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Microwave hydrodiffusion and gravity processing of *Sargassum muticum*

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ABSTRACT

An aqueous based extraction process was developed to obtain soluble fractions from *Sargassum muticum*. Microwave hydrodiffusion and gravity (MHG) and subsequent rehydration and extraction stages (MHG-W) in an open system, hot pressurized water extraction or autohydrolysis (A) and enzyme aided extraction (E) were selected on the basis of their simplicity and the possibility of using water as solvent. The performance of these combined technologies using biorenewable solvents to extract antioxidants was compared. A preliminary selection of MHG extraction conditions suggested that 600 W was the optimal irradiation power. A multistage process based on an initial solvent free MHG stage followed by a sequence of water extraction stages (MHG-W) proved suited to selectively obtain phenolic compounds in shorter times than conventional processes. The yield of total solubles was lower than with conventional solvent extraction, but the phenolic content of the extracts and their ABTS radical scavenging properties were enhanced. The microscopic observations of extracted tissues confirmed the impact of the microwave and hot pressurized water processes on the algal cell wall integrity.

1. Introduction

Marine macroalgae are potential renewable and sustainable sources of active compounds and, among them, brown algae are attracting interest in relation to their biological and antioxidant properties [1–3]. Particular attention deserved components exclusively found in brown algae, such as phlorotannins, fucose-containing sulphated polysaccharides and the xanthophyll fucoxanthin. Phlorotannins are dehydropolymerizates of phloroglucinol (1,3,5-trihydroxybenzene) and can be classified into phlorethols, fucols, fucophlorethols, eckols, fuhalols and carmalols. To incentivize utilization of renewable resources with non food uses, the valorization of components from the invasive alga *Sargassum muticum* was proposed. An early study on this brown alga provided information on the identification of phloroglucinol, diphlorethol pentaacetate, bifuhalol, trifuhalol A and trifuhalol B [4]. The crude and fractionated *S. muticum* solvent extracts, enriched in phenolic compounds, are efficient free radicals scavengers and possess antimicrobial, anti-inflammatory and cytoprotective properties [2,5].

Growing attention is being paid to the use of green solvents for the separation of purified valuable compounds using novel technologies or improving existing methods to avoid the excessive consumption of organic solvents, energy and time in extraction processes. Conventional solvent extraction [6] and fractionation [2], high pressure water extraction under subcritical conditions or autohydrolysis [7], and other alternative processes (centrifugal partition extraction, supercritical fluid extraction and pressurized liquid extraction) [8] have been proposed to obtain phenolic compounds from *S. muticum*.

Microwave-assisted extraction (MAE), based on the rapid heating of solvent and sample due to the direct effect of microwaves on molecules by ionic conduction and dipole rotation, is widely employed in the analysis and the extraction of natural compounds [9,10]. Despite the increased interest and recent advances [11], the mechanism of extracting plant secondary metabolites by microwave irradiation is still under study [12]. The process may be performed either in closed vessels, which can operate at pressure higher than atmospheric, or in open vessels, which operate at atmospheric pressure [13]. Open-vessel microwave operation can be more effective in relation to increased safety, the possibility of processing larger samples, the lower cost of the equipment and the milder conditions, suitable for thermolabile species [14]. Solvent

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free microwave hydrodiffusion and gravity extraction (MHG) is a novel green, efficient, economical and environment-friendly approach [15–18]. This technique is suited for applications at analytical scale [19] and continuous industrial operation can be performed if an active transport mechanism is used to feed the extractor [20]. MHG is particularly suited for high moisture content materials. However, it has not been previously applied to seaweed. The possibility of processing wet material avoiding sample drying is attractive to cut drying costs and because *S. muticum* phlorotannins and fucoxanthin content and extractability can be negatively affected by temperature [21,22].

Enzymatic degradation of vegetal cell walls has received attention as an alternative to chemical and mechanical conditioning stages to aid in the release of components found in organelles. Although enzyme assisted extraction of valuable compounds from terrestrial sources, i.e. oils [23] or juices [24] is well known, the application to macroalgal biomass was relatively recent and no reports with *S. muticum* are available. Commercial hydrolytic activities proved highly beneficial [1,25], since the complex cell wall algal polysaccharides limit the solvent accessibility to some solutes.

In the present work, aqueous based technologies (microwave hydrodiffusion and gravity, autohydrolysis and enzyme aided extraction) were combined for the extraction of soluble phenolic fractions with antioxidant properties from this brown alga.

2. Materials and methods

2.1. Material

S. muticum specimens, collected in Cape Estai (Pontevedra, Spain) in June 2012, were cleaned from epiphytes and sand, washed with tap water, frozen and stored in closed plastic bags at −20 °C until use. Defrosted algae were ground and used for extraction experiments.

2.2. Conventional aqueous extraction (CSE)

Wet ground algae (10 g, moisture content 87 wt%) were contacted with distilled water using liquid to solid ratios of 5, 10, 15 and 20 (v:w). Extraction was performed at 97 ± 2 °C protected from light during 1 h. The suspension was vacuum filtered through 0.45 μm membranes to recover a liquid phase that was characterized for phenolic content and antiradical properties. Extractions were performed at least three times, and the mean values were recorded.

2.3. Solvent free microwave hydrodiffusion and gravity (MHG) extraction

An open vessel multimode microwave extractor (NEOS-GR, Milestone Srl, Italy) operating at 2.45 GHz and maximum power of 900 W, with a 1.5 L Pyrex extraction vessel, was used. During solvent free microwave hydrodiffusion and gravity (MHG) extraction experiments, temperature was monitored by an external infrared automatic temperature sensor. Ground wet *S. muticum* (100 g, moisture content 87 wt%) was placed on the extraction vessel and irradiated at selected power and time. As in other open equipments, the maximum temperature was determined by the boiling point of the solvent at atmospheric pressure [13]. The extract drained by gravity on a condenser outside the microwave irradiation cavity where it was collected, cooled down to room temperature, vacuum filtered through 0.45 μm pore size membranes and stored at −20 °C in the absence of light until analysis.

2.4. Microwave hydrogravity and water assisted extraction (MHG-W)

The raffinate or exhausted solid resulting after MHG was reextracted with water in several cycles (MHG-W). Extraction experiments were performed by rehydrating the raffinate with an amount of distilled water equal to the volume drained in the previous stage, confirming that no solvent drained when the wet algae were placed into the vessel. Extractions were performed at least three times, and the mean values were reported.

2.5. Microwave assisted ethanol extraction in an open system (MWG-E)

Extraction of the wet ground alga (100 g, moisture content 87 wt%) was performed with 96% ethanol at 400 W during 10 min in the open vessel multimode NEOS-GR microwave extractor; a second stage on the rehydrated raffinate from the first stage was performed under the same conditions. Another extraction using 50% aqueous ethanol in three stages under the same irradiation power and time was carried out. No additional stages were performed since no extract was released.

2.6. Enzyme assisted extraction (E)

Enzyme aided extraction was performed in triplicate independent assays in 500 mL Erlenmeyer flasks, placed in an orbital shaker (INNOVA 4000, New Brunswick) using 10 g of wet alga at a liquid to solid ratio 25 (v:w). Ground wet *S. muticum* was suspended in sodium acetate-acetic acid buffer pH 4.5 and preheated at 50 °C. A commercial enzyme (Viscozyme, Novozymes) was added at an enzyme to substrate ratio of 5% (w/w, wb). For the kinetic study, the homogeneous suspension was periodically sampled, and samples were heated at 100 °C for 10 min to inactivate the enzyme. The extracts were centrifuged (4500 rpm, 8 min) to separate the solid residue, the supernatant was filtered through 0.45 μm membranes and the filtrate was stored at −20 °C in the absence of light until analysis.

2.7. Autohydrolysis (A)

The ground wet algae or the residual solid (30 g) obtained after five MHG-W stages were mixed with water (liquid:solid ratio 30:1 w:w) and heated in a Parr reactor (Parr Instr. Co., Moline, IL) under non isothermal operation, up to reach 190 °C. These conditions had been previously optimized to maximize the phenolic extraction yield and the antiradical properties of the extracts [7]. The vessel was then cooled and opened and the liquid and solid phases were separated by filtration. The liquid phase was further characterized.

2.8. Analytical methods

The phenolic content was measured by the Folin-Ciocalteu method, and expressed as grams of gallic acid equivalents (GAE). The scavenging of ABTS radical (2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonate)) was assessed with the TEAC (Trolox Equivalent Antioxidant Capacity) method. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The $ABTS^{\bullet+}$ solution was diluted with PBS (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. After addition of 1.0 mL of diluted $ABTS^{\bullet+}$ solution to 10 μL of antioxidant compounds or Trolox standards in ethanol or PBS the absorbance was read at 734 nm and 30 °C. The percentage was calculated as a function of the concentration of extracts and Trolox.

The phenolic profile of the extracts obtained during MHG-W at each irradiation power was qualitatively analyzed using an Agilent HPLC 1100 instrument equipped with a Waters Spherisorb ODS-2 column (5 mm, 250 mm × 4.6 mm) and diode-array detector (DAD detector), operating at 30 °C. The injection volume was 20 μL and the flow rate was 1 mL/min. A non-linear gradient of solvent A (acetonitrile/5% (v/v) formic acid in water, 10:90) and solvent B (acetonitrile/5% (v/v) formic acid in water, 90:10) was used as follows: 0 min, 100% A; 40 min, 85% A, 15% B [26].

2.9. Scanning electron microscopy (SEM)

Algal freeze-dried samples were fixed on aluminium stubs and sputter coated with gold in an Emitech K550X equipment and examined with a FEI Quanta 200 scanning electron microscope under vacuum conditions at an accelerating voltage of 12.5 kV.

3. Results and discussion

3.1. Solvent free microwave hydrodiffusion and gravity (MHG) extraction

3.1.1. Kinetics of MHG extraction

The microwave assisted extraction of plant secondary metabolites may be affected by solvent, solvent to sample ratio, power and time of irradiation, moisture content, particle size, temperature and number of extraction cycles [11]. Solvents differ in their ability to absorb energy and to convert it into heat, but the present work is limited to the green solvents water and ethanol. The degree of microwave absorption increases with the dielectric constant, which is three times higher for water than for ethanol, also the water dissipation factor is smaller. Thus, the aqueous system absorbs more microwave energy than it can dissipate, leading to an increased sample temperature that can induce cell rupture. In MHG the irradiation power and time are key factors. Generally there is an increase in the extraction yield with the irradiation power, which determines the energy supplied, the temperature reached in the sample and the partition of solutes between sample and solvent. However, high microwave power or too prolonged periods can affect thermostable solutes. The effect of irradiation power (200–900 W) on the extraction kinetics of the liquid drained and recovered, total