i>Ulva rigida</i>	<i>Gelidium microdon</i>	<i>Pterocladia capillacea</i>	Casein ^d	Ovalbumin ^e	Leguminous plant ^e
Aspartic acid	32.30 ± 0.35	5.63 ± 0.07	7.16 ± 0.04	11.49 ± 0.09	6.4	6.2	4.7–5.4
Glutamic acid	32.49 ± 0.10	7.63 ± 0.08	8.46 ± 0.04	35.39 ± 0.08	21.1	9.9	6.4–6.7
Serine	17.50 ± 0.07	3.27 ± 0.03	5.93 ± 0.04	6.42 ± 0.04	4.8	6.8	–
Glycine	12.07 ± 0.08	4.40 ± 0.04	4.10 ± 0.07	6.40 ± 0.08	1.6	3.4	–
Histidine ^b	2.15 ± 0.04	ND	13.31 ± 0.10	2.80 ± 0.03	4.0	3.8–4.0	4.1
Arginine ^b	14.11 ± 0.07	2.99 ± 0.02	8.48 ± 0.09	5.46 ± 0.06	3.7	11.7	13.0–14.0
Threonine ^b	18.21 ± 0.11	3.04 ± 0.04	5.94 ± 0.04	9.77 ± 0.07	3.7	3.0	4.0
Alanine	4.68 ± 0.09	1.27 ± 0.04	1.91 ± 0.03	1.71 ± 0.02	2.6	6.7	–
Proline	9.92 ± 0.10	2.32 ± 0.06	3.23 ± 0.04	4.12 ± 0.08	11.3	2.8	–
Tyrosine	8.35 ± 0.16	6.28 ± 0.09	7.22 ± 0.08	5.36 ± 0.06	4.1	1.8	2.3–2.6
Valine ^b	14.21 ± 0.11	2.74 ± 0.04	5.17 ± 0.07	4.46 ± 0.06	6.0	5.4	4.5
Methionine ^b	6.55 ± 0.13	1.43 ± 0.04	4.17 ± 0.06	4.21 ± 0.09	0.5	3.1	1.2–1.4
Leucine ^b	22.11 ± 0.20	14.51 ± 0.11	3.89 ± 0.03	2.75 ± 0.06	8.8	6.2	7.3
Isoleucine ^b	13.64 ± 0.10	2.07 ± 0.04	2.07 ± 0.04	18.61 ± 0.11	4.7	4.8	3.6
Phenylalanine ^b	8.45 ± 0.06	1.78 ± 0.03	4.42 ± 0.05	5.24 ± 0.06	4.8	4.1	2.4
Lysine ^b	11.34 ± 0.09	2.38 ± 0.04	5.32 ± 0.08	5.34 ± 0.06	7.4	7.7	6.4–6.5
Total AA ^c	228.08 ± 2.63 ^a	59.67 ± 1.03 ^d	90.78 ± 1.30 ^c	129.53 ± 2.19 ^b	95.50	87.40	59.90
Total EAA ^c	110.77 ± 1.29 ^a	28.87 ± 0.14 ^c	52.77 ± 0.79 ^b	58.64 ± 0.85 ^b	43.60	49.80	46.50
EAA (%)	48.57	48.38	58.13	45.27	45.65	56.98	77.63

^aValues are mean ± SD ($n = 3$). Means with different superscripts (a to d) in the same row differ significantly ($P < 0.05$). ND, not detected.

^bEssential amino acids (EAAs).

^cExcluding tryptophan that is not detected in this methodology.

^dReferred in Barbarino and Lourenço (2005).

^eReferred in Fleurence (1999).

for *U. rigida* (26.0%) from Brittany, and a lower value was found in Brazilian *P. capillacea* (26.6%) (Barbarino and Lourenço 2005). High levels of those 2 AAs were found in most seaweeds used as human foods (Bocanegra and others 2009) and are responsible for their special flavor and taste (Mabeau and others 1992). As for *G. microdon*, levels of these 2 AAs seem to be lower in some red seaweed species, such as *Porphyra tenera* Kjellman or “nori” (19.0% of total AA content) (Bocanegra and others 2009).

In vitro protein digestibility. The AA profiles are important in evaluating the nutritive quality of proteins, and their digestibility is the primary determinant of the availability of its AAs. The protein digestibility of the studied algae, expressed as a relative percentage to that of the sodium caseinate normalized at 100%, was $89.39 \pm 1.00\%$, $86.58 \pm 0.39\%$, $84.05 \pm 0.48\%$, and $82.22 \pm 0.02\%$ for *G. microdon*, *U. rigida*, *P. capillacea*, and *U. compressa*, respectively (Figure 1).

The nutritional value and the high protein digestibility recorded in the studied algae suggested that they can be used as complementary sources of food proteins in human nutrition or for the development of balanced diets for sea-cultivated species such as fish and abalone. This, according to Fleurence (1999), would be a promising way for the exploitation of marine resources in Europe.

Lipids. The majority of marine algae have very low lipid content ranging from as low as 0.3% to 7.0% of DW (Yuan 2008), revealing a low source of nutritional energy comparable with land vegetables (Darcy-Vrillon 1993). The crude lipid content of the studied algae (Table 1) is within that range. The highest values were recorded for *P. capillacea* (4.3%) and *G. microdon* (2.4%), whereas the lowest content was found in *U. rigida* (1.0%) and *U. compressa* (1.7%). Similar results were reported for Tunisian *U. rigida* (0.8%) (Frikha and others 2011) and other *Ulva* species from Philippines (1.6% to 1.8%) (Portugal and others 1983).

Fatty acid groups. Although the lipid content of macroalgae is relatively low, they contain a relative higher level of PUFA than land vegetables (Darcy-Vrillon 1993). However, the macroalgae FA contents are variable, depending on both environmental and genetic differences (for example, Nelson and others 2002).

Table 3 shows the different FA groups of the studied algae, in percentage of the total FAME (tFAME). The total SFA content was higher in *U. rigida* (46.8% of tFAME), *P. capillacea* (39.1% of tFAME), and *U. compressa* (37.6% of tFAME) than in *G. microdon* (23.3% of tFAME). Concerning the total unsaturated FA (the dominant FA group in all studied algae), *G. microdon* showed a lower content of MUFA than *P. capillacea*, *U. rigida*, and *U. compressa* (7.5%, 17.9%, 22.1%, and 32.9% of tFAME, respectively), and the PUFA values were 29.6%, 31.7%, 44.4%, and 69.2% of tFAME for *U. compressa*, *U. rigida*, *P. capillacea*, and *G. microdon*, respectively. Clinical studies (for example, Ginsberg and others 1990) have shown that diets rich in MUFA and PUFA and low in SFA reduce the total cholesterol and low-density lipoprotein (LDL)-cholesterol plasma, which can be positively correlated with low incidence of heart disease, thrombosis, and atherosclerosis (Ortiz and others 2006). Thus, the obtained results on the FA groups of the studied algae justify their direct use in human nutrition or in the development of food supplements and/or pharmaceutical products.

Soluble carbohydrates. The soluble carbohydrate content showed a narrow range among the studied algae (Table 1). The highest values were found in *P. capillacea* (19.8%) and *G. microdon* (17.6%), followed by *U. rigida* (16.7%) and *U. compressa* (14.5%). Frikha and others (2011) reported a similar value for Tunisian *U. rigida* (16.7%). These authors also reported moderate carbohydrate content for Tunisian green and red algae (10.4% to 18.7%), and also the highest value was found in the red algae. However, Chakraborty and Santra (2008) reported higher carbohydrate content for *Ulva lactuca* Linnaeus (35.3%) and Foster and Hodgson (1998) reported 43.1% for *Gelidium pristoides* (Turner) Kuetzing.

According to Rosemberg and Ramus (1982), the algae carbohydrate synthesis is related to periods of maximum growth and increased photosynthetic activity. On the other hand, these periods were influenced by increased values of temperature, salinity, and sunlight intensity that explain the carbohydrate content variability of the studied algae as compared with some published results from other authors on the same genus.

Total, soluble, and insoluble dietary fiber. Macroalgae are a richer source of TDF (29.0% to 62.0% of DW) as compared to the most fruits and vegetables (Lordan and others 2011), with SDF and IDF accounting for 8.3% to 85.0% and 20.0% to 98.9% of TDF, respectively (Yuan 2008). Indeed, SDF and IDF were highly variable between species, among other factors, although IDF appeared to predominate. In addition, algae fiber presents different physicochemical and biological properties as compared to fiber of terrestrial plants and thus induces different physiological effects (Wong and Cheung 2000; Ortiz and others 2009; Rupérez and others 2014).

As shown in Table 1, the dietary fiber was the most abundant component in the studied algae; the amount of TDF, SDF, and IDF ranged from 33.7% to 41.0%, 15.5% to 19.2%, and 18.2% to 21.8%, respectively, and is within the ranges referred for macroalgae species. Similar results were reported for Hawaiian *P. capillacea* (McDermid and others 2005) and for French *U. rigida* (Godard and others 2009). Furthermore, the study of Godard and others (2009) has demonstrated that fiber from this algae prevents the fall of antioxidant defenses and the development of atherosclerosis in hamsters that were fed with a high cholesterol diet. The SDF/IDF ratio of the studied algae was 0.82, 0.85, 0.88, and 0.89 for *U. rigida*, *U. compressa*, *P. capillacea*, and *G. microdon*, respectively. Similar ratios were reported for other species (for example, Ortiz and others 2006), indicating that macroalgae contain dietary fiber with surplus-value (good balance of both insoluble and soluble fibers). Thus, these soluble fiber-rich algae can become important in the prevention and/or treatment of various chronic health

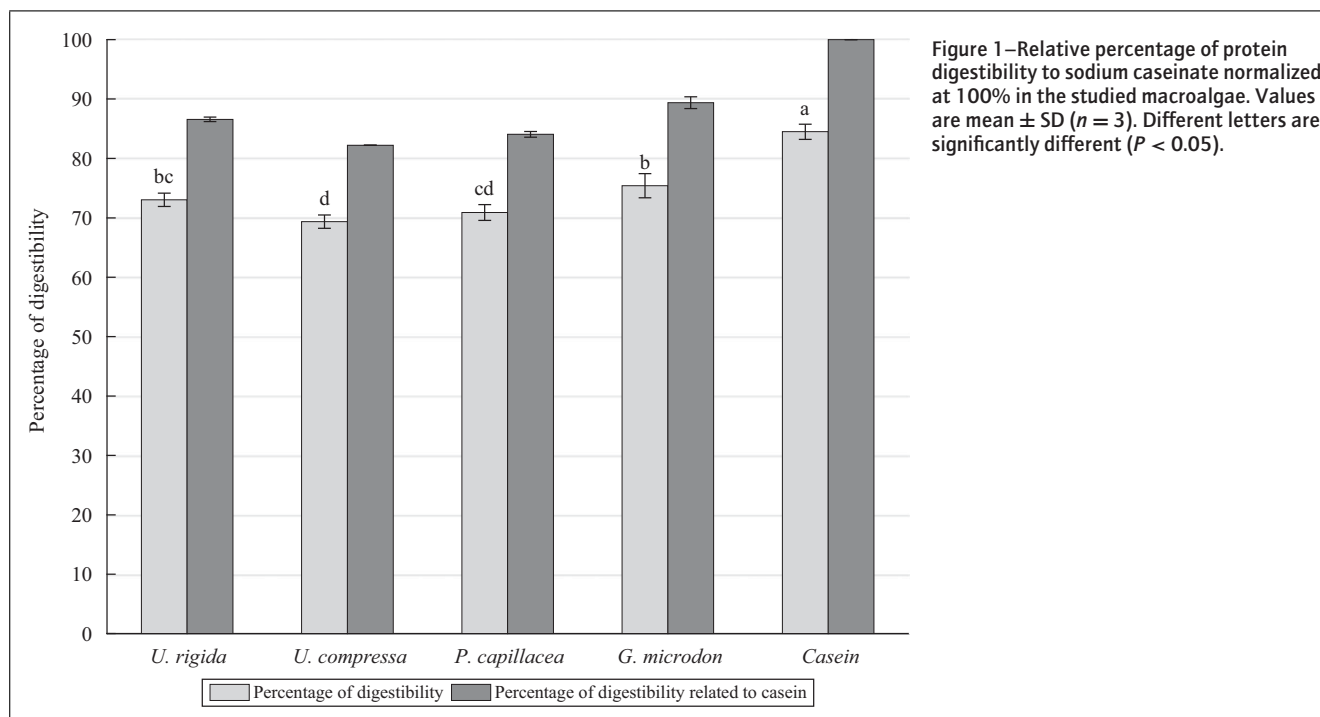


Table 3—Gas chromatography determination of fatty acid groups (% of total FAME) of the studied macroalgae.^a

Fatty acid groups ^b	Macroalgae species			
	<i>Ulva compressa</i>	<i>Ulva rigida</i>	<i>Gelidium microdon</i>	<i>Pterocladia capillacea</i>
Total saturated fatty acids (SFAs)	37.57 ± 2.19 ^b	46.79 ± 2.18 ^a	23.27 ± 1.41 ^c	39.09 ± 2.44 ^b
Total monounsaturated fatty acids (MUFAs)	32.85 ± 1.84 ^a	22.11 ± 1.17 ^b	7.50 ± 0.81 ^d	17.87 ± 1.10 ^c
Total polyunsaturated fatty acids (PUFAs)	29.57 ± 1.52 ^c	31.69 ± 1.42 ^c	69.22 ± 3.30 ^a	44.43 ± 2.17 ^b

^aValues are mean ± SD ($n = 3$). Means with different superscripts (a to d) in the same row differ significantly ($P < 0.05$). FAME, fatty acids methyl esters.

^bThe fatty acid profiles were previously referred by the authors (Paiva and others 2016c).

problems (constipation, colon cancer, cardiovascular disease, diabetes, hypertension, and obesity) which are accordingly associated with low fiber diets of the Western countries (Southgate 1990). However, further research on human trials needs to be undertaken before any health claims for functional ingredients based on seaweed dietary fiber complex can be proposed.

Ash. Ash content of marine algae is high (8.0% to 44.0% of DW) (Yuan 2008) compared to the most common vegetables (Ortega-Calvo and others 1993). The ash content of the studied algae (Table 1) was within the published range and was higher in *G. microdon* (20.7%), *U. rigida* (20.6%), and *U. compressa* (18.0%) than in *P. capillacea* (10.7%). Other reports for *Ulva* species indicated similar results, namely, for Tunisian *U. rigida* (25.7%) (Frikha and others 2011) and *U. lactuca* (21.3%) (Wong and Cheung 2000).

Biological activity assay

Angiotensin I-converting enzyme inhibitory activity. It is known that the ACE-inhibition has become a major target for the control of hypertension, one of the most common serious chronic health problems that affect about 25% of the world's adult population (Mittal and Singh 2010). However, synthetic ACE-inhibitors cause a number of undesirable side effects (Brown and Vaughan 1998). Therefore, searching for ACE-inhibitors from natural resources, such as marine organisms including macroalgae (for example, Wijesekara and Kim 2010), has become one of the major areas of research in the field of nutraceutical and pharmaceutical industries, and some ACE-inhibitory peptides have already been isolated from their enzymatic hydrolysates. Within this context, in order to evaluate the *in vitro* antihypertensive activity of the studied algae, we tested the potential to inhibit ACE activity from their enzymatic hydrolysates, after a prepurification using UF membrane cell dialyzer, and the results of the most active fractions (<1 kDa) are presented in Table 4. The comparison of the

Table 4—Angiotensin I-converting enzyme (ACE) inhibition of the Fr <1 kDa obtained by ultrafiltration of the macroalgae protein hydrolyzed by pepsin plus bromelain.^a

Macroalgae species	ACE-inhibition of Fr <1 kDa (IC ₅₀ , mg/mL)
<i>Ulva compressa</i>	0.099 ± 0.004 ^c
<i>Ulva rigida</i>	0.095 ± 0.003 ^c
<i>Gelidium microdon</i>	0.695 ± 0.042 ^a
<i>Pterocladia capillacea</i>	0.440 ± 0.035 ^b

^aValues are mean ± SD ($n = 3$). Means with different superscripts (a to c) in the same column differ significantly ($P < 0.05$). Fr, fraction. IC₅₀ represents the concentration of an inhibitor that is required for 50% inhibition of ACE activity (tested concentration = 2 mg/mL). Captopril, used as a positive control for ACE-inhibition, showed an IC₅₀ value of 0.163 ng/mL.

IC₅₀ values revealed the greatest anti-ACE activities in *U. rigida* (0.095 mg/mL) followed by *U. compressa* (0.099 mg/mL) and *P. capillacea* (0.440 mg/mL), whereas the lowest activity was found in *G. microdon* (0.695 mg/mL) with a significant difference between

Chlorophyta and Rhodophyta species ($P < 0.05$). Remarkable ACE-inhibitory activities of enzymatic hydrolysates from some macroalgae have also been reported by other authors, especially from brown and red algae (Samarakoon and Jeon 2012). These results suggest that *Ulva* peptide fractions might have a potential use as an ingredient for antihypertensive functional foods and nutraceuticals formulation. Furthermore, purified peptides could be efficiently generated from *U. rigida* protein hydrolysate, as recently demonstrated by the authors (Paiva and others 2016a).

Conclusion

Seaweeds remain a relatively underutilized sustainable resource for mining pharmaceutical, nutraceutical, and functional food benefits, but limited information is available on the nutritional and/or pharmacological value of the Azorean macroalgae. The locally abundant *U. compressa*, *U. rigida*, *G. microdon*, and *P. capillacea* species, evaluated in this study, clearly represent a rich source of high-quality nutrients and bioactive compounds. All the studied macroalgae are an excellent source of dietary fiber with surplus-value (good balance of both soluble and insoluble fibers), which may be important in body-weight control and prevention and/or treatment of various chronic health problems, such as gastrointestinal, cardiovascular, diabetes, and hypertension diseases. Ash content is also high in *Ulva* and *Gelidium* species, and can contribute with important microelements to human and animal nutrition that are rare or absent in some land vegetables. All the macroalgae also revealed to be rich sources of digestible proteins that contain, in general, acceptable amounts of 9 out of 10 EAAs as compared to terrestrial foodstuffs, which can be a complementary source of food proteins for human and animal nutrition. All the studied macroalgae had a low lipid content thus being a low source of nutritional energy. However, at the same time, they had a high-quality fat, comprising a relative higher level of PUFA as compared to land vegetables. *Ulva* and *Pterocladia* species also revealed to be rich sources of MUFA. The total unsaturated FA was the dominant FAs group in all species, suggesting that they can reduce the risk of heart disease, thrombosis, and atherosclerosis, since diets rich in MUFA and PUFA and low in SFA reduce the total cholesterol and LDL-cholesterol plasma.

The above results, allied with pristine seawaters in the Azores region, justify the direct use of the selected macroalgae in human nutrition as functional foods or its use as ingredients in many healthy low-fat food supplements that will enhance a nutritionally balanced human diet, as well as in pharmaceutical products with potential economic value. In addition, they can also be used for the production of low-cost nutritious foods for animal feed, especially for fish and other marine species of great commercial interest farmed by aquaculture. Furthermore, this study also revealed that ACE-inhibitory peptide fractions, efficiently generated from the protein hydrolysis, particularly from *Ulva* species, might have a

potential use as an ingredient for antihypertensive functional foods and nutraceuticals formulation.

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