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Green improved processes to extract bioactive phenolic compounds from brown macroalgae using *Sargassum muticum* as model

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ABSTRACT

A comparative study between "alternative" extraction processes such as centrifugal partition extraction (CPE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) and classical solid/liquid used in the laboratory are currently focusing on the efficiency (selectivity and productivity) to obtain bioactive phenolic compounds from the phaeophyte *Sargassum muticum* model. The choice of the best process was based on several measurements: (i) the total phenolic content measured by the colorimetric Folin–Ciocalteu assay, (ii) radical scavenger and antioxidant activities assessed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, and the β-carotene bleaching method and finally (iii) the method productivity. Irrespective of the solvent used in the processes, alternative methods are always sharply more effective than classical ones. With the exception of SFE which does not allow extracting the totality of the active phenolic compounds, two of the other extraction methods were particularly promising. First, CPE afforded the most important yields in concentrated phenolic compounds (PC) (22.90 ± 0.65%DW) also displaying the best activities (0.52 ± 0.02 and 0.58 ± 0.19 mg/mL for IC50 and AAC_{700}, respectively). Secondly, PLE using an EtOH:water mixture 75:25 (v/v) allowed a good PC extraction (10.18 ± 0.25%DW) with huge efficiency. Despite a lesser activity of the extracts (0.77 ± 0.01 and 1.59 ± 0.15 mg/mL for IC50 and AAC_{700}, respectively) PLE is a green process and potentially complies European norms requirements for the prospective valorization of phenolic compounds from *S. muticum* in Brittany.

1. Introduction

Phenolic compounds (PC) in brown algae, also called phlorotannins are secondary defense metabolites synthesized during development as components of algal cell-walls [1] or as chemical defense in response to abiotic and biotic stress conditions, such as UV radiation, grazing, bacterial infection and epiphytism, as well as in intra- and inter-specific communication [2–5]. As these compounds are produced in response to the production of reactive oxygen species (ROS), PC exhibits antiROS, i.e. antioxidative properties [6–8]. Structurally, phlorotannins are oligomers and polymers of 1,3,5-trihydroxybenzene (phloroglucinol) [2] can be considered as a pool composed by CP of different natures and/or polarities. In the last few years, natural antioxidants from plant and animal sources are actively investigated as replacements for synthetic antioxidants currently used as food additives. Conventional solvent solid/liquid extraction is frequently used to extract bioactive compounds from algae [9–16]. Nevertheless, this time-consuming technique often uses large volumes of toxic organic solvents. Advanced alternative processes exist and are already used for the extraction of various biomolecules, such as amino acids, fatty acids, natural pigments, polysaccharides, vitamins, toxins and other metabolites from seaweeds [17]. In particular, supercritical-fluid extraction (SFE), pressurized-liquid extraction (PLE) and centrifugal partition extraction (PCE) are frequently used for these classes of compounds [17–27].

Abbreviations: CPE, centrifugal partition extraction; PC, phenolic compounds; PLE, pressurized liquid extraction; ROS, reactive oxygen species; SFE, supercritical fluid extraction; TPC, total phenolic content

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In our ongoing investigations to quantitatively optimize and qualitatively refine phlorotannins extraction from brown algal tissues, we present here a comparative study of the alternative methods mentioned hereafter versus the classical solvent-extraction method still used in many laboratories. CPE has been widely used for the extraction of natural products [26,28–30]. This technique is very attractive in terms of selectivity, amenable to sample loading capacity and scaling-up ability [31–33]. SFE operates at low temperatures in oxygen-free conditions and uses supercritical CO_2 as the extractive agent. These characteristics make SFE an ideal technique for the extraction of natural antioxidants. On the other hand, PLE can use all kind of solvents at relatively high temperatures and pressures enough to keep them in liquid state; advantages associated to its use are the low solvent volumes employed and the short extraction times, compared to conventional processes. Moreover, high extraction yields have been commonly associated to this technique. Green (meaning that complies with the principles of Green Chemistry; [34]) and selective extraction methods such as SFE and PLE are now preferred to all others.

Sargassum muticum (Yendo) Fensholt was chosen as model organism to carry out these assays. This introduced brown macroalga has widely spread along the French Atlantic coasts and is currently one of the most accessible Sargassaceae species on the French Brittany shores. This large sustainable biomass could represent a viable biotechnological asset in the regional resource development programs. Moreover this species is known to produce interesting phenolic compounds [35] of low molecular weight [15] which can be extracted and purified more easily than bigger polymers and having already demonstrated biological activities such as antifouling and antioxidant ones [10,36,37]. The potential represented by *S. muticum* led us to carry out a study aimed at selecting the best extraction technique, i.e. which would maximize yield while minimizing the denaturation and the loss of original properties, of the polyphenols of interest. More specifically, we need: (i) to control and validate the quantity and the quality of PC extracted by each process (Folin and NMR profiles), (ii) to verify that active extracts are not lost during the process and finally (iii) to monitor the process productivity by taking into account the PC quantity obtained in regard of freeze-dried raw material quantity, solvent quantity and extraction time.

2. Materials and methods

2.1. Plant material

Thalli of *S. muticum* were collected in July 2011 at the Dellec (Plouzané), a semi-exposed field site on the western coast of Brittany (France). This month was chosen because phenolic-rich thalli of *S. muticum* are best collected in summer [4,10].

During collection, only the apical and median parts of the thalli were retained and the holdfast was left in place to allow regrowth and thus minimize collecting impact. Immediately after collection and epiphytes removal, seaweeds were washed first with filtered seawater then with distilled water in order to get rid of residual sediments and salts. The cleaned algal materials were then surface dried with blotting paper towel, chopped into fragments, freeze-dried, powder reduced with a Waring Blender and finally sieved at 250 μm.

2.2. Conventional solid/liquid extraction of phenolic compounds

2 g (dw) of finely ground algal material was placed in 250 mL flasks, and extracted using 150 mL volumes of the following solvent mixtures: hexane–EtOH 88:12 (v/v), ethanol 75% (EtOH–water 75:25) and 25% EtOH–water 25:75 or ethyl acetate 50% (AE 50:50) (v/v in distilled water). The choice of these different solvents was done in accordance to alternative approaches (see below). Each preparation was left under stirring at 40 °C for 3 h in the dark. The mixtures were then centrifugated using an Eppendorf 5810R centrifuge (Eppendorf A.G., Hamburg, Germany) at 4000 g for 10 min at 4 °C. The supernatants were then filtered on cotton wool and concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator (R-3000, Büchi, Flawil, Switzerland). About 10 mL of Milli-Q water was then added to each residue to give the crude extract. All extracts were then freeze-dried prior to further analyses (phenolic content quantification and NMR analyses). All the extracts were prepared in triplicate. In graphics, solid/liquid extraction was noted as “classical” extraction with, respectively, hexane–EtOH 88:12 (v/v), ethanol–water (75:25) and (25:75) or EA–water (50:50).

2.3. Alternative extraction of phenolic compounds

2.3.1. Centrifugal partition extraction (CPE)

A centrifugal partition extractor was used for experiments. The centrifugal partition extractor is a prototype built by Kromaton Technology (Annonay, France) for the GEPEA Laboratory using the laboratory knowledge in CPE hydrodynamics and design. The apparatus contains a rotor of 7 circular partition disks engraved with a total of 231 partition asymmetric twin cells arranged circumferentially and connected together by ducts. The stationary phase was maintained inside the column by a centrifugal acceleration generated by the rotation around a single vertical shaft. The volume of the column is 279 mL. The rotation speed can be adjusted from 200 to 1200 rpm, producing a centrifugal acceleration up to 160 g. The mobile phase was pumped through the stationary phase with a 3 pistons Armen Instrument Preparative Pump (Vannes, France). Two operating modes of elution are possible with a biphasic system: the descending mode, when the mobile phase is the heavy one and the ascending mode in the opposite case. For extraction, the solvent (organic phase) is kept stationary while the aqueous phase is pumped in the descending mode. In this work the potential of the CPE apparatus for the extraction of phenolic compounds was examined. For this, 1 L of a mixture of ethyl acetate (EA):distillated water (50:50) was prepared: 2 phases were obtained, an aqueous one saturated in EA and an organic one saturated in water. At first, an algal suspension was prepared as follows: 2 g of freeze-dried algal powder were put in suspension in 150 mL of aqueous phase; the obtained suspension was ultrasounds homogenized during 15 min. The organic phase was used as the stationary phase while the algal suspension was pumped as the mobile phase. The extraction was performed at room temperature, using the descending mode, at 800 rpm, during 3 min, with a flow rate of 75 mL/min. Operating conditions were chosen according to earlier preliminary assays and following different requirements: (1) the extraction solvent has to be stationary and the aqueous phase must to be pumped in the fault; (2) the centrifugal acceleration should not be too strong (to avoid particles sedimentation in the column) and (3) solvent retention in the column ought to be rather good (> 50% volume of the column). In graphics, CPE extraction was noted as “alternative” extraction with EA–water (50:50) as extracting solvent.

2.3.2. Supercritical fluid extractions (SFE)

A Suprex PrepMaster (Suprex, Pittsburgh, PA) supercritical fluid extractor was used for the SFE experiments; the extractor was equipped with a dual piston pump for CO_2. For the experiments, 2.5 g of algae were mixed with 2.5 g of sea sand (as dispersing agent, to avoid system clogging) and the mixture was loaded into a 20 mL stainless-steel extraction cell. The extraction cell was fitted with glass wool at the inlet and outlet. Ethanol was pumped using a Jasco PU2080 HPLC pump (Jasco Inc., Easton, PA) and mixed at high pressure with supercritical CO_2 (SC-CO_2). Compressed fluid mixture was fed to the heater prior entering the extraction cell. The flow rate was controlled using a needle valve as variable restrictor. All the extractions were performed using CO_2 modified with 12% ethanol at 15.2 MPa (2204 psi) and 60 °C during 90 min. Extracts were collected in a glass vessel cooled by ice. In graphics, SFE was noted as "alternative" extraction with CO_2–EtOH (88:12) as extraction solvent.

2.3.3. *Pressurized liquid extraction (PLE)*

Different PLE extraction experiments were performed using an accelerated solvent extractor system (ASE 200, Dionex Corporation, Sunnyvale, CA) equipped with a solvent controller to keep solvent ratios at desired level. In all cases 2.5 g of freeze dried algae were loaded into a 11 mL stainless steel extraction cell fitted with cellulose filter at the inlet and outlet. Two solvent mixtures composed of water and ethanol were used, but with different ratios 75:25 and 25:75. In the first step, the extraction cell was filled with the selected solvent and the pressure was increased to the desired level. Initial heat-up time was then applied depending on the extraction temperature. The heat-up time was automatically fixed by the equipment and corresponded to 6 min for 120 °C. A static extraction process was carried out with all valves shut closed for the specified extraction time (20 min), during this period pressure was kept at 1500 psi (10.3 MPa). After the static stage of extraction, the cell and the tubing were rinsed (with 60% of the cell volume) using the fresh extraction solvent. Then, all the solvent present in the system was purged using N_2 gas. The extract from this stage was collected in a vial and pressure of the unit was released. A thorough rinse of the system was applied between the two successive extractions to avoid any carry-over from one experiment into the next. PLE parameters were chosen according to earlier studies [38,39] which allowed defining temperature, duration, pressure and solvents to be used during extraction. High temperatures are generally more effective [17,38,40] but similar to the SFE process, temperature between 100 and 150 °C was preferred. The extraction duration remained unchange with regard to previous studies carried out on this device in the same context: 20 min was sufficient to extract molecules [17,40]. The chosen pressure was about 10 MPa as for the other studies at the chosen temperature. As for the used solvents, it was demonstrated that ethanolic mixtures were very effective for the obtaining of active extracts [38–40]. As no information existed on the exact PC polarity in *S. muticum*, two mixtures of different polarities were thus tested: 25 and 75%. In graphics, PLE extraction was noted as "alternative" extraction with EtOH 75 or 25% as extraction solvent.

Chemicals for SFE and PLE were obtained as follow: ethanol and washed sea sand (0.25–0.30 mm diameter) were supplied by Panreac Quimica S.A. (Barcelona, Spain). CO_2 (Premier quality) and N_2 (Technical quality) were obtained from Carburos Metálicos (Air Products Group, Madrid, Spain). Deionized water was obtained using a Milli-Q system from Millipore (Molsheim, France).

2.4. *Quantity and quality of extracts*

2.4.1. *Determination of the total phenolic contents and Folin–Ciocalteu assay*

The total phenolic content of all extracts was colorimetrically (Labsystems, Multiskan MS, Finland) determined with a microplate adapted Folin–Ciocalteu assay [41], which is known to be less affected by interfering compounds; these last substances, however, are thought to account for less than 5% of the Folin–Ciocalteu-reactive compounds in brown seaweeds [42]. Phloroglucinol (1,3,5-trihydroxybenzene, Sigma, Saint Quentin Fallavier, France) was used as a standard, and concentrations were determined in each extract by freeze-drying three aliquots of 1 mL. Total phenolic contents (TPCs) were expressed as percentages of phenolic compounds per dry weight (DW) in the aliquot.

2.4.2. *NMR analysis of extracts*

The global structural composition of crude extracts was assessed by means of ^1H NMR analyses on a Bruker Avance 400 using standard pulse sequences available in the Bruker software (Bruker, Wissembourg, France). All spectra were recorded in MeOD, at room temperature. Chemical shifts were expressed in parts per million. HR-MAS NMR spectra were acquired on a DRX 500 spectrometer (Bruker BioSpin, Wissembourg, France) equipped with an indirect HR-MAS $^1H/^{31}P$ probe head with gradient *Z* at 25 °C. A typical proton ^1H HR-MAS NMR spectrum consisted of 64 scans with pre-saturation of the water peak. The HR-MAS spectrum allows to have an idea of composites initially present in the studied seaweed. NMR profiles unambiguously point out PC within extracts, with peak(s) between 5.5 and 6.5 ppm corresponding to signals visible in HR-MAS NMR (see following paragraph) and not hampered by very large mannitol signals (polyol visible between 3.6 and 3.9 ppm). Indeed, the absence of mannitol will facilitate the phenolic compounds purification after extraction.

2.5. *Activity measurements of extracts*

2.5.1. *DPPH radical scavenging assay*

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay modified according to [10] was used to determine the radical scavenger activities in extracts. Ascorbic acid (Sigma, Saint Quentin Fallavier, France), α-tocopherol (Sigma, Saint Quentin Fallavier, France), butylated hydroxyl-anisole (BHA), butylated hydroxyl-toluene (BHT) (respectively 2(3)-*t*-butyl-4-hydroxyanisole and 2,6-di-*tert*-butyl-4-methylphenol, Sigma, Saint Quentin Fallavier, France) were used as positive controls. The protocol was microplate adapted for a faster use and to decrease sample preparation time. Briefly, various dilutions of the extracts were prepared in triplicate before addition to 12 μL aliquots of 150 μM of DPPH radical (222 μL). The mixture was stored in the dark for 60 min prior to absorbance measurement at 540 nm. Distilled water was used as a negative control. All samples were assayed in triplicate. Antioxidant activity was expressed as IC50 (the concentration of substrate that causes a 50% loss of the DPPH activity). Furthermore, the lower the IC50, the stronger the antioxidant activity. Extracts with an IC50 higher than 10 mg/mL were considered as non-active extracts.

2.5.2. *β-Carotene bleaching method*

The antioxidant activity of extracts and controls was measured by the β-carotene bleaching microplate adapted method, modified in accordance with literature [43,44]. 2 mL of a β-carotene solution in chloroform (0.1 mg/mL) were added to round-bottom flasks containing 20 mg of linoleic acid and 200 mg of Tween 40. After evaporation with a rotavapor, oxygenated distilled water (50 mL) was added, and the mixture was shaken to form a liposome solution. This mixture was added to 12 μL of extracts, or positive controls (α-tocopherol, BHA and BHT), or negative controls (distilled water and ethanol). The absorbance of the solution at 450 nm was measured immediately ($t=0$ min) and after 2 h at 50 °C ($t=120$ min). All samples were assayed in triplicate. Antioxidant activity was expressed through the antioxidant activity coefficient (AAC) as follows:

$$\mathrm{AAC} = \left[\frac{A_{s(120)} - A_{c(120)}}{A_{c(0)} - A_{c(120)}}\right] \times 1000 \tag{1}$$

where $A_{s(120)}$ is the absorbance of the antioxidant mix at $t = 120$ min, $A_{c(120)}$ is the absorbance of the control at $t = 120$ min, and $A_{c(0)}$ is the absorbance of the control at $t = 0$ min. Since the positive controls, BHA and BHT, had an average AAC of 700, this value was arbitrarily chosen to express the antioxidant activity as AAC_{700} [10]. AAC_{700} was then the concentration of substrate needed to obtain an AAC value of 700. A high AAC_{700} was considered as indicative of a weak antioxidant activity.

2.6. Productivity of each assay

The difficulty here was to be abled to compare all these different processes, not using either the same volume of solvent or the same extraction times. To achieve this, we chose to introduce an additional productivity component that takes into account the proportion of PC quantity extracted per mass of algal lyophilisate, against the extraction time and against the solvent volume/amount used for every process. Productivity was calculated as follows:

$$\mathrm{productivity} = TPC\ ExT^{-1}\ SolQ^{-1} \tag{2}$$

where *TPC* is the total phenolic content in milligram PC per gram of algal DW, *ExT* is the extraction time in minute, and *SolQ* is the solvent quantity in litre. Productivity is then expressed in milligram of PC per gram of dried alga per minute and per liter of solvent.

2.7. Ranking of the processes using a score calculation

A score was attributed to every variable considered: TPC (%DW), IC50 (antiradical activity, DPPH), AAC_{700} (antioxidant activity, β-carotene bleaching test) and productivity according to the results of two-way ANOVAs. The score corresponded to the number of groups generated by the post hoc Tukey HSD test as follows: when 3 groups were highlighted, then the most efficient obtained a score of 3, the second a 2 and, finally, the less efficient obtained a 1. When data was common to two groups its score was the mean of the two values. In case of 4 groups, the score was calculated as follows: ‘a’, ‘b’, ‘(a, b)’ and ‘c’; then ‘a’ had a 3, ‘b’ had 2, ‘(a, b)’ had 2.5 and ‘c’ had 1. Then, for clarity, the scores were converted to a mark between 0 and 10. Finally, all these scores were averaged and the final mark, attributed to each process (classical versus alternative) gave an indication of the most interesting process by taking into account all parameters.

2.8. Statistics

All analyses were carried out in triplicate, and their results are presented as mean values ± standard deviation (SD), using the Statistica 8 (StatSoft®) software for PC. Homogeneity of variance was tested with the Brown–Forsythe test at the 0.05 risk error. The data which did not satisfy the criteria of normality and homoscedasticity for parametric tests were square root transformed. Afterwards, two way ANOVA was performed on TPC, antiradical activity (IC50, DPPH test), antioxidant activity (AAC_{700}, β-carotene bleaching test) and productivity, using methods (classical or alternative) and solvents as fixed factors. When ANOVA demonstrated significant difference, a post hoc Tukey HSD test was carried out to know which means contributed to the effect.

3. Results

3.1. Distinction between processes using PC contents

The different extraction processes had a significant effect on the TPCs from algal samples ($p < 0.001$, Table 1; Fig. 1). The highest TPCs were measured on EA–water extracts (22.90 ± 0.65%DW and 18.59 ± 0.52%DW for the alternative and classical extraction method, respectively); on the other hand, the lowest ones were determined on the alternative extraction method using CO_2–EtOH and the classical method using hexane–EtOH extracts (3.45 ± 0.31%DW and 3.72 ± 0.18%DW with $p < 0.001$ respectively). The observed differences in TPC between the “alternative” and “classical” processes are lower than 5%DW. Only CPE leads to a better extraction yield than the classical method ($p < 0.001$). In this case, with EA as extraction solvent, a three-phase extraction was achieved and only the organic phase was retained for the tests: the aqueous phase did not contain PC and had a very low activity (Tanniou unpublished data).

3.2. Distinction between processes using NMR analysis

The presence of phenolic compounds (between 5.5 and 6.5 ppm) together with mannitol in great quantities (between 3.6 and 3.9 ppm) can be noticed (Fig. 2). Phenolic compounds are

Table 1
Variation in TPC (%DW), IC50 (antiradical activity, DPPH test) and AAC_{700} (antioxidant activity, β-carotene bleaching test) in extracts from *Sargassum muticum* according to the extraction method.

Variable	Statistical test		Fixed factors		
			Method	Solvent	Method × solvent
TPC (%DW)	Two way Anova	*df*	1	3	3
		F	16.16	1423.74	105.98
		p	$p < 0.001$	$p < 0.001$	$p < 0.001$
	Tukey $p < 0.05$		C > A	EA > E_{75} = E_{25} > E_{12}	
IC50 (mg/mL)	Two way Anova (square root)	*df*	1	3	3
		F	57.18	161.28	14.52
		p	$p < 0.001$	$p < 0.001$	$p < 0.001$
	Tukey $p < 0.05$		C > A	E_{12} > E_{25} = E_{75} = AE	
AAC_{700} (mg/mL)	Two way Anova	*df*	1	3	3
		F	245.21	446.31	205.50
		p	$p < 0.001$	$p < 0.001$	$p < 0.001$
	Tukey $p < 0.05$		C > A	E_{25} > E_{75} > E_{12} = EA	

Method: C, Classical; A, Alternative. Solvent: EA, EA–water 50:50; E_{12}, CO_2–EtOH or hexane–EtOH 88:12; E_{75}, EtOH–water 75:25; E_{25}, EtOH–water 25:75.

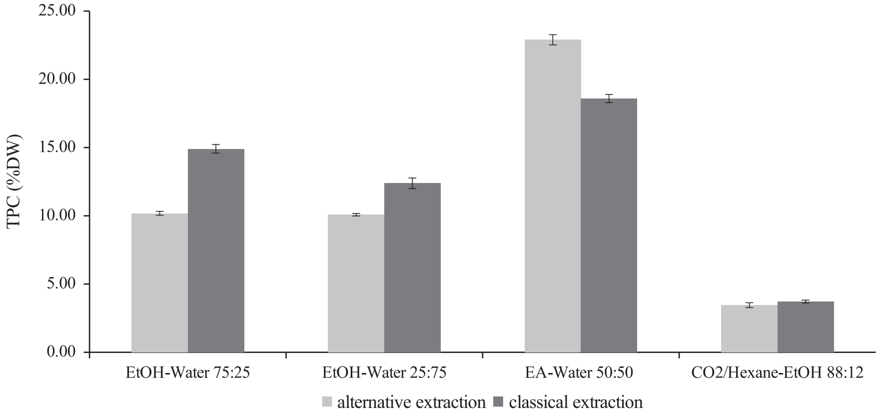

Fig. 1. Total phenolic content (TPC) in extracts from *Sargassum muticum* according to the different extraction methods—"alternative": CPE, PLE and SFE using respectively AE–water 50:50, EtOH–water 75:25 or 25:75 and CO_2–EtOH 88:12 (v/v) as extraction solvents or "classical": Solid/Liquid extraction using AE–water 50:50, EtOH–water 75:25 or 25:75 and hexane–EtOH 88:12 (v/v) as extraction solvents.

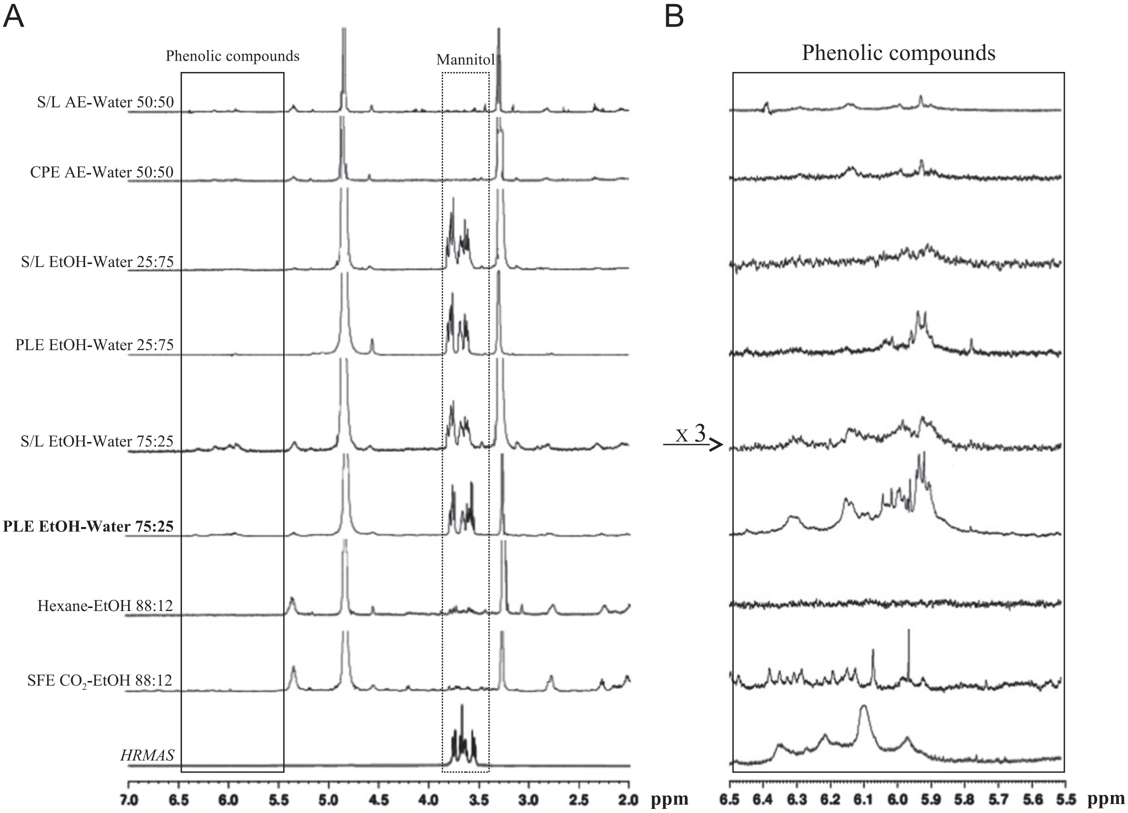

Fig. 2. (A) initial *S. muticum* HR-MAS and ^{1}H NMR extracts profiles in the 2–7 ppm area (B) enlargement (× 3) of the phenolic area between 5.5 and 6.5 ppm. For the EA 50 S/L extraction sample only the organic phase was represented.

present in the extracts, irrespective of the solvents and of the method used except in case of the hexane–EtOH S/L extraction where no phenolic compound was extracted. However phenolic signals are somewhat different between the HR-MAS NMR profiles and vary according to the extraction protocols. Indeed, as the selected processes used different polar solvents, the part of the PC pool extracted during the process will then not be the same according to the process; the idea was previously to be able to get back the majority of compounds composing this pool in a single extraction. For PLE and CPE techniques the PC pools visible on NMR spectra are quite the same between the "alternative" and the "classical" extraction. With CPE two areas of signals are detectable: at about 6.1 ppm and 5.9 ppm for the alternative and classical extraction. With 25% EtOH, for alternative and classical extractions, two areas are also visible: at about 6 and 5.9 ppm. When polarity increases using EtOH 75% as extraction solvent, two other peaks are visible at 6.1 and about 6.3 ppm. For the hexane–EtOH S/L extraction, no aromatic compounds are visible on NMR spectra. Finally, using SFE, phenolic compounds appear very different to those obtained with the other methods: many small signals are visible in the area between 5.9 and 6.4 ppm, showing that different types (differentiated by their polarity) of compounds were extracted in relation with the type of process and/or solvent used. According to NMR spectra, the technique which allows extracting the great majority of phenolic compounds initially present in *S. muticum* seems to be the PLE EtOH–water 75:25 (v/v).

A large peak corresponding to mannitol can be noticed on the HR-MAS NMR spectra as well as on the ^{1}H NMR spectra obtained on the various extracts, except when EA or hexane–EtOH and CO_2–EtOH mixture were used as extraction solvents.

3.3. *Monitoring of activities in extracts between various processes*

According to the DPPH test (Table 2), the antioxidant activity (IC50) in the EA extracts (0.52 ± 0.03 and 0.45 ± 0.01 mg mL^{-1} at $p < 0.001$, for the alternative and classical extraction methods, respectively), were significantly higher ($p < 0.001$) than those obtained using other solvents. According to the ANOVA, EA extracts (classical and alternative methods) and EtOH:water 75:25 and 25:75 (classical method) permitted to obtain activities similar to the four positive controls BHA, BHT and Vitamins C and E. On the other hand, the lowest yields were in CO_2–EtOH and hexane–EtOH extracts (28.31 ± 4.20 and 2.60 ± 0.20 with $p < 0.001$ for the alternative and classical extraction methods, respectively) (Tables 1 and 2). The classical S/L method always allowed a better scavenging activity in extracts than with the alternative processes ($p < 0.001$).

The antioxidant activity determined by the β-carotene bleaching method (AAC_{700}) was the highest in hexane–EtOH extracts ($2.46 \times 10^{-4} \pm 5.28 \times 10^{-6}$ mg mL^{-1} with $p < 0.001$ for the classical method) and EA extracts (0.58 ± 0.33 and 0.18 ± 0.01 mg mL^{-1} with $p < 0.001$ for the alternative and classical extraction method, respectively) (Tables 1 and 2). According to the ANOVA, EA extracts (classical and alternative methods) and hexane–EtOH and CO_2–EtOH (classical and alternative method) allowed activities equivalent to positive controls BHA, BHT and Vitamin E.

3.4. *Productivity*

The productivity varied in relation with the method and solvent ($p < 0.001$ for both factors, Tables 3 and 4). Alternative processes were more efficient than classical ones ($p < 0.001$). The PLE methods allowed obtaining the best productivity (200.13 ± 5.56 and 146 ± 2.40 mg PC g^{-1} DW min^{-1} L^{-1}, for the EtOH–water 75:25 and 25:75, respectively, Table 3) with a greater efficiency for the richest EtOH (75:25) mixture ($p < 0.001$, Tables 3 and 4). CPE productivity was only 2.5% (4.22 ± 0.11 mg PC g^{-1} DW min^{-1} L^{-1}) while other processes came to less than 0.1% that of PLE ($7.35 \times 10^{-3} \pm 5.04 \times 10^{-4}$ to 0.66 ± 0.09 mg PC g^{-1} DW min^{-1} L^{-1}, i.e. the classical hexane–EtOH 88:12 and classical EA extraction, respectively). Thus the alternative methods were unquestionably more effective in term of productivity than the classical methods ($p < 0.001$).

3.5. *Ranking of the processes*

All the results were combined and assigned by a score as shown in Table 5. Processes using the EA as extraction solvent came first, then processes using EtOH–water mixtures and finally processes using more apolar solvents like hexane–EtOH and

Table 3
Mean productivity according to the extraction method (SD: standard deviation). The best productivities were underlined in the table.

Method	Solvent	Productivity (mg TPC g^{-1} DW min^{-1} L^{-1}) mean values ± SD	Tukey HSD ($p < 0.05$)
Alternative			
PLE	EtOH–water 75:25	200.13 ± 5.56	(a)
PLE	EtOH–water 25:75	146.79 ± 2.40	(b)
CPE	EA–water 50:50	4.22 ± 0.11	(c)
SFE	CO_2–EtOH 88:12	0.74 ± 0.07	(e)
Classical			
S/L	EtOH–water 75:25	1.53 ± 0.06	(d)
S/L	EtOH–water 25:75	1.79 ± 0.10	(d)
S/L	EA–water 50:50	0.66 ± 0.09	(e)
S/L	hexane–EtOH 88:12	< 0.01	(f)

Table 2
Mean values of IC50 (antiradical activity, DPPH test) and AAC_{700} (antioxidant activity, β-carotene bleaching test) measured on extracts from *Sargassum muticum* according to the extraction method (SD: standard deviation). The most active extracts were underlined.

Variable	Method	Solvent	Mean values ± SD	Tukey HSD ($p < 0.05$)
IC50 (mg/mL)	Alternative			
	PLE	EtOH–water 75:25	0.77 ± 0.01	(d,e)
	PLE	EtOH–water 25:75	0.86 ± 0.02	(e)
	CPE	EA–water 50:50	0.52 ± 0.03	(b,c,d)
	SFE	CO_2–EtOH 88:12	non-active	(g)
	Classical			
	S/L	EtOH–Eau 75:25	0.67 ± 0.29	(c,d,e)
	S/L	EtOH–Eau 25:75	0.65 ± 0.03	(c,d,e)
	S/L	EA–Eau 50:50	0.45 ± 0.01	(a,b,c)
	S/L	hexane–EtOH 88:12	2.60 ± 0.20	(f)
	Positive controls	BHA	0.27 ± 0.01	(a,b)
		BHT	0.40 ± 0.01	(a,b,c)
		Vit C	0.19 ± 0.01	(a)
		Vit E	0.28 ± 0.02	(a,b)
AAC_{700} (mg/mL)	Alternative			
	PLE	EtOH–water 75:25	1.59 ± 0.27	(d)
	PLE	EtOH–water 25:75	3.50 ± 0.31	(e)
	CPE	EA–water 50:50	0.58 ± 0.33	(b,c)
	SFE	CO_2–EtOH 88:12	0.72 ± 0.22	(c)
	Classical			
	S/L	EtOH–water 75:25	1.61 ± 0.24	(d)
	S/L	EtOH–water 25:75	1.92 ± 0.05	(d)
	S/L	EA–water 50:50	0.18 ± 0.01	(a,b)
	S/L	Hexane–EtOH 88:12	< 0.01	(a)
	Positive controls	BHA	0.22 ± 0.01	(a,b,c)
		BHT	0.22 ± 0.01	(a,b,c)
		Vit E	0.21 ± 0.01	(a,b,c)

Table 4
Variation in the productivity according to the type of solvent and the extraction method.

Variable	Statistical test		Fixed factors		
			Method	Solvent	Method × solvent
Productivity (mg TPC g^{-1} DW min^{-1} L^{-1})	Two way Anova (square root)	*df*	1	3	3
		F	9893.68	3390.59	3294.14
		p	$p<0.001$	$p<0.001$	$p<0.001$
	Fisher LSD		A > C	$E_{75} \geq E_{25} \geq EA = E_{100}$	

Method: C, Classical; A, Alternative. Solvent: EA, EA–water 50:50; E_{12}, CO_2–EtOH or hexane–EtOH 88:12; E_{75}, EtOH–water 75:25; E_{25}, EtOH–water 25:75.

Table 5
Classification of processes for the extraction of phenolic compounds in *Sargassum muticum*, using a score calculation in regard of statistical tests carried out on data. The best process was underlined in the table.

Method	Solvent	Phenolic content	Activities		Productivity	Score
			DPPH	*β* caroten		
Alternative						
PLE	EtOH–water 75:25	3.33	5.00	5.00	10.00	6/10
PLE	EtOH–water 25:75	3.33	2.50	8.75	8.33	6/10
CPE	EA–water 50:50	10.00	8.75	8.75	6.67	9/10
SFE	CO_2–EtOH 88:12	1.67	7.50	2.50	3.33	4/10
Classical						
S/L	EtOH–water 75:25	6.67	5.00	8.75	5.00	6/10
S/L	EtOH–water 25:75	5.00	5.00	8.75	5.00	6/10
S/L	EA–water 50:50	8.33	8.75	10.00	3.33	8/10
S/L	Hexane–EtOH 88:12	1.67	10.00	5.00	1.67	5/10

CO_2–EtOH mixtures, the latter being the least efficient as extracting solvent for phenolic compounds in *S. muticum*.

4. Discussion

This study dealt with the effect of different extraction methods on the TPC and antioxidant activities of *S. muticum* extracts. All extracts obtained by the different classical and alternative processes were made up of over 10% PC except for those obtained using CO_2–EtOH and hexane–EtOH mixtures as extracting solvents, thereby demonstrating that both the method of extraction and the choice of solvents have a significant effect on the PC extraction. CPE, which afforded the best yields in PC extraction could be used as a pre-purification step in, as only its organic phase is exploited (aqueous phase is PC-avoid) (Tanniou unpublished data). For the classic S/L phenolic extraction, numerous solvents of different polarities have been used, with usually methanol or acetone as the solvents more appreciated for seaweeds [3]. In this work, we decided to use ethanol-based solvents and ethyl acetate to be able to compare with alternative processes involving both types of solvents (ethanol for PLE conditions and ethyl acetate for CPE conditions). Although ethanol has been reported as an inefficient PC solvent in “classic” conditions [13, Tanniou unpublished data], its use under PLE conditions has been reported in the literature together with hexane and methanol [17,39]. In our study ethanol–water 75:25 and 25:75 mixtures yield the same TPC in duplicated extracts. Similar trends were found with another brown macroalga *Himanthalia elongata*, in which comparable yields were obtained when extracting with ethanol and with water [17]. Similar phenolic compounds were extracted whatever the used EtOH–water mixture but the extracts did not have the same polarity, being more polar than the extracts obtained with an EtOH–water 25:75 mixture.

In our study, TPC was expressed in percentage dry weight of seaweed and a maximal value of 4.5%DW was obtained for *S. muticum* whatever the process. Different TPCs were obtained on the same species by other authors who found values between 0.2 and 6%DW [15,45]. TPC depends on the environment parameters and/or of the seaweed thallus part kept for extraction [45]. Here, to have a homogeneous algal material, the totality of the thallus was kept for extraction. In the same way algae were collected in summer when they usually produce the maximum of PC [4,15]. However this quantity varies between sites (depending on environmental conditions). All these considerations could explain why TPCs were different between these experimental studies. For the same species, following sites, seasons and thallus portion, PC contents vary, together with the polarity of compounds extracted. Indeed compounds within the pool of PC in *S. muticum* have not the same polarity even if they are all of fucophlorethols type (Tanniou unpublished data-2D RMN) and the proportion of a specific compound within the pool is going to vary also in relation with the environmental data of the considered site as well as with the season (not yet published data).

Different types of phenolic compounds were present within the extract from *S. muticum* representing a PC pool. Our interest was to find a method which would optimize the extraction of this PC pool both qualitatively (all chemical species) and quantitatively (no activity denaturation). Folin test quantified PC and NMR profiles allowed, first to ensure that PC were present within the extract (disregarding interfering aromatics in the colorimetric assay), and hence to check if the extracted pool was similar to the one initially present in the alga. Finally, ^{1}H NMR was used to obtain a phenolic fingerprinting of the overall extracts to find out which compounds should be removed in a future PC purification. Thus, compared to the HR-MAS NMR spectrum used as reference, the almost totality of compounds initially present in *S. muticum* was extracted thanks to the PLE EtOH 75 process. Conversely, NMR spectra showed very different compound profiles between SFE and the pool of PC initially present in the algae (as evidenced in the HR-MAS NMR profile). Regarding the hexane–EtOH control which presents a polarity close to the CO_2–EtOH mixture used in

SFE, the observed difference in NMR profiles can be explained by the mixture polarity which would not allow the extraction of the same PC as with the other techniques. Indeed, the hexane–EtOH mixture is too apolar to allow a PC extraction. It is important to underline also the fact that for the obtaining of RMN spectra, extracts were all dissolved in Deutered Methanol (MeOD), not allowing entire PC pool solubilization but only the soluble part, having the same polarity. MeOD was chosen in regard with results showing that the majority of *S. muticum* PC had an affinity for the MeOH, as demonstrated by Tanniou (unpublished data) using the solid phase extraction (SPE) process but also cited by [4,10,45]. It also allowed the alignment of NMR spectra to obtain a global view of the differences between the various extraction processes.

Even if it was reported by many authors that PC are sensitive to high temperatures, other authors showed the opposite and demonstrated the positive effect of the increase of temperature on the extraction yield [17,46, Tanniou unpublished data]. This phenomenon can also depend on exposure time, of very limited duration in our case to reduce temperature degradation. Pressure level can also impact PC; indeed some authors used more important pressures (20–40 MPa) [38,47] than those used in our study (15 Mpa) without reporting any modification of their end products. Moreover, other works reported extraction times four times longer than ours, i.e. 6 h [47] compared to 1 h 30 min here. Our results, especially for CPE and PLE, are really promising in terms of efficiency.

Concerning active compounds, one should retain that the choice of the extraction solvent is important in preserving PC activity. In our study, the EA–water mixture always produced the most active extracts, whatever the method used. In this respect, conventional methods afforded better activities than alternative ones. As mentioned, high temperature used by some processes can have a detrimental effect on the PC. In fact, for the EtOH:water and hexane–EtOH mixtures, there was a decrease in activity between conventional and alternative processes, suggesting that the temperature may play a negative role on the extracted compounds activity. Indeed, in other studies, an activity decrease with an increase of temperature has been reported in PLE [17], but this was not confirmed as a general trend for other solvents. EA extracts had very interesting activities similar to those obtained from positive controls (IC50: 0.52 ± 0.03 and 0.45 ± 0.01 mg mL^{-1} and AAC_{700}: 0.58 ± 0.33 and 0.18 ± 0.01 mg mL^{-1} for the alternative and classical extraction methods, respectively) and higher than those obtained for the same species with other extraction solvents [10]. $\mathrm{CO_2}$–EtOH and hexane–EtOH extracts had DPPH and good β-carotene activities (IC50: 28.31 ± 4.20 and 2.60 ± 0.20 mg mL^{-1}, and AAC_{700}: 0.72 ± 0.22 and $2.4610^{-4} \pm 5.2810^{-6}$ mg mL^{-1} for the alternative and classical extraction methods, respectively). Regarding NMR profiles, β-carotene activity was due to non-polar compounds such as lipids rather than PCs. As already shown in the literature, active extracts shown by the DPPH test were not necessarily identical to those identified by the β-carotene test [10]. Indeed, β-carotene test targets lipophilic compounds activity while the DPPH is less selective [44]. In our study, the most active extracts (EA:water classical and CPE) shown by the DPPH were also shown by β-carotene, suggesting a lipophilic nature to the active compounds, with an affinity for non-polar solvents. Fractions of the phenolic pool may undergo oxidative polymerization in the apoplasm, and thus be integrated as components of the parietal macromolecular complex, upon which their original solubility and solvent affinity characteristics are completely modified [48]. Similarly, if we consider only at the solvent effect, the more polar the used solvent is, the less activity shown by β-carotene test.

We also noticed that active extracts with DPPH are also the ones containing more PC with regard to the Folin–Ciocalteu assay. This report was also made by other authors [45,49–51]. It could let believe in a direct correlation between CP and antioxidant activity, even if several authors denied this and attribute activities to other secondary metabolites produced by the seaweed [52]. The Folin–Ciocalteu component of the test used to quantify PC not only measures total phenol content, but also reacts with any reducing substances. This reagent therefore measures the reducing capacity of the totality of the sample, and not only the level of phenolic compounds [53]. Thus it is not surprising to find a correlation between the DPPH results and those obtained by the Folin–Ciocalteu assay. In our case this procedure remained interesting to permit a comparison between our results and those obtained in the literature.

Compared with other processes, solvent extraction with EA resulted in the highest TPCs and antioxidant activities. Our results clearly demonstrated that CPE could concern an initial separation or purification of the extract. Indeed, only the organic phase of the biphasic extract (composed of 20.74 ± 0.32% PC) was kept here. The NMR spectra showed none or negligible amounts of mannitol, a major interfering polar component. This process thus allowed obtaining within minutes a fraction with a huge content of PC. However, ethyl acetate is not recommended in subsequent processing steps because of its inherent toxicity. Our study underlined that alternative processes were much more effective than the classic S/L extraction. Indeed they can often save time and solvent used even if the final yields in PC are somewhat inferior, with the exception of CPE. Regarding the productivity in standard S/L extraction, it increased with solvent polarity, which would mean that polar solvents allowed extracting more phenolic compounds. Others authors made the same report connecting the extraction yield with the increase of solvent polarity [17], but at the expense of including large amounts of mannitol which upsets accurate yield estimates, the large ^{1}H NMR signals of the latter interfering with the more upfield phenolic signals and spoiling the central part of the spectrum baseline (see Fig. 2, NMR).

If the main interest is on active compounds characterization, one would better focus on a process allowing phenolics extraction in large quantities, e.g. CPE. On the other hand, if a tradeoff between activity and market regulations for food products is sought, a process using water:ethanol mixtures that better complies with stringent European standards is more appropriate. In this respect, PLE allowed obtaining rich PC fractions produced in a very effective way may be a sound candidate for industrial applications, being both more tractable for scaling-up and certainly more green, notwithstanding the seasonal availability of the natural resource, in this case an invasive species.

For further analyses, to still improve the extraction process, it would be interesting to combine some of these processes. SFE and PLE are well suited for on-line and off-line combination with analytical techniques such as liquid chromatography. For this study, on-line coupling is not needed since sample analysis was not performed afterwards and the selection of the extraction process was based on in vitro measurements using micro-plates. On the other hand, the combination of extraction techniques with in-cell cleanup (solid-phase extraction, SPE) has been proposed recently for the optimization of the extraction of selected analytes (phenolics) in different algae; the on-line SPE/SFE technique helped improving the selectivity of the extraction process [54]. Similarly, PLE was suggested in combination with in-cell SPE to isolate the target compounds by retention in the SPE material of either the target compounds or the interferences [55].

Finally, maintenance considerations are to be considered for sustainable operability despite the excellent productivities reached by alternative processes such as SFE, PLE and CPE, we must not forget that these processes have very long cleaning operations that should also be taken into account. Similarly the energy cost (high pressure and high temperature energy-intensive) together with the price of such apparatus to realize such processes must not to be neglected.

5. Conclusion

Our study demonstrates for the first time the unquestionable usefulness of using alternative, solvent-free and green methods for the non-denaturing extraction of brown algal polyphenols, using the brown macroalga *S. muticum* as model. Several extraction solvents and processes were tested here to obtain active phenolic compounds from *S. muticum* samples collected in Brittany (France). In particular, CPE and PLE seemed the most promising for the extraction of polyphenols that are endowed with useful antioxidant potential. First, CPE afforded the most important yields in concentrated phenolic compounds, also displaying the best associated activities. Secondly, PLE using an EtOH:water mixture 75:25 (v/v) allowed a good PC extraction with huge efficiency. Despite a lesser activity of the extracts, PLE is green (environmentally-safe) and potentially complies with the European requirements for the prospective valorization of phenolic compounds from *S. muticum* in Brittany.

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Appendix A. Supporting information

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