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Anti-proliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga *Sargassum muticum* collected on North-Atlantic coasts

Lidia Montero[a], Andrea P. Sánchez-Camargo[a], Virginia García-Can˜ as[a], Anaëlle Tanniou[b], Valérie Stiger-Pouvreau[b], Mariateresa Russo[c], Luca Rastrelli[d], Alejandro Cifuentes[a], Miguel Herrero[a,], Elena Ibánez[a]*

[a] *Laboratory of Foodomics, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain*
[b] *LEMAR UMR 6539 CNRS UBO IRD IFREMER, Université de Bretagne Occidentale (UBO), Institut Universitaire Européen de la Mer (IUEM), Technopôle Brest-Iroise, Rue Dumont d'Urville, Plouzané 29280, France*
[c] *Dipartimento di Agraria, Università Mediterranea di Reggio Calabria, loc. Feo di Vito, 89122 Reggio Calabria, Italy*
[d] *Dipartimento di Farmacia, Università di Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Italy*

* Corresponding author. Tel.: +34 910 017 946; fax: +34 910 017 905.
E-mail address: m.herrero@csic.es (M. Herrero).

ABSTRACT

In the present work, the phlorotannin composition of different *Sargassum muticum* samples collected at different locations along the North Atlantic coasts as well as the bioactivities related to these com- ponents were investigated. After pressurized liquid extraction, the samples collected at the extreme locations of a latitudinal gradient from Portugal and Norway, were found to be the richest on total phe- nols and, particularly, on phlorotannins, containing up to 148.97 and 5.12 mg phloroglucinol equivalents g^{-1} , respectively. The extracts obtained from these locations were further purified and chemically characterized using a modified HILIC × RP-DAD-MS/MS method. The application of this methodology allowed the tentative identification of a great variability of phlorotannins with different degrees of polymerization (from 3 to 11) and structures, determined for the first time in *S. muticum*. The most-abundant phlorotan- nins on these samples were fuhalols, hydroxyfuhalols and phlorethols, showing also particularities and important differences depending on the geographical location. Afterwards, the antiproliferative activ- ity of these extracts against HT-29 adenocarcinoma colon cancer cells was studied. Results revealed that the richest *S. muticum* samples in terms of total phlorotannins, i.e., those from Norway, presented the highest activity, showing a good cytotoxic potential at concentrations in the medium micromolar range.

Introduction

Phlorotannins are polyphenolic compounds widely recognized to be exclusive from brown seaweeds (Phaeophyceae) [1]. This particular type of polyphenols comprises a very heterogeneous group of polymeric compounds with a great chemical variability [2]. The interest of these compounds is related to their associated bioactivities, such as antioxidant [3–5], anti-inflammatory [6], anti-bacterial [7,8], antidiabetic [9] or anti-adipogenic [10], among others. Moreover, their potential anti-proliferative activity has been pointed out by several researches [11–13]. Phlorotannin con- tent in brown algae can reach up to 15% of dry weight, depending on species, and they may be found in free form or forming complexes with different components of the cell walls, such as alginic acid [14]. From a purely chemical point of view, phlorotannins are made up of phloroglucinol (1,3,5-trihydroxybenzene) units with varying degrees of polymerization that may be linked through different bonds forming several structures and types, namely: fuhalols and phlorethols, which contain ether linkages; fucols, with phenyl link- ages; fucophlorethols in which both ether and phenyl linkages are present; and eckols, that possess a benzodioxin linkage. Although their presence in brown algae is widely accepted, it is rather difficult to find studies in which the complete characterization of such complex polymeric structures is carried out. In fact, several approaches have been attempted for the structural elucidation of phlorotannins in their native form; for instance, Stiger-Pouvreau et al. [15] employed one- and two-dimensional nuclear magnetic resonance (NMR) (1H, heteronuclear multiple bond correlation) together with

in vivo NMR (high-resolution magic-angle spinning, HR-MAS NMR) analyses, to structurally elucidate and fingerprint phlorotannin signals in different Sargassaceae species.
Results revealed that these techniques were useful for discriminating among species, giving a differentiated profile but only determining the class of phlorotannins in the sample, without elucidating the entire structure of any compound. In a recent work carried out in our laboratory [16], a new comprehensive two-dimensional liquid chromatography coupled to DAD and tandem mass spectrometry (LC × LC-DAD-MS/MS) methodology was developed based on the coupling of a hydrophilic-interaction chromatography (HILIC)-based separation in the first dimension and an RP-based separation in the second dimension that allowed the separation and identification of more than 50 compounds in a *Cystoseira abies-marina* brown algal extract. By using this approach, phlorotannins containing from 5 to 17 phloroglucinol units were identified in this sample [16]. The application of this methodology to *S. muticum* could therefore imply a definitive step forward for the characterization of its phlorotannin composition.
S. muticum is an invasive brown macroalga widely spread along the European Atlantic coasts [17] Although native from Japan, this macroalga grows well in a variety of different environments, being in fact, one of the most readily available Sargassaceae species in Europe. Considering its availability and the fact that the presence of phlorotannins in *S. muticum* composition has been already confirmed [5,8], this seaweed has been pointed out as a potential sustainable source of bioactive compounds.
Different methods have been tested to extract phlorotannins from brown algae; the classical procedure [1] involves a solid–liquid extraction with large volumes of aqueous mixtures of ethanol or methanol for a long time. New green processes have been previously shown to be suitable for the extraction of bioactive compounds from a variety of different natural sources [18]; among them, centrifugal partition extraction (CPE), super- critical fluid extraction (SFE) and pressurized liquid extraction (PLE) have been employed, and compared to classical solid- liquid extraction, to obtain bioactive phenolic compounds from *S. muticum* [5]. Results demonstrated that PLE can be employed with advantages for obtaining extracts rich in phenolic com- pounds from brown algae, with high efficiency and complying with the rules of green chemistry. On the other hand, in a recent work carried out in our laboratory, enzyme-assisted extraction (EAE) was studied and compared to an optimized PLE process to try to increase the recovery of phenolic compounds from *S. muticum* [19]. This study showed that EAE did not significantly improve the results directly attainable through the use of PLE.
Thus, in the present work, the previously optimized PLE process [19] was applied to the extraction of phlorotannins from *S. muticum* samples collected at 13 different locations along the North-Atlantic coasts (Portugal, Spain, France, Ireland and Norway) with the aim to study the influence of the growing conditions on the chemical composition of the extracts. The extracts were characterized in terms of total phenol content, total phlorotannin content and antioxidant activity. Besides, a comprehensive two-dimensional liquid chromatography (LC × LC) method was optimized and applied to the richest samples to chemically characterize for the first time the native complex phlorotannin composition of *S. muticum*. Moreover, these extracts were also assayed to test their potential anti-proliferative activity against human colon cancer cells.

1. Materials and methods

1.1. *Samples and chemicals*

Samples of the brown alga *S. muticum* were collected from April to May 2011 in 13 different sites of five European Atlantic coast countries (Portugal, Spain, France, Ireland and Norway) as already described in Tanniou et al. [8]. The algae were rinsed firstly with filtered seawater and then with distilled water to remove the resid- ual sediments and salts. After that, the samples were dried with absorbent paper and cut into fragments before their freeze-drying. Finally the dry material was powdered and sieved at 250 µm.
The solvents employed were HPLC-grade. Acetonitrile, ethanol, methanol and acetone were acquired from VWR Chemicals (Barcelona, Spain), whereas dichloromethane was acquired from Fluka AG (Buchs, Switzerland) and ethyl acetate from Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA).

Gallic acid, phloroglucinol, acetic acid, formic acid, 2,4-dime- thoxybenzaldehyde (DMBA), 6-hydroxy-2,5,7,8-tetramethylch-oman-2-carboxylic acid (Trolox) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma–Aldrich (Madrid, Spain). The Folin–Ciocalteu phenol reagent was provided by Merck (Darmstadt, Germany). Hydrochloric acid was obtained from Probus (Barcelona, Spain). For inhibition of cell proliferation assays, dry purified extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich) at the appropriate concentrations and stored as aliquots at −80 °C until use.

1.2. *Pressurized liquid extraction (PLE)*

Firstly, extractions of freeze-dried and ground *S. muticum* samples from 13 different localizations along the North-Atlantic coasts were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. For each extraction, an 11 mL stainless steel extraction cell was employed to load the sample. The extraction cell bottom was loaded with 1 g of sea sand, followed by 1 g of dried brown alga being mixed with the same quantity of sea sand. Subsequently, 1 g of sea sand was added on top as dispersive agent. Before the static extraction period, an instrumentally preset warming-up time of 6 min was used. The extraction conditions applied were based on a previous optimization [19], including the use of ethanol:water (95:5) as extraction solvent at 160 °C and 10.3 MPa for 20 min. Each extraction was carried out by duplicate. After the extraction process, the ethanol was removed by evaporation (Rotavapor R- 210, Buchi Labortechnik AG, Flawil, Switzerland) and finally, the extracts were freeze-dried (Labconco Corporation, MO) and kept in the darkness at −20 °C until analysis.

1.3. *Phlorotannins purification procedure*

In order to obtain concentrated phlorotannin extracts, a purification protocol previously reported by Stiger-Pouvreau et al. [15] was applied to the *S. muticum* samples from Norway and Portugal. The dried extracts were re-dissolved in water and submitted to a liquid-liquid extraction with dichloromethane (1:1, *v/v*) in order to eliminate the lipidic compounds and chlorophylls present in the extract, repeating this step several times until a colorless non- polar fraction was obtained. After that, successive precipitations of proteins and carbohydrates were carried out with acetone and ethanol, respectively, ending with the elimination of the organic solvent using a gentle stream of nitrogen.
Finally, phlorotannins were extracted from the water fraction with three rinses with equivalent volumes ofethyl acetate. The ethyl acetate fractions were pooled and the solvent was evaporated under a stream of nitrogen.

2.4. In vitro determinations

2.4.1. Total phenol content (Folin–Ciocalteu method)

Total phenol content of the PLE extracts and the purified extracts were measured by the Folin–Ciocalteu method developed by Kosar et al. [20] with some modifications. Briefly, 10 µL of sample (10 mg mL^{-1} in methanol) were transferred to 600 µL of water, and then 50 µL of undiluted Folin–Ciocalteu reagent were added. After 1 min, 150 µL of 20% (*w/v*) Na_2CO_3 were added and the volume was made up to 1 mL with water. The reaction was incubated at 25 °C for 2 h and then 300 µL of the mixture were transferred to a 96-well microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader Powerwave XS (Bio Tek Instruments, Winooski, VT) and compared to a phloroglu- cinol calibration curve (62.5–2000 µg mL^{-1}). The phenolic content was expressed as mg of Phloroglucinol Equivalents (PGE) per g extract.
Moreover, total phenol content was also expressed as % dry weight of algae. All analyses were done by triplicate.

2.4.1 Total phlorotannin content (DMBA assay)

To estimate the total phlorotannin content of the algal PLE and purified extracts, the DMBA colorimetric assay was employed [6]. DMBA solution was prepared just prior to use by mixing equal vol- umes of 2% DMBA reagent in acetic acid (*w/v*) and 6% hydrochloric acid in acetic acid (*v/v*). A total of 50 μL of sample (5 mg mL^{-1}) was mixed with 250 μL of DMBA solution in a 96-well microplate and the reaction was conducted at room temperature for 60 min in the dark. Then, the absorbance was read at 515 nm using a microplate spectrophotometer reader Powerwave XS (Bio Tek, Winooski, VT, USA). Water was used as blank and control samples without DMBA solution were also included. A calibration curve using phloroglu- cinol (PG) (0.1–46.0 μg mL^{-1}) was employed to estimate the total phlorotannin content. All samples, blanks, and controls were prepared in triplicate. Data are presented as the average of triplicate analyses expressed as milligram phloroglucinol equivalents (PGE) per gram of dry extract.

2.4.3. Trolox equivalents antioxidant capacity assay (TEAC)

The antioxidant capacity of the algal extracts was estimatedwith the TEAC assay following the ABTS method based on the pro-cedure described by Re et al. [21]. ABTS•+radical was producedby mixing 7 mM ABTS and 2.45 mM potassium persulfate allowingtheir reaction during 16 h in the dark at room temperature. Theaqueous ABTS•+solution was diluted with 5 mM phosphate buffer(pH 7.4) until an absorbance of 0.7 (±0.02) at 734 nm was achieved.10 _L of sample (5 different concentrations ranging from 0.25 to2 mg mL−1) and 1 mL of ABTS•+solution were mixed in an eppen-dorf vial and 300 _L of the mixture were transferred into a 96-wellmicroplate. The absorbance was measured at 734 nm every 5 minduring 45 min in a Powerwave XS microplate spectrophotometerreader (BioTek). Trolox was used as reference standard and resultswere expressed as TEAC values (mM Trolox g−1extract). These val-ues were obtained from five different concentrations of each extracttested in the assay giving a linear response between 20 and 80% ofthe blank absorbance. All analyses were done in triplicate.

2.5. Anti-proliferative activity against human colon cancer cells

Human colon cancer cell line HT-29 was used in order to measure the anti-proliferative activity of Norway and Portugal purified extracts. HT-29 cells obtained from the ATCC (American Type Culture Collection, LGC Promochem, UK) were grown in McCoy's 5Amedium supplemented with 10% heat-inactivated fetal calf serum, 50 U mL−1penicillin G, and 50 U mL−1streptomycin, at 37◦C in humidified atmosphere and 5% CO2. Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay. Briefly, HT-29 cells were seeded onto 96-well culture plates at 10,000 cells cm−2, and permitted to adhere overnightat 37◦C. Cells were treated with the vehicle (medium with 0.2%DMSO) or different concentrations of Sargasssum muticum purified extracts (from 12.5 to 100 _g mL−1) for 24, 48 and 72 h.After incubation with the extracts, the medium was aspirated and0.5 mg mL−1of MTT reagent (Sigma–Aldrich) was added to the cells and incubated for 3 h at 37◦C in humidified 5% CO2/air atmosphere. The medium was then removed, and the purple formazan crystals were dissolved in 100 _L of DMSO. The absorbance at 570 nm was measured in a microplate reader (MultiskanTMFC Microplate Photometer, Thermo Fisher Scientific, Vantaa, Finland). Results are shown as the mean ± 95% confidence interval of at least three independent experiments, each performed in triplicate. Cell viability at the beginning of the treatment (time zero) was used to calculate the following parameters related to cell proliferation: GI50 (50%growth inhibition), TGI (total growth inhibition), and LC50 (50%cell death). These parameters were calculated according to the NIH definitions [22].2.6. Comprehensive two-dimensional liquid chromatography(LC × LC) analysis of phlorotannins

2.6.1. Instrumentation

An Agilent 1200 series liquid chromatograph (Agilent Technolo-gies, Santa Clara, CA) equipped with an autosampler and a diodearray detector was used in the first dimension (D1) of the LC × LC setup. In order to obtain more reproducible low flow rates and gradients, a Protecol flow-splitter (SGE Analytical Science, Milton Keynes, UK) was placed between the first dimension pump and the autosampler. An additional LC pump (Agilent 1290 Infinity)was coupled to perform the second dimension (D2) separations. Both instruments were hyphenated through an electronically con-trolled two-position ten-port switching valve equipped with two30 _L injection loops. An Agilent 6320 Ion Trap mass spectrometer equipped with an electrospray interface was online coupled andoperated in negative ionization mode using the following conditions: dry temperature, 350◦C; mass range, m/z 90–2200 Da; dry gas flow rate, 12 L min−1; nebulization pressure, 40 psi. The LC datawere elaborated and visualized in two and three dimensions using LC Image software (version 1.0, Zoex, Houston, TX, USA).2.6.2. LC × LC separation conditionsSamples of purified extracts of S. muticum from Portugal andNorway were prepared at 50 mg mL−1in methanol/acetonitrile(3:7, v/v) from the dry extract obtained as described in Section 2.3.D1 separation was run on a Lichrospher diol-5 (150 × 1.0 mm,5 _m d.p., HiChrom, Reading, UK) column, following the separation conditions developed in our previous work [16]. Briefly, the mobile phases were (A) acetonitrile/acetic acid (98:2, v/v) and (B)methanol/water/acetic acid (95:3:2, v/v/v) eluted at 15 _L min−1according to the following gradient: 0 min, 0% B; 3 min, 0% B; 5 min,7% B; 30 min, 15% B; 70 min, 15% B; 75 min, 25% B; 85 min, 25% B.The injection volume was 5 _L.D2 consisted on 78 s-repetitive gradients during the whole LC × LC separation, being modulation time of the switching valve also 78 s. In the D2 separation an Ascentis Express C18(50 × 4.6 mm,2.7 _m, d.p., Supelco, Bellefonte, CA) partially porous column witha C18precolumn was employed, using as mobile phases water (0.1%formic acid, A) and acetonitrile (B) eluted according to the follow-ing repetitive gradients: 0 min, 0% B; 0.1 min, 0% B; 0.3 min, 5% B;0.8 min, 70% B; 0.9 min, 90% B; 1.0 min, 0% B. The flow rate was 3 mL min−1. UV–vis spectra of the second dimension eluent were registered in the range of 190–550 nm using a sampling rate of20 Hz, while 280 nm was the wavelength used to monitor the separations. The MS was operated under negative ESI mode. The flow eluting from the second dimension column was split before the MS instrument, so that the flow rate entering the MS detector was500 _L min−1.2.7. Statistical analysis IBM SPSS Statistics software v.19 was employed for data elab-oration and statistical analysis using a level of significance set at 95%. One-way analysis of variance (ANOVA), together with Student–Newman–Keuls test, was employed to group samples, based on statistically significant differences. Mean values were compared using the Tukey's test and differences were considered statistically significant if $p < 0.05$.3.

3- Results and discussion

3.1. Influence of growing conditions on phlorotannin content.

In order to study the influence of the particular growing conditions on the chemical composition of the S. muticum extracts, an optimized PLE procedure was applied to 13 different algal samples collected along the North-Atlantic coasts, including Portugal, Spain, France, Ireland and Norway locations. The extraction procedure applied included the use of ethanol:water (95:5, v/v) as extraction solvent at 160◦C and 10.3 MPa for 20 min. The extracts obtained were firstly characterized in terms of total phenol content, total phlorotannin content and antioxidant activity. Table 1summarizes the results obtained; as can be clearly observed, the extraction yield gives a first hint regarding the different compositions of the macroalgae studied, not only between countries and distant geographical locations but also within some areas, as in the case of the samples collected on the French coast. The highest extraction yields were obtained with the samples collected in Norway, whereas those collected in Spain possessed the lowest amount of dried matter. As for total phenols, samples collected in Norway possessed the highest amount of total phenols, followed by those harvested in Portugal. These values were significantly higher than the ones obtained for the rest of locations. S. muticum samples collected in France possessed the lowest amount of total phenols, less than half of those of the richest samples. Although smaller, statistically significant differences ($p < 0.05$) were also observed among the samples collected in the same country, except those collected in Spain and Norway, that could be considered

statistically similar ($p > 0.05$). Next, total phlorotannin contents were estimated in the different extracts; the trend found was rather similar to that of total phenols, that is, the samples collected in Norway and Portugal were the richest. In any case, significant differences were observed between these two countries. For the samples collected from the rest of countries, the values found were closer and lower than those found in the Norwegian samples. As in the case of total phenols, intra-country variations ($p < 0.05$) were observed for the total phlorotannin contents, excepting for those from Spain and Portugal. Previous fingerprints obtained by HR-MAS NMR and FT-IR for the same samples yielded similar conclusions about inter-and intra-site variability [23].Lastly, the antioxidant capacity of the obtained extracts was determined using the TEAC assay. A good correlation was found between the TEAC values obtained and the total phenols and total phlorotannin contents determined in the extracts. In agreement, the most active extracts were obtained from the samples collected in Norway and Portugal, whereas the rest of extracts possessed lower antioxidant capacity (Table 1). Results obtained in the present work were, in general, well in accordance with those previously presented on S. muticum samples [8] in terms of percentage of total phenols per g of dried algae (Table 1). Samples collected in Norway and Portugal were those with higher % of total phenols with respect to the initial dry algae employed, whereas the rest of countries possessed very similar contents (Table 1). However, in the present work, a higher content was found for Norwegian samples compared to those harvested in Portugal. These small differences observed between the two studies could be due to the different extraction process employed to achieve the phenolic-rich extracts.

Looking at the results summarized in this section, it seemed clear that the algae growing in the most extreme locations of this latitudinal gradient along the North-Atlantic coasts, i.e., Norway and Portugal, were the most interesting from a chemical composition perspective. For this reason, the samples collected in those countries (four geographical locations in total) were selected to carry out an in-depth chemical characterization as well as to study their potential anti-proliferative activity. This activity has already been related to the presence of phenolic compounds in general and phlorotannins in particular [12,13].To do that, a purification protocol was applied as described in Section 2.3 in order to further enrich the selected extracts in those compounds prior analysis. This purification protocol was properly optimized, studying the most influencing variables. Todo that, different approaches were studied, including precipitation with several solvents (hexane, dichloromethane, ethyl acetate), use of SPE (different stationary phases) and molecular weight fractionation or combinations thereof. The optimum conditions were selected according to the best results observed in the two-dimensional LC analysis and involved a sample clean-up with dichloromethane followed by precipitations using acetone and ethanol finishing with an ethyl acetate extraction. After this procedure, the total phlorotannin content of the enriched extracts was more than double for Norwegian samples and around 1.5 times the initial content in Portuguese samples, being 11.730 ± 0.141,12.461 ± 0.264, 5.906 ± 0.324 and 6.4217 ± 0.355 mg PGE g^{-1}forthe samples N1, N2, P1 and P2, respectively.3.2. Chemical characterization of phlorotannins by LC × LC-ESI-MS/MS.

Although the determination of total phlorotannins using the DMBA colorimetric method is able to provide a general estimation of the phlorotannin amount found in the extract, this assay is notable to provide any information about the chemical structure of those compounds. The structural variability known in phlorotannins (see introduction), that includes 5 chemical classes, could be therefore linked to their bioactivity. For this reason, in order to elucidate the compounds present in the most active S. muticum extracts, a LC × LC approach was used. We have previously pre-sented the ability of this analytical tool to separate and tentatively identify phlorotannins of other brown algae, C. abies-marina, reaching degrees of polymerization of 17 phloroglucinol units(PGU) [16]. By using a combined HILIC separation in the first dimension (D1) and a RP-based separation in the second dimension (D2), 52 different phlorotannins could be effectively separated in C. abies-marina extracts. Considering that C. abies marina is a closely related species to S. muticum, both belonging to the family Sargassaceae within the class Phaeophyceae, this method was employed as starting point for the optimization of the separation of the purified phlorotannins' extracts from S. muticum collected in Portugal and Norway. Different experimental conditions were tested in the two dimensions; D1 optimization included the use of microbore silica, diol and amino columns as well as different combination of acetonitrile and 10 mM ammonium acetate at several pH (6.6 and 8.0). The second dimension optimization was based on the study of several columns (i.e, C18monolithiccolumn, and C18and PFP short partially porous columns)

using different flow rates, mobile phases and analysis time. As for the combination of the two dimensions, once the conditions were optimized separately, several transfer volumes (20, 30 and 50 _L)and modulation times (1.3, 1.5 and 2.0 min) were studied. After the optimization of all the separation conditions, the diol column was selected for the D1 HILIC. Moreover, the best separation conditions for the D2 included the use of a short C18partiallyporous column adapting the gradient employed to the complexity and composition of the samples studied (see Section 2.6.2).

Figs. 1 and 2 show the two-dimensional plots obtained (280 nm)for the samples collected in Norway and Portugal, respectively, under optimized the experimental conditions. At first sight it is possible to observe that the profiles obtained were significantly different as expected considering the different locations and associated environmental conditions such as sea surface temperature(8–10◦C for Norway and 18–20◦C for Portugal) and photosynthetically available radiation (35–40 mol photons m−2day−1for Norway and 40–45 mol photons m−2day−1for Portugal) [8]. Tables 2 and 3summarize the data corresponding to the separated compounds in the Norwegian and Portuguese samples, respectively, as well as the tentative assignments of the peaks observed; a first com-parison between samples shows important differences in terms of phlorotannin composition. For instance, samples harvested in Norway were more complex in terms of number of components. As can be observed in Table 2,phlorotannins with a polymerization degree up to 10 units were found. Even if the separation performance achieved was quite satisfactory, the identification of phlorotannins is a very tough taskdue to their huge chemical variability. In these samples, fuhalol and hydroxyfuhalol-type phlorotannins were the most frequently elucidated components, although phlorethols with diverse degree of polymerization were also present (peaks 16, 35 and 43). These two types of phlorotannins are characterized by being formed from phloroglucinol units linked through ether bonds; the difference between phlorethols and fuhalols is the presence of one or more additional hydroxyl groups on the terminal monomer unit [24]. As shown in Table 2, different fuhalols were found inthe Norwegian samples, starting from a degree of polymerization of 3 to 10, containing additional hydroxyl group(s). Moreover,other polymers were also elucidated containing up to 4 additional hydroxyl groups. Polymers containing 4–7 phloroglucinolunits were the most-frequently found (see Fig. 1 and Table 2).Interestingly, although the profiles were not identical between sitesin Norway, they were quite similar, being peaks 2, 19, 24 and34 among the most intense peaks in both samples, corresponding to trifuhalol (degree of polymerization (DP) = 3, 1 hydroxylgroup), tetrafuhalol (DP = 4, 1 hydroxyl group), dihydroxytetra-fuhalol (DP = 4, 3 hydroxyl group) and pentafuhalol (DP = 5, 1hydroxyl group). Sample N2 also contained other very intense peaks (peaks 5, 6) for which an assignment was not attained. In any case, the fragmentation pattern observed for the different peaks was very important for their identification, as they followed a typical fragmentation showing different losses of phloroglucinol units and hydroxyl groups, which helped to achieve a tentative identification. An example is shown in Fig. 3 with the MS/MS fragmentation pattern of dihydroxyheptafuhalol in sample N1 (peak 63). It is also important to note that the relative position of a peak in the 2D plane can be used as a tool for identification since LC × LC allows obtaining2D patterns in agreement with differential retention in each dimension. For instance, D1 separation shows a distribution according to an increase on degree of polymerization while D2 retention implies that highly hydrophobic compounds elute later, making possible the discrimination, between doubtful identifications, based on peak position. Therefore, this enhanced identification capability of unknown peaks is one of the strongest advantages of 2D approaches over one-dimensional ones. In the case of the Portuguese samples, less complex 2D plots were obtained (Fig. 2). In this case, the differences between the samples collected in the same country were also more marked. Anyway, peaks 14 and 26 were among the most-intense peaks in both samples, corresponding to an unidentified compound and to a pentafuhalol (DP = 5, 1 hydroxyl group), respectively. From the information summarized in Table 3 it is possible to infer that fuhalols and hydroxyfuhalols were also the predominant phlorotannins in these Portuguese samples.

Different degrees of polymerization were found, from 3 to 11, whereas the additional hydroxyl groups associated to these structures were up to 4 in some cases. Although the presence of hydroxyfuhalols in S. muticum has been reported [25], it is worth to mention that there are no previous studies describing in such detail the phlorotannin content in S. muticum samples nor in any other algal sample containing a similar variability on fuhalols, hydroxyfuhalols and phlorethols composition, which gives a clear idea of the difficulty of this task.

In this regard, the potential of LC × LC-MS/MS to separate and to tentatively identify such a complex mixture is demonstrated in the present work. NMR spectra (HMBC analysis) obtained with algae collected in different North-Atlantic sites allowed only the determination of phlorethols in the phlorotannin fraction of S.muticum [8,15]. Thus, considering the complexity of the polyphenols fraction (as demonstrated by the phlorotannins profile shown in Figs. 1 and 2), we can conclude that the pressurized liquid extraction (PLE) combined to a LC × LC-DAD-MS/MS methodology applied appears a promising tool for the complete separation and identification of phlorotannins in algal samples. This multidimensional tool offers a series of advantages that one-dimensional approaches cannot provide, thanks to the simultaneous use of different separation mechanisms. In fact, the gains obtained through this two-dimensional approach may be illustrated from the peak capacity values achieved for the separations. In this regard, a theoretical peak capacity (nc2D) of 1050 was obtained for N1 sample whereas P1 separation reached a peak capacity of 906; measurements were done according to Li et al. [26] in order to consider the D2 time cycle as well as the influence of under sampling of the D1 eluate. Moreover, to stress the importance of reoptimizing the LC × LC separation according to the sample studied, the values found with the present method and that previously developed [19]were compared for the same sample (N1); results showed that a50% increase on separation performance was obtained (nc2D699 vs1050).At present, very little is known about the relationship between phlorotannin structure (degree of polymerization, type of bond, branching) and bioactivity [4]. From the published data as well as from the results included in this work, it seems that the antioxidant activity of these components may be more related to their relative abundance than to their different structure [4]. Nevertheless ,one should note that in Norwegian populations, a higher variety of smaller compounds compared to Portuguese populations was detected. This observation is in agreement with previous reports that demonstrated that small phlorotannins possess higher antioxidant activity than highly polymerized compounds [27]. Other reports have pointed out some discrepancies between antioxidant activity and other activities, such as hepatoprotective activity, showing that some phlorotannins that presented good antioxidant activity did not possess similar hepatoprotective activity[28]. Besides, to further study the possible relationship betweenphlorotannin structure and antiproliferative activity, the four purified S. muticum extracts (collected in Norway and Portugal) were assayed in order to observe their potential effects against a human colon cancer cell line, HT-29.3.3. Antiproliferative activity of phlorotannins from S. muticum. To determine the antiproliferative effect of the phlorotannins- enriched extracts, HT-29 cells were incubated with increasing concentrations of extracts (from 0 to 100 _g mL−1) for 24, 48 and72 h and cell proliferation was analyzed by the MTT assay. As can be observed in Fig. 4, after 24 h incubation, the concentration dependence of the antiproliferative activity of the extracts was significant. The extracts demonstrated different in vitro antiproliferative effects on HT-29 colon cancer cells. In general, the most enriched extracts Fig in phlorotannins (those obtained from Norway, sites 1 and 2; N111.730 ± 0.141 and N2 12.461 ± 0.264 mg PGE g−1) exerted higher antiproliferative activity. For instance, incubation with N1 andN2 extracts at a concentration of 50 _g mL−1, reduced cell proliferation by roughly 50% after 24 h, while extracts obtained from Portugal, sites 1 and 2 (P1 and P2 samples, total phlorotannin con-tent 5.906 ± 0.324 and 6.4217 ± 0.355 mg PGE g−1, respectively.)did not exert any appreciable effect at this concentration. Furthermore, cell viability was totally reduced after treatments withN1 and N2 extracts at concentrations close to 100 _g mL−1at anyof the assayed times, while the same concentration of P1 and P2extracts reduced cell viability down to ca. 40% after 24 h incubation. In order to characterize in more detail the antiproliferative e activity of these extracts, the growth inhibition (GI50), the total growth inhibition (TGI) (as an indicator for cytostaticity), and the lethal concentration (LC50) (as an indicator for the cytotoxic level of effect), were also determined at 24, 48 and 72 h incubation times. As it is shown in Table 4, the phlorotannin-enriched extracts exert different in vitro cytostatic and cytotoxic effects depending on the type of extract and concentration, exhibiting maximum inhibitory activity after 24 h exposure. In particular, N1 and N2 demonstrated cytotoxic potential at concentrations in the medium micromolar range. The comparison of the results obtained in the present study with those reported in literature for brown seaweeds-derived extracts is not straightforward due to limited information available about chemical phlorotannin composition and differences in the in vitro cell models used. However, the parameter values obtained in the present study seem to be close to the antiproliferative concentration levels of brown seaweeds extracts published in various reports for other in vitro cell studies [11,29,30]. As an example, He et al. [31]

reported cell viability inhibition values of36.9 and 60.5% for the brown seaweed Saccharina japonica extracts containing phlorotannins (2.19 and 1.28 mg g−1, respectively) on hepatocellular carcinoma cells following a exposure time of 24 h at the concentration of 60 _g mL−1. Interestingly, data summarized in Table 4 also showed that N1 extract, which contained lower phlorotannin concentration than N2 extract (see Table 1 for the values of TPC and total phlorotannins for the purified N1, N2, P1 andP2 extracts), exerted slightly higher effect on cell proliferation thanN2 extract, provided by the lower values obtained for the parameters at the different incubation times. According to the chemical differences in the phlorotannin fraction observed between these extracts, these results suggest that either phlorotannins with selective bioactivity or the presence of other compounds in the extracts might be responsible for the differential antiproliferative effective-ness observed between N1 and N2. In this regard, further work isrequired to elucidate the active constituents responsible for this differential effectiveness between phlorotannin-enriched extracts.

4. Conclusions

In the present work, the phlorotannin composition of different S. muticum samples collected at different locations along theEuropean Atlantic coast was investigated. After PLE extraction ,the samples collected at the extreme locations of a latitudinal gradient along North Atlantic coasts, i.e., Portugal and Norway, were found to be the richest in total phenols and, particularly, inphlorotannins. The extracts obtained from these locations were further purified and chemically characterized using a modified HILIC × RP-DAD-MS/MS method. The application of this method-ology allowed the tentative identification of phlorotannins with great chemical variability containing different degrees of polymerization and structures; fuhalols, hydroxyfuhalols and phlorethols were the most-abundant phlorotannins on these samples, showing also particularities and important differences depending on the geographical location. This is the first time that these complex structures are separated and characterized with such detail. After-wards, the antiproliferative activity of these extracts against HT-29adenocarcinoma colon cancer cells was studied. Results revealed that Norwegian samples of S. muticum presented the highest activity, showing a good cytotoxic potential at concentrations in the medium micromolar range.

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Table 1
Extraction yield (%), total phenol content (mg PGE g^{-1} extract), total phlorotannin content (mg PGE g^{-1} extract) and antioxidant activity (mmol TE g^{-1} extract, TEAC assay) of the brown macroalga *Sargassum muticum* PLE extracts according to their collection location. Values presented are mean ± sd. Superscripts mean groups not statistically different ($p > 0.05$) for each response.

Location		Extraction yield (%)	Total phenol content (mg PGE g^{-1})^{A}	Total phenol content (% DWalgae)^{B}	Total phlorotannins (mg PGE g^{-1})^{C}	TEAC value (mmol TE g^{-1})^{D}
	F1	23.7 ± 0.3	75.43 ± 1.56^{a}	1.783 ± 0.029^{d}	3.297 ± 0.337^{c}	0.983 ± 0.018^{c,d}
France	F2	17.5 ± 0.4	58.19 ± 3.31^{b}	1.016 ± 0.070^{a}	2.606 ± 0.036^{a}	0.652 ± 0.013^{a}
	F3	27.4 ± 2.5	78.55 ± 0.51^{a}	2.150 ± 0.157^{f}	3.380 ± 0.163^{c}	1.091 ± 0.017^{e}
	P1	24.9 ± 0.1	145.02 ± 3.05^{e}	3.604 ± 0.060^{h}	4.088 ± 0.239^{d}	2.101 ± 0.047^{g}
Portugal	P2	23.2 ± 0.5	119.27 ± 1.39^{f}	2.768 ± 0.068^{g}	4.127 ± 0.129^{d}	1.828 ± 0.025^{f}
	S1	18.7 ± 0.1	79.29 ± 134^{a}	1.481 ± 0.030^{b,c}	2.629 ± 0.091^{a}	0.919 ± 0.023^{b}
Spain	S2	16.2 ± 0.2	77.19 ± 1.64^{a}	1.248 ± 0.022^{a,b}	2.477 ± 0.139^{a}	0.943 ± 0.033^{b,c}
	S3	15.5 ± 0.1	80.46 ± 1.38^{a,c}	1.245 ± 0.026^{a,b}	2.567 ± 0.075^{a}	0.923 ± 0.016^{b}
	I1	21.3 ± 0.7	86.60 ± 2.44^{c}	1.844 ± 0.064^{d,e}	2.933 ± 0.090^{b}	1.106 ± 0.020^{d}
Ireland	I2	21.8 ± 0.6	78.39 ± 5.57^{a}	1.710 ± 0.156^{c,d}	2.670 ± 0.335^{a}	0.889 ± 0.011^{b}
	I3	22.3 ± 1.0	94.08 ± 0.96^{d}	2.098 ± 0.077^{e,f}	3.462 ± 0.095^{c}	1.068 ± 0.007^{e}
Norway	N1	31.5 ± 0.3	148.97 ± 0.85^{e}	4.696 ± 0.034^{i}	5.115 ± 0.145^{e}	2.297 ± 0.050^{h}
	N2	31.6 ± 1.4	146.44 ± 4.54^{e}	4.639 ± 0.274^{i}	4.839 ± 0.134^{f}	2.090 ± 0.032^{g}

[A] mg phloroglucinol equivalents g^{-1} extract.
[B] % dry weight algae.
[C] mg phloroglucinol equivalents g^{-1} extract.
[D] mmol Trolox equivalents g^{-1} extract.

Table 2
Tentative peak assignment of the compounds separated by LC × LC-ESI-MS/MS found in the *S. muticum* samples collected in Norway.

Peak	Identification	D2 t_R (s)	Total t_R (min)	$[M-H]^-$	Main MS/MS fragments detected
1	Trifuhalol	40.30	27.97	389.0	375, 265, 245
2	Trifuhalol	41.85	30.60	389.0	375, 265, 245
3	Trifuhalol	42.00	34.50	389.1	375, 265, 245
4	NI*	45.50	34.56	448.5	415, 385, 321, 245, 196
5	NI	47.10	34.59	566.6	533, 389, 306, 244, 193
6	NI	50.75	34.65	564.3	526, 437, 373, 331, 202
7	NI	55.95	34.73	571.9	526, 449, 383, 319, 261, 193
8	NI	57.30	34.76	625.0	581, 498, 388, 258
9	Hydroxytetrafuhalol	45.30	35.86	529.8	512, 404, 389, 343, 262
10	Hydroxytetrafuhalol	47.10	35.89	529.1	513, 405, 387, 345, 264
11	NI	50.40	35.94	572.6	538, 511, 446, 318, 164
12	Pentafuhalol	53.70	36.00	637.1	621, 513, 385, 262
13	Tetrafuhalol	47.15	37.19	513.0	391, 373, 264, 245, 219
14	Hydroxytetrafuhalol	48.40	37.21	529.4	483, 465, 401, 389, 262, 245
15	Tetrafuhalol	41.60	38.39	513.4	389, 265, 245
16	Pentaphlorethol	52.05	38.57	621.5	603, 493, 357, 245
17	Hydroxytetrafuhalol	52.15	38.58	529.4	513, 389, 262
18	NI	55.25	38.32	590.5	570, 545, 466, 437, 401, 245
19	Tetrafuhalol	43.50	38.43	513.8	389, 263, 245
20	Tetrafuhalol	45.25	38.45	513.3	389, 263, 245
21	Tetrafuhalol	49.50	38.53	513.8	387, 263, 245
22	NI	52.40	38.57	683.3	648, 555, 509, 415, 387, 263, 245
23	NI	57.85	38.66	590.3	573, 547, 446, 333, 245, 195
24	Dihydroxytetrafuhalol	44.45	39.74	545.4	525, 513, 484, 403, 389, 375
25	NI	48.35	39.81	683.9	651, 557, 509, 387, 621, 245
26	NI	49.80	39.83	683.1	652, 543, 389, 302, 263, 245
27	NI	51.30	39.86	588.8	571, 522, 441, 380, 278, 246
28	Hydroxytetrafuhalol	40.20	40.97	529.5	465, 403, 389, 341, 263, 245
29	Hydroxytetrafuhalol	43.35	41.02	529.3	511, 403, 389, 263, 245
30	Pentafuhalol	46.45	41.07	637.3	621, 513, 373
31	Pentafuhalol	50.55	41.14	637.5	633, 513, 273
32	NI	52.30	41.17	807.3	775, 681, 541, 509, 385, 244
33	Pentafuhalol	44.05	42.33	637.3	621, 513, 497, 389
34	Pentafuhalol	46.30	42.37	637.4	513, 374
35	Hexaphlorethol	51.50	42.46	745.3	727, 619, 603, 371, 355, 309
36	NI	51.65	42.46	807.2	775, 757, 681, 509, 385, 245
37	Hydroxypentafuhalol	45.35	43.66	653.8	638, 527, 513, 466, 389, 263, 245
38	Dihydroxypentafuhalol	42.60	44.91	669.6	623, 527, 465, 403, 385, 341, 261
39	Dihydroxypentafuhalol	43.60	44.93	669.6	623, 543, 527, 465, 402, 385, 341, 260
40	Hydroxypentafuhalol	48.65	45.01	653.6	637, 527, 387, 245

41	Hexafuhalol	50.10	45.03	761.6	637
42	Hexafuhalol	50.40	45.04	761.3	745, 637, 498, 389, 245
43	Heptaphlorethol	53.00	45.08	869.2	851, 745, 728, 603, 245
44	Hexafuhalol	53.30	45.09	761.3	747, 637, 621, 513, 497, 245
45	Dihydroxypentafuhalol	47.15	46.29	669.8	621, 541, 527, 463, 401, 337, 271
46	Dihydroxyhexafuhalol	50.00	46.33	793.1	775, 731, 651, 527, 511, 403, 387
47	Hydroxyhexafuhalol	43.65	47.53	777.7	651, 637, 529, 511, 387, 261, 245
48	Dihydroxyhexafuhalol	46.00	47.57	793.3	775, 669, 653, 527, 403, 389, 262
49	Hydroxyhexafuhalol	47.85	47.60	777.3	763, 655, 529, 515, 388
50	NI	50.60	47.64	947.3	915, 821, 651, 527, 385
51	NI	54.00	47.70	894.2	830, 766, 625, 568
52	Dihydroxyhexafuhalol	49.15	48.92	793.7	777, 652, 589, 554, 511, 390, 311
53	Trihydroxyhexafuhalol	47.75	50.20	809.7	791, 765, 747, 667, 543, 527, 405
54	Dihydroxyheptafuhalol	51.90	50.27	917.1	873, 855, 838, 791, 775, 731, 713, 651, 513, 387
55	Octafuhalol	54.94	50.32	1009.2	994, 968, 887, 872, 747, 621
56	Dihydroxyhexafuhalol	42.80	51.41	793.3	667, 653, 529, 403, 387, 263
57	Dihydroxyhexafuhalol	47.80	51.49	794.2	774, 667, 653, 529, 403, 387, 263
58	Hydroxyheptafuhalol	51.70	51.56	901.8	857, 775, 761, 637, 511, 387
59	Dihydroxyhexafuhalol	49.50	52.83	793.9	773, 668, 653, 529, 403
60	Trihydroxyheptafuhalol	53.05	52.88	933.8	889, 793, 747, 651, 525, 385
61	Nonafuhalol	53.25	52.89	1133.9	1115, 1007, 993, 885, 869, 760, 745, 620
62	Nonafuhalol	56.15	52.94	1133.7	1117, 1009, 995, 887, 870, 853, 761, 745, 622
63	Dihydroxyheptafuhalol	45.45	52.76	917.5	874, 777, 651, 527, 387
64	Trihydroxyheptafuhalol	51.90	52.87	933.4	914, 889, 792, 748, 650, 529
65	Trihydroxyoctafuhalol	54.20	52.90	1057.2	1008, 915, 793, 652, 527, 387
66	Dihydroxyheptafuhalol	45.25	54.05	917.5	899, 874, 791, 775, 651, 527, 387
67	Dihydroxyheptafuhalol	51.75	54.16	917.7	873, 791, 777, 653, 527, 387
68	Trihydroxyoctafuhalol	54.05	54.20	1057.3	
69	Decafuhalol	56.75	54.25	1257.7	1239, 1133, 1117, 1007, 885, 624, 573, 387
70	Dihydroxyheptafuhalol	47.30	55.39	917.1	897, 873, 791, 777, 731, 653, 527, 389
71	Dihydroxyoctafuhalol	54.90	55.52	1041.3	979, 915, 901, 853, 777, 731, 651, 637, 528, 389
72	Decafuhalol	57.50	55.56	1257.7	
73	Dihydroxynonafuhalol	55.00	56.82	1165.7	1146, 1040, 1025, 917, 899, 777, 653, 637, 389

* NI, not identified.

Table 3
Tentative peak assignment of the compounds separated by LC × LC-ESI-MS/MS found in the *S. muticum* samples collected in Portugal.

Peak	Identification	D2 t_R (s)	Total t_R (min)	[M−H]−	Main MS/MS fragments
1	Trifuhalol	40.70	31.88	389.0	263, 245
2	Trifuhalol	41.90	34.50	389.0	263, 245
3	NI	45.00	38.45	447.8	429, 385, 323, 311, 261
4	Trifuhalol	47.20	38.49	389.0	375, 265, 250
5	NI	48.15	38.50	568.4	552, 537, 443, 305
6	Trifuhalol	42.45	39.71	389.0	375, 251
7	NI	50.50	39.84	570.6	551, 511, 443, 305, 263
8	NI	47.20	41.09	526.8	507, 491, 401, 387, 357, 263, 245
9	NI	48.30	41.11	536.3	520, 475, 411, 333, 268
10	Tetrafuhalol	43.65	42.33	513.7	437, 389, 265, 251
11	Tetrafuhalol	40.30	43.57	513.0	475, 438, 391
12	NI	41.60	43.59	520.0	499, 439, 389, 319, 251
13	Tetrafuhalol	43.55	43.63	513.0	499, 437, 389, 263
14	NI	44.45	43.64	516.3	437, 427, 389, 297, 251
15	NI	48.70	43.73	685.1	651, 633, 509, 387, 245
16	NI	49.55	43.73	685.3	651, 633, 557, 509, 387, 263, 245
17	NI	52.35	43.77	590.4	572, 511, 465, 426, 325, 245
18	Dihydroxytetrafuhalol	44.65	44.94	545.7	529, 513, 389
19	Hydroxytetrafuhalol	40.30	46.17	529.7	403, 389, 277, 262, 245
20	NI	48.55	46.19	687.2	653, 579, 525, 388, 244
21	NI	41.80	46.20	531.4	513, 487, 403, 391, 341, 263, 245
22	NI	43.35	46.22	531.0	515, 486, 405, 391, 363, 307, 265, 245
23	NI	46.55	46.28	646.7	633, 529, 501, 387, 245
24	NI	50.95	46.35	646.5	633, 607, 525, 509, 387, 343, 263, 245
25	Dihydroxypentafuhalol	42.20	47.50	671.0	653, 637, 627, 544, 466, 247
26	Pentafuhalol	46.20	47.57	637.3	623, 513, 373
27	Trihydroxyhexafuhalol	51.55	47.66	809.5	791, 775, 637, 511, 387
28	NI	52.95	47.68	734.3	715, 689, 607, 566, 437, 285
29	Dihydroxypentafuhalol	44.20	48.84	669.0	651, 625, 607, 465, 403, 263
30	Dihydroxypentafuhalol	45.60	48.86	671.3	653, 637, 627, 467, 467, 405, 349
31	Hydroxypentafuhalol	41.40	50.09	653.2	633, 527, 513, 387, 263, 245
32	Hydroxypentafuhalol	42.80	50.11	653.8	637, 527, 513, 387, 263, 245
33	NI	44.20	50.14	655.3	636, 611, 529, 515, 469, 388, 341, 262, 245
34	NI	48.65	50.21	657.0	633, 621, 524, 483, 370, 263
35	NI	50.25	50.24	780.5	638, 611, 532, 388, 217
36	NI	53.05	50.28	877.6	859, 832, 797, 661, 612, 520
37	NI	46.20	51.47	661.2	612, 555, 509, 367, 263
38	NI	47.20	51.49	663.7	647, 574, 537, 505, 374
39	Dihydroxyhexafuhalol	47.70	52.80	793.1	775, 749, 731, 527, 483, 465, 385
40	Dihydroxyhexafuhalol	50.65	52.84	793.2	775, 749, 731, 527, 511, 483, 387, 245
41	NI	53.95	52.89	895.4	
42	NI	44.15	54.02	781.4	763, 701, 499, 437, 263
43	Hydroxyhexafuhalol	50.90	54.08	949.1	
44	Dihydroxyhexafuhalol	49.45	54.12	793.5	777, 730, 634, 513, 485
45	NI	51.05	54.15	948.1	775, 749, 652, 607, 528, 510, 483, 431, 389
46	Dihydroxyheptafuhalol	49.20	55.42	917.3	900, 874, 856, 714, 634, 513
47	Dihydroxyheptafuhalol	52.00	55.47	917.6	898, 873, 791, 777, 634, 513, 403
48	Dihydroxynonafuhalol	55.20	55.52	1165.3	
49	Dihydroxyheptafuhalol	48.00	56.70	919.2	900, 874, 856, 837, 714, 634, 513, 265
50	Dihydroxynonafuhalol	51.90	56.77	1165.7	
51	Dihydroxyhexafuhalol	53.20	56.80	793.1	777, 749, 653, 529, 403, 387, 263
52	Trihydroxyheptafuhalol	53.30	58.09	933.5	914, 871, 773, 667, 651, 623, 511
53	Dihydroxyoctafuhalol	54.60	58.09	1043.1	
54	Nonafuhalol	56.30	58.14	1134.9	
55	Decafuhalol	54.55	60.71	1257.7	
56	Endecaphlorethol	57.70	62.06	1365.3	

[*] NI, not identified.

Table 4
Antiproliferative *in-vitro* effect of *S. muticum* extracts on HT-29 cells. Values represent the concentrations in μg mL^{-1} of each extract that caused 50% growth inhibition (GI50), total growth inhibition (TGI), and 50% cell death (LC50). Results are shown as the mean ± SEM of three independent experiments, each performed in triplicate. Superscripts mean groups not statistically different ($p > 0.05$) for each response.

Sample	Incubation time (h)	GI_{50} (μg mL^{-1})	TGI (μg mL^{-1})	LC_{50} (μg mL^{-1})
N1	24	32.2 ± 1.7^a	41.7 ± 1.4^a	53.5 ± 0.9^a
	48	37.2 ± 1.4^a	$45.5 \pm 0.8^{a,b}$	55.0 ± 0.2^a
	72	36.4 ± 4.7^a	$46.3 \pm 2.7^{a,b}$	59.4 ± 4.2^a
N2	24	37.0 ± 2.3^a	$46.7 \pm 1.3^{a,b}$	57.9 ± 0.1^a
	48	40.3 ± 2.5^a	$49.2 \pm 1.9^{a,b}$	57.8 ± 0.2^a
	72	46.5 ± 1.1^a	56.1 ± 3.2^b	74.0 ± 2.6^b
P1	24	77.7 ± 4.4^b	NC*	NC
	48	72.4 ± 2.7^b	NC	NC
	72	81.8 ± 3.1^b	NC	NC
P2	24	72.0 ± 3.3^b	NC	NC
	48	75.8 ± 2.9^b	NC	NC
	72	83.8 ± 4.6^b	NC	NC

*NC, not calculated.

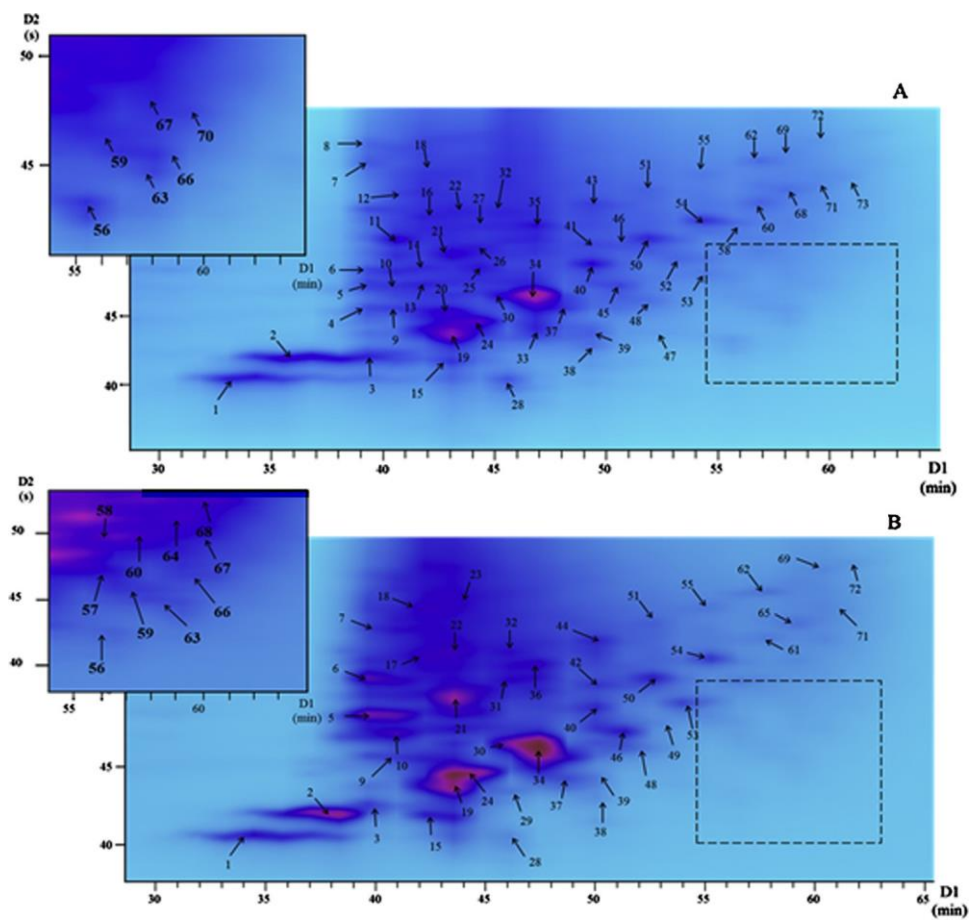

Fig. 1. 2D plot (280 nm) of the *Sargassum muticum* enriched extracts from the samples collected in Norway (A, sample N1 and B, sample N2) obtained using the optimized HILIC × RP-MS/MS method. For peak identification, see Table 2.

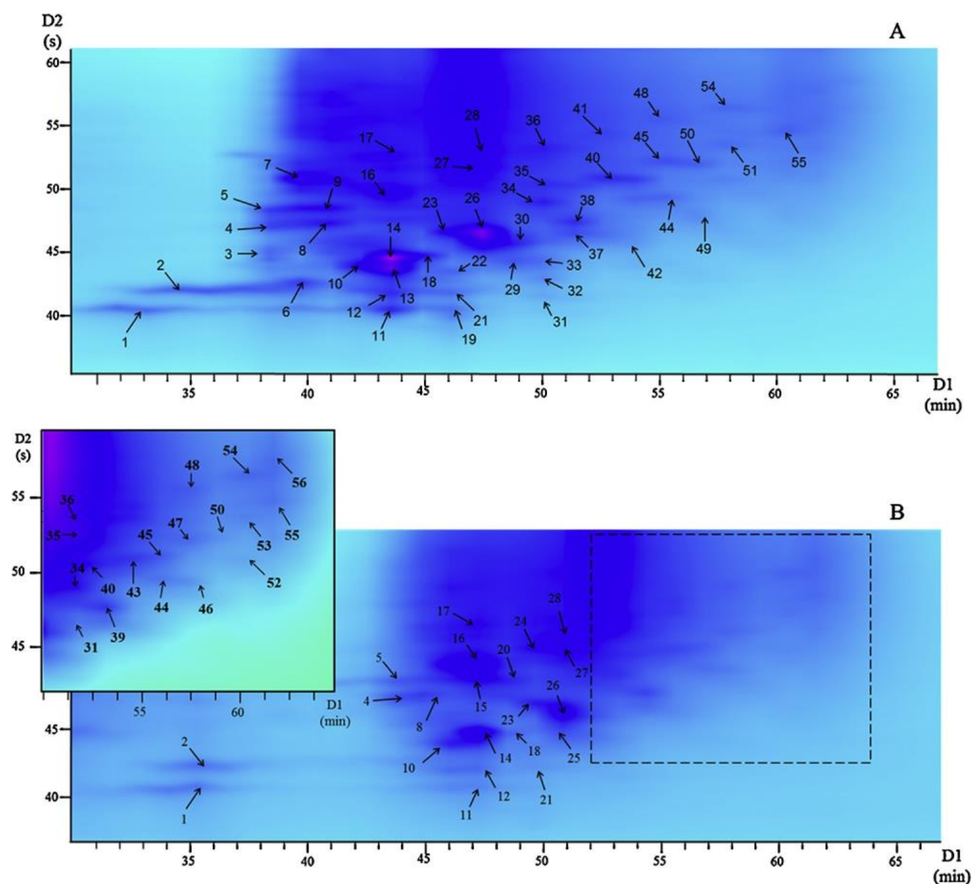

Fig. 2. 2D plot (280 nm) of the *Sargassum muticum* enriched extracts from the samples collected in Portugal (A, sample P1 and B, sample P2) obtained using the optimized HILIC × RP-MS/MS method. For peak identification, see Table 3.

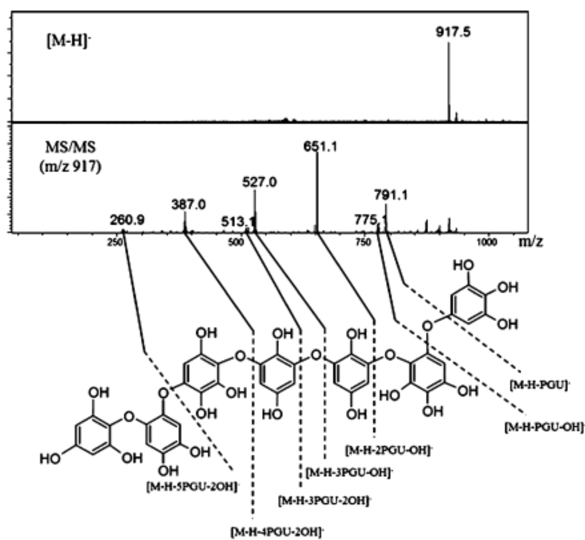

Fig. 3. MS spectrum and MS/MS fragmentation pattern of dihydroxyheptafuhalolin sample N1 (peak 63, fuhalol with DP = 7 with two additional hydroxyl groups) aswell as the tentatively proposed chemical structure. PGU: phloroglucinol units.

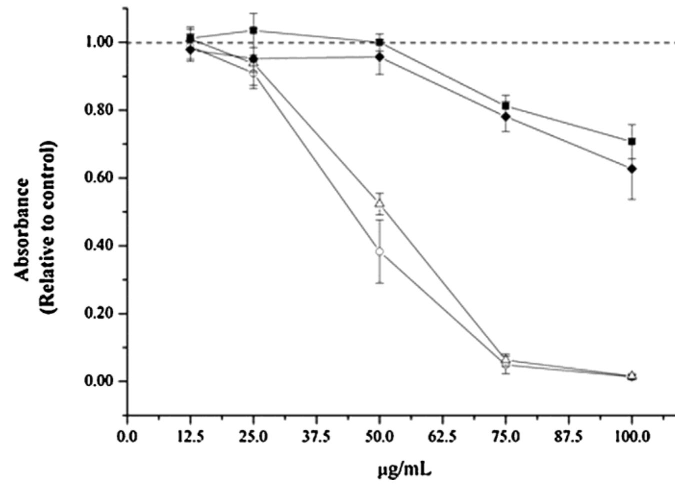

Fig. 4. HT-29 cell viability upon treatment for 24 h with different concentrations of N1 (circle), N2 (triangle), P1 (square) and P2 (diamond) extracts. Error bars are given as 95% confidence interval.

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