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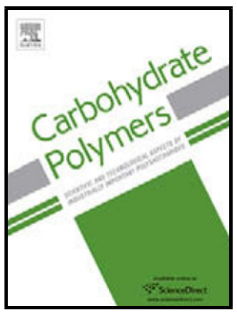

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Microwave-assisted extraction of sulfated polysaccharides (fucoidan) from brown seaweed

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ABSTRACT

Sulfated polysaccharides (fucoidan) were recovered from brown seaweed *Fucus vesiculosus* by microwave-assisted extraction (MAE). Different conditions of pressure (30 to 120 psi), extraction time (1 to 31 min), and alga/water ratio (1/25 to 5/25 g ml^{-1}) were evaluated during this process aiming to establish a condition to maximize the extraction results. The alga degradation (%), total sugar yield (%), and SO_3 content (%) were also determined to each experimental condition. All the studied variables presented significant ($p<0.05$) influence on fucoidan yield. MAE at 120 psi, 1 min, using 1 g alga/25 ml water was the best condition for the fucoidan recovery. L-fucose was the main constituent of this polysaccharide, which also contained xylose and galactose. MAE under optimum reaction conditions was an effective method to recover fucoidan from *Fucus vesiculosus*. This method required short extraction times, and non corrosive solvents, resulting in reduced costs and being an environmentally friend technique.

Keywords: Seaweed; *Fucus vesiculosus*; Microwave-assisted extraction; Sulfated polysaccharides; Fucoidan

1. Introduction

Marine algae, or seaweeds, contain several physiologically bioactive compounds with important economical relevance, such as polysaccharides and iodine organic products, macro and micro elements, vitamins and unsaturated fatty acids (Bhakuni & Rawat, 2005; Craigie, 2010). Brown seaweeds are the second most abundant group of marine algae comprising about 2,000 species. Among them, *Ascophyllum spp*., *Fucus spp*., *Laminaria spp*., *Sargassum spp*., and *Turbinaria spp*. are the most commonly used on industrial level (Hong, Hien, & Son, 2007). Recent studies have demonstrated that brown algae contain biologically active substances that can be used as anticoagulant, antithrombotic, anti-inflammatory, anti-tumor, contraceptive, and anti-viral, for the treatment of several diseases (Synytsya et al., 2010; Wang et al., 2010a). Such properties have been attributed to the sulfated polysaccharides fucoidans in the algae cell wall structure (Ellouali, Boisson-Vidal, Durand, & Jozefonvicz, 1993; Berteau & Mulloy, 2003; Queiroz et al., 2008).

Fucoidans may constitute up to 25–30% of the alga dry weight, depending on the seaweed specie and, to a lesser extent, on season. These polysaccharides are composed by α–1,3-backbones or repeating disaccharide units of α–1,3- and α–1,4-linked fucose residues with branchings attached at C2 positions. Depending on the structure of the main chain, fucoidans may be sulfated at C4, C2 or in both positions of the fucose units. Besides fucose, fucoidans may also contain mannose, xylose, galactose, and rhamnose sugars, and uronic acids (Kusaykin et al., 2008; Rodríguez-Jasso, Mussatto, Pastrana, Aguilar, & Teixeira, 2010).

Sulfated polysaccharides are generally extractable with hot water, dilute acid, or dilute alkali, by using large solvents volume and long extraction times (Marais & Joseleau, 2001; Rioux, Turgeon, & Beaulieu, 2007; Wang, Zhang, Zhang, & Li, 2008; Yang, Chung, & You 2008). In the last decade, microwave-assisted extraction (MAE) has been successfully applied

for extraction of numerous biologically active compounds from a wide variety of natural resources (Sosa-Ferrera, Santana-Rodríguez, & Mahugo-Santana, 2005; Périno-Issartier, 2010; Wang et al., 2010b; Martins, Aguilar, de la Garza-Rodriguez, Mussatto, & Teixeira, 2010). This technique consists in the penetration of microwave energy into the material structure, which produces a volumetrically distributed heat source due to molecular friction resulting from dipolar rotation of polar solvents and from the conductive migration of dissolved ions, accelerating the mass transfer of target compounds. In general, the compounds are extracted more selectively and quicker by this technique, with similar or better yields in comparison with conventional extraction processes, using less energy and solvent volume, thus being more environmentally friend (Eskilsson & Björklund, 2000; Srogi, 2006; Bélanger & Paré, 2006). Only few works report the use of microwave-based techniques for extraction of compounds (alkaline galactans, carrageenans, and agar) from seaweeds (Uy, Easteal, Farid, Keam, & Conner 2005; Navarro, Flores, & Stortz, 2007; Chhatbara, Meena, Prasada, & Siddhanta, 2009; Sousa, Alves, Morais, Delerue-Matos, & Gonçalves, 2010).

The present study evaluated the extraction of sulfated polysaccharides (fucoidan) from *Fucus vesiculosus* seaweed by MAE technique. An experimental design was applied to verify the influence of pressure, extraction time and alga/water ratio in the response of fucoidan yield, and the condition able to maximize the extraction yield was established. The percentage of alga degradation, total sugar yield in the hydrolysates after MAE, and SO_3 content were also determined to each experimental condition. Characterization of the recovered fucoidan was performed by HPLC, FTIR, and TGA/DSC analyses.

2. Material and methods

2.1. Chemicals

Anthrone reagent was purchased from Prolabo, Normapur, Merck; 3,5-dinitrosalicylic acid from Fluka, Chemika, and Coomassie Plus (Bradford) assay kit was from Thermo Scientific Co. Other reagents were all of analytical grade.

2.2. Alga collection and sample preparation

Fucus vesiculosus seaweed was collected from the Praia Norte, Viana do Castelo, Portugal, during September 2009. After collected the algal material was washed with fresh water in order to remove salt, sand and epiphytes, dried at 35 ºC, and milled using a home blender. Particles lower than 1000 μm were not used in experiments. Milled material was kept in plastic bags at room temperature for use in the extraction experiments. Material samples were analyzed to determine the moisture and ash contents (AOAC official methods). The total sugars content present in the alga composition was determined after sulfuric acid hydrolysis for 2 h under vigorous agitation.

2.3. Extraction procedure

MAE experiments were performed in a digestion oven model MDS-2000 (CEM Corporation, Matthews, NC). For each experiment, reaction vessels interconnected with tubing were placed in the sample holder, a rotating carousel. One of the vessels was equipped with pressure sensor that measured and controlled the set point within the cell.
For the extraction reactions, milled seaweed was suspended in the desired amount of distilled water and placed into the extraction vessel. The suspensions were irradiated under different pressures, for times varying between 1 and 31 minutes. Conditions of alga/water ratio,

pressure and time used in each experiment are shown in Table 1. After irradiation, the vessels were immediately cooled in ice bath and the suspensions were filtrated through nylon fiber to separate the residual alga, which was dried at 35 °C, weighted to determine the residual amount obtained (value that was also used to calculate the alga degradation, % AD), and stored. An aliquot of each obtained hydrolysate was taken for total sugar quantification (% TS-A_{MAE}). Subsequently, 1% (w/v) $CaCl_2$ solution was added to the liquid fraction and the mixture was maintained overnight at 4 °C for alginate removal. The fraction obtained by ionization of $CaCl_2$ was separated by filtration. Double volume of ethanol absolute was added to the resultant filtrate and the mixture was stored at 4 °C for 8 h. Ethanol-precipitated polysaccharide was recovered by centrifugation (8,500 rpm, 15 min, 4 °C), dried at 35 ºC, milled and stored for further analyses. Fucoidan extraction yield (% Fuc), alga degradation (% AD), and total sugar yield of hydrolysates after microwave-assisted extraction (% TS-A_{MAE}), were calculated according to Eq.1-3, where WM_{OH} is the dry mass weight obtained after ethanol precipitation; WA is the alga weight used in each experiment; WA_{MAE} is the dry alga weight recovered after MAE; TS_H_{MAE} is the mg of total sugars in the hydrolysates obtained after MAE; and TS_A is the mg of total sugars in the alga *Fucus vesiculosus* (35.12 mg TS/100 mg alga).

$$\%Fuc = \frac{WM_{OH}}{WA} \times 100 \quad (1)$$

$$\%AD = \left(\frac{WA - WA_{MAE}}{WA} \right) \times 100 \quad (2)$$

$$\%TS - A_{MAE} = \left(\frac{TS\_H_{MAE}}{TS\_A} \right) \times 100 \quad (3)$$

2.4. Characterization of the recovered fucoidan

A mass of 10-15 mg of the recovered fucoidan was submitted to hydrolysis with 4 N HCl (2 ml) at 121 °C for 2 h. After the hydrolysis reaction, the total sugar content in the liquid fraction was determined by the anthrone method (using glucose as standard), and the content of sulfate groups was determined by turbidity through the barium chloride–gelatin method (Dodgson, 1961). All absorbance measurements were performed in triplicate.

For the determination of monosaccharides content by HPLC, 10-15 mg of the recovered fucoidan was hydrolyzed with 2 M trifluoroacetic acid (0.5 ml) at 121 °C for 2 h, in glass tubes sealed with N_2. After reaction, the tubes were cooled in ice-water bath, centrifuged (5000 rpm, 5 min), and the liquid fraction was neutralized to pH 7 with 2 M NaOH. Resulting samples were then injected into the HPLC system. A Jasco chromatograph system equipped with a refraction-index detector and a MetaCarb 87P (300 × 7.8 mm) column at 80 °C was used for the sugars determination. Deionized water was used as mobile phase at a flow rate of 0.4 ml min^{-1}.

Micrographs of seaweed samples before and after extraction were obtained by scanning electron microscopy using a Nova NanoSEM 200 microscope. For the analyses, the samples were fixed on a specimen holder with aluminum tape and then sputtered with gold in a sputter-coater under high vacuum condition. Images were obtained at magnification of 2000 fold.

Thermal gravimetric analysis (TGA) data were taken with a thermo balance model TGA-50 (Shimadzu Corporation, Kyoto, Japan) in a nitrogen atmosphere. Differential scanning calorimetry (DSC) analyses were performed using a Modulate DSC-50 (Shimadzu Corporation, Kyoto, Japan). Mass samples of 10-13 mg were run from room temperature to 600 °C, at a rate of 10 °C min^{-1}.

Infrared analysis spectroscopy (FTIR) was carried out on a Perkin-Elmer 16 PC spectrometer (Boston, USA) using 16 scans and frequency range of 400-4000 cm^{-1}. For FT-IR

174 measurement, the polysaccharide was ground with spectroscopic grade potassium bromide
175 (KBr) powder and then pressed into 1 mm pellets. The vibration transition frequencies of each
176 spectrum were baseline corrected and the absorbance was normalized between 0 and 1.
177
178 *2.5. Experimental design*
179
180 A 2^3 full experimental design with four replicates at the centre point was used to
181 evaluate the effects of the variables pressure (X_1; psi), time (X_2; min), and alga/water ratio (X_3;
182 g ml^{-1}) on the extraction of fucoidan under MAE conditions. For statistical analysis, the
183 variables were coded according to Eq. 4, where each independent variable is represented by x_i
184 (coded value), X_i (real value), X_0 (real value at the centre point), and ΔX_i (step change value).
185 The real and coded values of the variables are given in Table 1. Low and high factors were
186 coded as -1 and +1; the centre point was coded as 0.

187 $$x_i = (X_i - X_0) / \Delta X_i \quad (4)$$

188 Four assays at the centre point of the design were carried out to estimate the random
189 error needed for the analysis of variance, as well as to examine the presence of curvature in
190 the response surfaces. The fucoidan yield (Y_1; % Fuc), alga degradation (Y_2; % AD), total
191 sugar yield of hydrolysates after MAE (Y_3; % TS-A_{MAE}), and the sulfate content (Y_4; % SO_3)
192 were taken as dependent variables or responses of the experimental design. The results were
193 analyzed by analysis of variance (ANOVA), and the responses and variables (in coded unit)
194 were correlated by response surface analysis to obtain the coefficients of Eq. 5.

195 $$Y_i = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad (5)$$

196 In Eq. 5, Y_i represents the response or dependent variable; a_0 is the interception
197 coefficient; x_1, x_2 and x_3 are the coded levels of the three variables (pressure, time and alga
198 mass/water volume ratio), and a_1, a_2, a_3, a_{12}, a_{13}, a_{23} are the regression coefficients. The

statistical significance of the regression coefficients was determined by Student's *t*-test, and the proportion of variance explained by the models was given by the multiple coefficient of determination, R^2. Statistica 5.0 was the software used for data analysis.

3. Results and discussion

3.1. Alga characterization

Fucus vesiculosus contained a moisture content of 15.95 ± 0.08% (w/w). This value is higher than those reported to other marine algae such as *Laminaria* (6.64%) and *Gigartina* (9.86%) (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010), and is a positive aspect considering the alga use in MAE because the moisture content is closely related to the absorption efficiency of microwaves by the immersed target material. The water molecules convert the microwave energy into heat, resulting in a sudden rise of the temperature inside the material. The temperature keeps rising until the internal pressure exceeds the capacity of expansion of the matrix thus creating an explosion at the intermolecular level. As a consequence, the substances that are located within these chemical systems migrate to the surrounding medium that traps and dissolves them (Bélanger & Paré, 2006).

Ashes in *Fucus vesiculosus* corresponded to 18.32 ± 0.83% (w/w), a high value currently found in seaweeds, but much higher than those generally observed in terrestrial vegetables. Ashes content comprises the minerals present in the material. Although the minerals present in *Fucus vesiculosus* ashes were not determined here, brown seaweeds have been reported to have high chloride content, small amounts of fluoride, nitrate and phosphate, and trace amounts of nitrite and bromide. Due to the significant mineral content present in their chemical composition, several seaweeds have been used as food supplement to help

meet the recommended daily intakes of some minerals and trace elements (Gómez-Ordóñez et al., 2010)

Total sugars content in *Fucus vesiculosus* was 35.12 ±0.02% (w/w). This value is lower than those reported by Rioux et al. (2007) for brown seaweeds, and probably, it is a consequence of the period in which the alga was harvested. Algae generate their biomass reserve after the rapid grow phase in spring in order to survive the winter where hardly any photosynthesis occurs. As a consequence, a larger amount of polysaccharides is found during the winter season. In the present study, the alga was harvested in September (autumn season), which was not the best collection period.

3.2. Operational variables affecting fucoidan extraction by MAE

Several studies report MAE as a technique able to produce biopolymers with high molar mass at significantly shorter heating times than conventional extraction methods (Chen, Liu, Jiang, & Zeng, 2005; Leonelli & Mason, 2010). Considering this aspect and the structural and chemical complexity of sulfated polysaccharides, MAE was used in the present study to extract fucoidan from algal material. It was also expected that by using this method fucoidan would undergo degradation. In this work the microwave energy over the target material was controlled under pressure parameter, because one of the most frequent problems of heating by microwave fields is the temperature measurements, which are complicated by the presence of high intensity electromagnetic fields (Kustov & Sinev, 2010).

The used extraction conditions, including pressure, extraction time and algae/water ratio were selected based on previous studies for the production of other heteropolymers by MAE, such as pectin from citric peels or sugar beet pulp (Fishman, Chau, Hoagland, & Ayyad, 2000; Fishman, Chau, Cooke, & Hotchkiss, 2008). Pressure conditions, particularly, were

249 evaluated until the maximal operational value allowed by the equipment. Fig. 1 shows the
250 pressure profiles against heating time of microwave irradiation for a sample load of 1 g per 25
251 ml of water. Heat stages rates were estimated measuring the ramp up and ramp down through
252 the heating and cooling phases between the isothermal periods of the extraction procedures.
253 The equivalent temperature used to each pressure (after the system has reached a saturated
254 vapor behavior) was estimated using tables of water liquid-vapor phase and corresponded to
255 122, 152 and 172 °C for 30, 75, and 120 psi, respectively. As can be seen in Fig. 1, the
256 samples reached the hydrothermal stage (constant pressure) in less than 2 min, showing
257 similar pressure increment with heating rates of around 103-128 °C min^{-1}. On the contrary,
258 the pressure reduction showed that cooling rates were dependent of the quantity of time that
259 the sample was irradiated at the isothermal stage. As a consequence, the compounds
260 hydrolysis is also influenced at the cooling phase.
261 Moreover, the time required to attain the desired pressure was also dependent of the
262 number of vessels processed simultaneously in the equipment, and therefore, the number of
263 vessels should be chosen in order to minimize the time needed to reach the set conditions and
264 to avoid a ‘‘bumping’’ phenomenon during the extraction (Eskilsson & Björklund, 2000).
265 The solid/liquid ratio, i.e., the ratio between alga mass and water volume used for the
266 reactions, is also an important parameter to be considered in MAE. The product recovery by
267 conventional extraction methods is usually increased when using high solvent volumes
268 (Eskilsson & Björklund, 2000); however, similar behavior may not occur in MAE. For this
269 reason, different solid/liquid ratios varying from 1/25 to 5/25 g ml^{-1} were evaluated in the
270 present study. Table 1 shows the conditions of pressure, reaction time and alga/water ratio
271 used in each experimental MAE assay, and the respective fucoidan yield, alga degradation,
272 total sugar yield and sulfate content obtained. Great variation in all the responses was

observed according to the used experimental condition. Fucoidan yield, for example, was increased in up to 17 times, by varying the MAE conditions.

All the studied operational variables affected the extraction process, presenting significant main effects and/or interactions for all of the evaluated responses (Table 2). For fucoidan yield, the alga/water ratio presented a significant main effect ($p<0.05$) of negative signal, which reveals that the fucoidan yield was increased when using an alga/water ratio of 1 g/25 ml. Although the pressure and extraction time have not shown significant main effects for fucoidan yield, interaction between these variables was highly significant ($p<0.01$) for this response. When observing the main effects of these two variables for the other responses, it can be observed that pressure had a significant main effect of positive signal for all of them, which suggests that the extraction results were improved when the pressure was increased. As a consequence, since the interaction between pressure and reaction time had a significant negative effect for fucoidan yield response, it can be concluded that the use of lower reaction times favored the extraction process. This analysis is in agreement with the results presented in Table 1, which shows that the highest fucoidan yield (18.22%) was obtained when the highest pressure (120 psi) and the lowest extraction time (1 min) and alga/water ratio (1 g/25 ml) were used (conditions of the assay 5).

Similar behavior was reported by (Latha, 2007) during the biopolymers extraction by MAE. According to this author, the particle concentration increase promotes a strong absorption of the microwave energy near the surface of the vessel, and low penetration depth of microwave radiation, which reduces the percentage of extraction. On the other hand, the pressure increase promotes the temperature raise in a direct proportion. As a consequence, the extraction rate increases due to the viscosity and surface tension reduction (Eskilsson & Björklund, 2000).

297 In the present study, despite the pressure increase has favored the fucoidan yield,
298 equipment limitations did not allow to evaluate pressure values higher than 120 psi.
299 Additionally, the use of alga/water ratios lower than 1/25 g ml^{-1} might not be economically
300 advantageous for the process since it would increase the costs for fucoidan recovery from the
301 liquid phase. Therefore, the optimal MAE conditions for fucoidan extraction from *Fucus*
302 *vesiculosus* were established in the studied range of operational values. An analysis of
303 variance of the obtained data for linear models gave high values for the coefficient of
304 determination R^2 (between 0.84 and 0.95), which show a close agreement between
305 experimental results and the theoretical values predicted by the first-order polynomials. A
306 multiple regression analysis was then performed to fit first-order polynomial equations to the
307 experimental data points. The fucoidan yield (Y_1, %), alga degradation(Y_2, %), total sugar
308 yield of hydrolysate (Y_3, %), and the sulfate content (Y_4, %) were correlated as a function of
309 extraction pressure (x_1), time (x_2) and alga/water ratio (x_3) (coded values) used for MAE,
310 resulting in Eqs. 6, 7, 8, and 9, respectively.
311
312 $$Y_1 = 10.30 + 1.29x_1 + 0.05x_2 - 2.58x_3 - 4.17x_1x_2 + 0.46x_1x_3 + 0.53x_2x_3 \quad (R^2 = 0.84) \quad (6)$$
313 $$Y_2 = 46.19 + 7.50x_1 + 5.96x_2 - 4.72x_3 - 2.74x_1x_2 - 2.89x_1x_3 - 3.24x_2x_3 \quad (R^2 = 0.92) \quad (7)$$
314 $$Y_3 = 11.64 + 2.79x_1 + 1.81x_2 - 9.26x_3 - 2.51x_1x_2 - 2.02x_1x_3 - 1.44x_2x_3 \quad (R^2 = 0.95) \quad (8)$$
315 $$Y_4 = 24.34 + 3.06x_1 + 4.17x_2 + 1.37x_3 + 0.81x_1x_2 + 0.92x_1x_3 + 1.19x_2x_3 \quad (R^2 = 0.94) \quad (9)$$
316
317 Three-dimensional response surfaces described by the above-mentioned first-order
318 polynomials were well fitted to the experimental data points through flat surfaces, confirming
319 the suitability of the proposed linear models to explain the responses variations in the studied
320 range of values. Fig. 2 represents the variations in fucoidan yield according to the pressure
321 and alga/water ratio used for extraction. As can be seen, the flat surface clearly indicates a

region where the value of this response is maximized, which corresponds to the use of 120 psi, and 1/25 alga/water ratio (g ml^{-1}) during 1 min of extraction. The highest fucoidan extraction yield (18.22% in a dry weight basis) is in good agreement with the values reported by Rioux et al. (2007) during the extraction of *F. vesiculosus* by 3 sequential hydrolysis steps (each one of 3 h) at 70 °C. Moreover, this value was higher than those reported for fucoidan obtained from other sources extracted by hydrothermal conventional procedures under temperatures between 25 and 70 °C and times of 2-6 h (Zvyagintseva et al. 1999; Duarte, Cardoso, Noseda, & Cerezo, 2001; Navarro, D. A., Flores, M. L., & Stortz, C. A., 2007). Additionally, Yang et al. (2008) evaluated the hydrolysis of sulfated polysaccharides of *U. pinnatifida* testing twice microwave for 30-120 sec and founded that microwave heating around 30-60 seconds only was more effective in improving the polymer dissolution.

Fig. 3 shows the alga structure before and after MAE under optimum conditions. As can be seen, the untreated sample (Fig. 3A) presented closed cells and rough surfaces, which were mostly destroyed after MAE (Fig. 3B). A less destructive effect of destruction in the alga structure was observed after MAE under milder pressure conditions (Fig. 3C). Such facts evidence the importance of the pressure increase on the extraction process, as commented before. The alga structure after MAE under high pressure (120 psi, Fig. 3B) was formed by a very rough surface with many cavities, suggesting that microwave radiation had the power on cuticular layer destruction, as observed also by other authors (Chen et al., 2005).

3.3. Characterization of the extracted fucoidans

3.3.1. Compositional analysis

The fucoidans obtained in all the experimental MAE conditions were characterized regarding the monosaccharide and sulfate contents (Table 3). L-Fucose was the only

monosaccharide found in all the samples. Galactose was also present in most of the samples, but xylose was only present in some of them. The results presented in Table 3 suggest that the pressure used for extraction had a strong influence on the fucoidan composition, since the galactose contents in the fucoidan structure were increased when the pressure used for extraction was increased to 120 psi; and only fucose was present in the fucoidans obtained at 30 psi. Similarly, xylose was only present in structures obtained at 120 psi. Under the optimum MAE conditions, a fucoidan structure composed predominantly by fucose, followed by significant proportion of xylose and minor galactose content was obtained (Table 3, assay 5). This is in agreement with literature data that report that fucoidan from *F. vesiculosus* has a heterogeneous and branched structure (Marais & Joseleau, 2001).

Besides the monosaccharide content, the conditions used for MAE affected also the fucoidans sulfating degree (Table 3). However, high sulfate content (> 20%) was found in practically all the fucoidan samples, which is an advantageous aspect since sulfate groups have been reported to have important biological functions such as anti-HIV activity; and such activity is potentially increased when the sulfating degree is increased (Schaeffer & Krylov, 2000). Additionally, the presence of non-sulfate monosaccharide units in polysaccharides branches is reported to annul the anticoagulant effect of the polysaccharide (Costa et al., 2010). The ratio between total sugars and sulfate content (TS/SO_3, Table 3) is considered an indicator of the anticoagulant activity of fucoidan polysaccharides (Wang et al., 2008). In the present study, most of the experiments showed TS concentrations similar or higher than SO_3 concentrations.

Fucoidan polymers from other sources had comparable amounts of sulfates (19–30%) and monosaccharide composition with fucose as the major sugar in the extracted fucoidans (50-90 % mol) and lower amounts of galactose and xylose (Zvyagintseva et al. 1999; Duarte et al. 2001; Rioux et al. 2007). However, it is important emphasizing that chemical

372 composition of fucoidan polymers is significantly dependent on species, anatomical regions,
373 growing conditions, extraction procedures and analytical methods.
374
375 *3.3.2. Thermal analysis*
376 TGA and DSC curves of fucoidan extracted under optimum MAE conditions are
377 showed in Fig. 4. Three different stages were well defined during these analyses. The first one
378 was basically associated with the weight loss (moisture) due to dehydration, which covered a
379 temperature range between 25 °C and 110 °C. Subsequently, pyrolysis reactions of the sample
380 started at 120 °C. The second stage started at 195 °C and consisted in the devolatilization of
381 the sample, with evolution of the volatile matter mainly occurring between 220 °C and 490
382 °C. Finally, the third stage began close to 500 ºC and was maintained up to 600 °C. The
383 remaining mass at the end of this process (around 50% of the original fucoidan mass)
384 corresponds to the ash content in the sample. This residual mass is probably constituted by
385 sulfates, phosphates and carbonates, which are minerals usually found in polysaccharides
386 structures like fucoidan (Anastasakis, Ross, & Jones, 2011).
387
388 *3.3.3. FTIR analysis*
389 Fucoidan obtained under optimum MAE conditions, as well as fucoidan samples
390 obtained under other evaluated extraction conditions, were analyzed by FTIR to determine
391 the specific absorption bands present in the recovered products. The FTIR spectra in Fig. 5
392 clearly show that all the evaluated samples exhibited absorption bands typical of fucoidans.
393 The absorption band at 1240–1255 cm^{-1} (S=O stretching) confirmed the presence of sulfate
394 in the recovered polysaccharides. The sharp band at 840 cm^{-1} and the shoulder at 820 cm^{-1}
395 (C-S-O) suggest a complex pattern of substitution, primarily at C-4 position (axial C-4

substitution of α-linked L-fucopyranose) with other substitution at C-2 or/and C-3 (equatorial positions) in lower amount (Marais & Joseleau, 2001; Wang et al., 2010a)

4. Conclusions

In summary, MAE under optimum reaction conditions was an effective method to recover fucoidan from *Fucus vesiculosus*. This method required short extraction time and use of non corrosive solvents, resulting in reduced costs when compared to the conventional extraction techniques. Additionally, MAE can be considered a more environmentally friend technique than the traditional extraction processes, since it requires lower energy consumption and generates less wastes. For all these reasons, MAE was considered a potential method to obtain fucoidan from brown seaweed.

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542 **Figure captions**

543

544 **Fig. 1.** Pressure profiles as a function of radiation time during MAE of *Fucus vesiculosus* for
545 fucoidan recovery; relation between heating and cooling rates (°C/min). Sample load: 1 g
546 alga/ 25 ml water. A) 30 psi (122 °C) / 1min; B) 30 psi (122 °C) / 31min; C) 75 psi (152 °C) /
547 16min; D) 120 psi (172 °C) / 1min; and E) 120 psi (172 °C) / 31min. ΔH: heat rate (1); ΔC:
548 cool rate (2).

549

550 **Fig. 2.** Response surface fitted to the experimental data points corresponding to the fucoidan
551 yield during MAE of *Fucus vesiculosus*.

552

553 **Fig. 3.** Scanning electron micrographs of *Fucus vesiculosus*: (A) untreated sample; (B)
554 sample obtained after MAE at 120 psi, 1 min, using 1 g alga/ 25 ml water; (C) sample
555 obtained after MAE at 30 psi, 31 min, using 1 g alga/ 25 ml water. Magnification: 2000-fold.

556

557 **Fig. 4.** TGA and DSC thermograms of fucoidan sample obtained under optimum MAE
558 conditions.

559

560 **Fig. 5.** Infrared analysis spectroscopy (FTIR) of fucoidan samples obtained by MAE of
561 *Fucus vesiculosus* under different operational conditions.

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Highlights

- Fucoidan was recovered from brown seaweed by microwave-assisted extraction.
- Pressure, extraction time, and alga/water ratio affected the fucoidan yield.
- Extraction conditions able to maximize the fucoidan recovery were established.
- Recovered fucoidan was constituted by fucose, xylose and galactose.
- MAE with short extraction time was effective to recover fucoidan from seaweed.

Table 1

Experimental conditions used for MAE of *Fucus vesiculosus* according to a 2^3 full experimental design. Real and (coded) values of the operational variables pressure (x_1), extraction time (x_2) and alga/water ratio (x_3), and results obtained for the responses fucoidan yield (Y_1; % Fuc), alga degradation (Y_2; % AD), total sugar yield of hydrolysates after MAE (Y_3 ; % TS-A_{MAE}), and sulfate content (Y_4; % SO_3).

	Variables [a]						Responses			
Assay	x_1		x_2		x_3		Y_1 (% Fuc)	Y_2 (% AD)	Y_3 (% TS-A_{MAE}) [b]	Y_4 (% SO_3)
1	30	(-1)	1	(-1)	1/25	(-1)	6.25	28.82	9.42	20.08
2	30	(-1)	1	(-1)	5/25	(+1)	1.08	27.92	1.39	16.87
3	30	(-1)	31	(+1)	1/25	(-1)	15.61	48.99	24.52	22.76
4	30	(-1)	31	(+1)	5/25	(+1)	8.60	42.57	3.59	27.63
5	120	(+1)	1	(-1)	1/25	(-1)	18.22	51.36	27.62	21.09
6	120	(+1)	1	(-1)	5/25	(+1)	10.93	46.33	4.39	24.88
7	120	(+1)	31	(+1)	1/25	(-1)	6.93	67.98	25.54	30.31
8	120	(+1)	31	(+1)	5/25	(+1)	5.74	42.59	3.68	35.55
9	75	(0)	16	(0)	3/25	(0)	12.53	48.76	9.65	23.07
10	75	(0)	16	(0)	3/25	(0)	13.24	50.51	10.01	22.57
11	75	(0)	16	(0)	3/25	(0)	12.16	47.02	8.56	24.99
12	75	(0)	16	(0)	3/25	(0)	12.36	51.40	11.36	22.33

[a] pressure (x_1): psi; time (x_2): min; alga/water ratio (x_3): g ml^{-1}. [b] % TS-A_{MAE} was calculated by the ratio between mg of total sugars in the hydrolysates obtained after MAE, and mg of total sugars in the alga (35.12 mg/100 mg).

Table 2

Effect estimates (EE), standard errors (SE) and level of significance (*p*) for fucoidan yield (Y_1; % Fuc), alga degradation (Y_2; % AD), total sugar yield of hydrolysates after MAE (Y_3; % TS-A_{MAE}), and sulfate content (Y_4; % SO_3) obtained after MAE of *Fucus vesiculosus* according to a 2^3 full experimental design.

Variables	Y_1 (% Fuc)		Y_2 (% AD)		Y_3 (% TS-A_{MAE}) [a]		Y_4 (% SO_3)	
	EE ± SE	*p*	EE ± SE	*p*	EE ± SE	*p*	EE ± SE	*p*
x_1	2.57 ± 1.99	0.2521	14.99 ± 3.19	0.0053 ***	5.58 ± 2.19	0.0512 *	6.12 ± 1.31	0.0055 ***
x_2	0.10 ± 1.99	0.9618	11.93 ± 3.19	0.0134 **	3.63 ± 2.19	0.1580	8.33 ± 1.31	0.0014 ***
x_3	-5.17 ± 1.99	0.0482 **	-9.44 ± 3.19	0.0315 **	-18.51 ± 2.19	0.0004 ***	2.67 ± 1.31	0.0970
x_1x_2	-8.34 ± 1.99	0.0085 ***	-5.49 ± 3.19	0.1460	-5.02 ± 2.19	0.0700 *	1.61 ± 1.31	0.2733
x_1x_3	0.93 ± 1.99	0.6609	-5.78 ± 3.19	0.1298	-4.03 ± 2.19	0.1245	1.84 ± 1.31	0.2188
x_2x_3	1.07 ± 1.99	0.6147	-6.47 ± 3.19	0.9816	-2.88 ± 2.19	0.2446	2.38 ± 1.31	0.1288

Significance level: 99% (***); 95% (**); 90% (*). x_1: pressure (psi); x_2: time (min); x_3: alga/water ratio (g ml^{-1}).

[a] % TS-A_{MAE} was calculated by the ratio between mg of total sugars in the hydrolysates obtained after MAE, and mg of total sugars in the alga (35.12 mg/100 mg).

Table 3

Monosaccharide and sulfate composition of fucoidan isolated from *Fucus vesiculosus* by MAE under different operational conditions according to a 2^3 full experimental design. Monosaccharide amount are expressed as the percent of the total sugar content in the sample, in moles.

Assay	Pressure (psi)	Extraction time (min)	Alga/water ratio (g ml^{-1})	Fucose (% mol)	Galactose (% mol)	Xylose (% mol)	TS/SO_3*
1	30	1	1/25	100.0	0.0	0.0	1/1.00
2	30	1	5/25	100.0	0.0	0.0	1/0.89
3	30	31	1/25	100.0	0.0	0.0	1/0.89
4	30	31	5/25	82.3	17.6	0.0	1/1.07
5	120	1	1/25	53.8	10.8	35.3	1/0.77
6	120	1	5/25	57.4	42.5	0.0	1/0.96
7	120	31	1/25	27.1	42.9	29.9	1/1.84
8	120	31	5/25	39.1	60.8	0.0	1/2.11
9	75	16	3/25	49.0	50.9	0.0	1/1.12
10	75	16	3/25	49.8	50.1	0.0	1/0.93
11	75	16	3/25	53.6	46.3	0.0	1/0.96
12	75	16	3/25	57.6	42.3	0.0	1/1.02

* TS/SO_3 = (mg TS/100 mg fucoidan)/(mg SO_3/100 mg fucoidan). TS: total sugars.

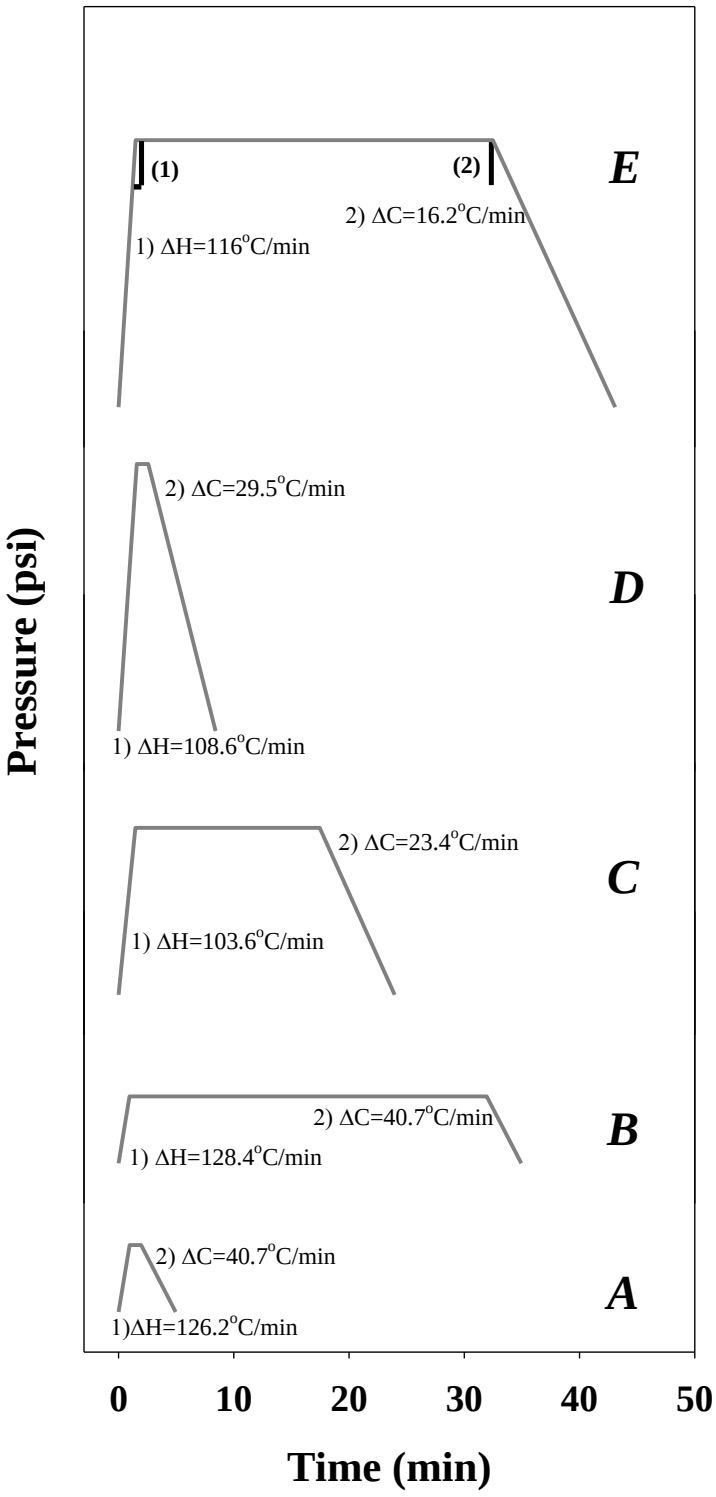

Fig. 1.

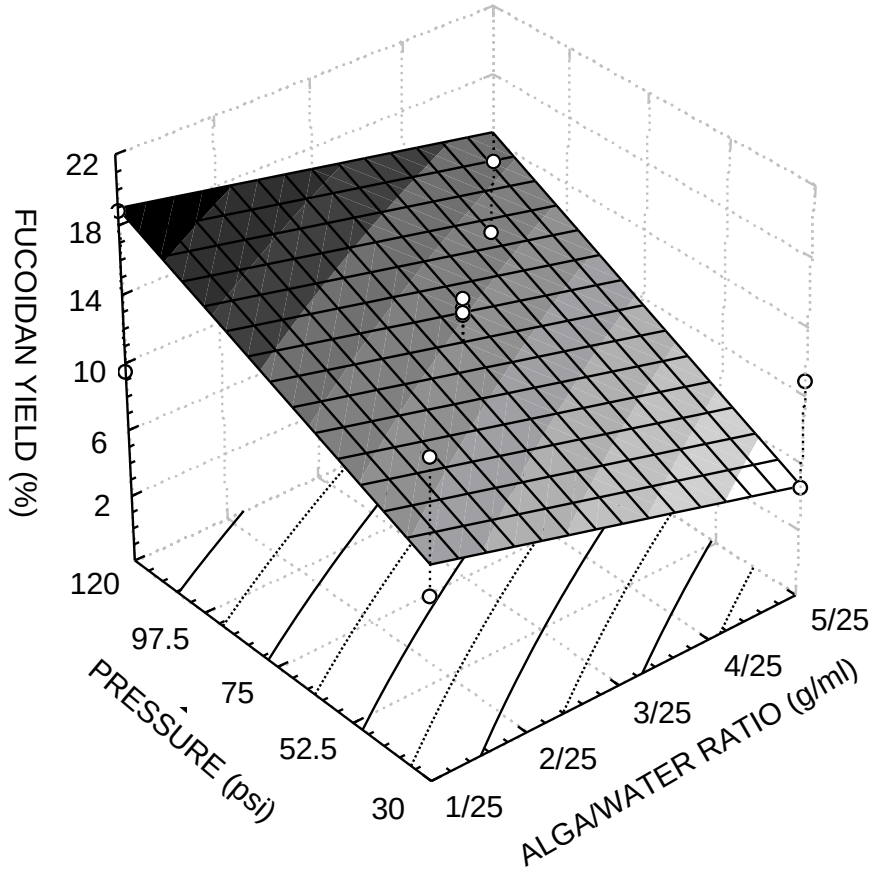

Fig. 2.

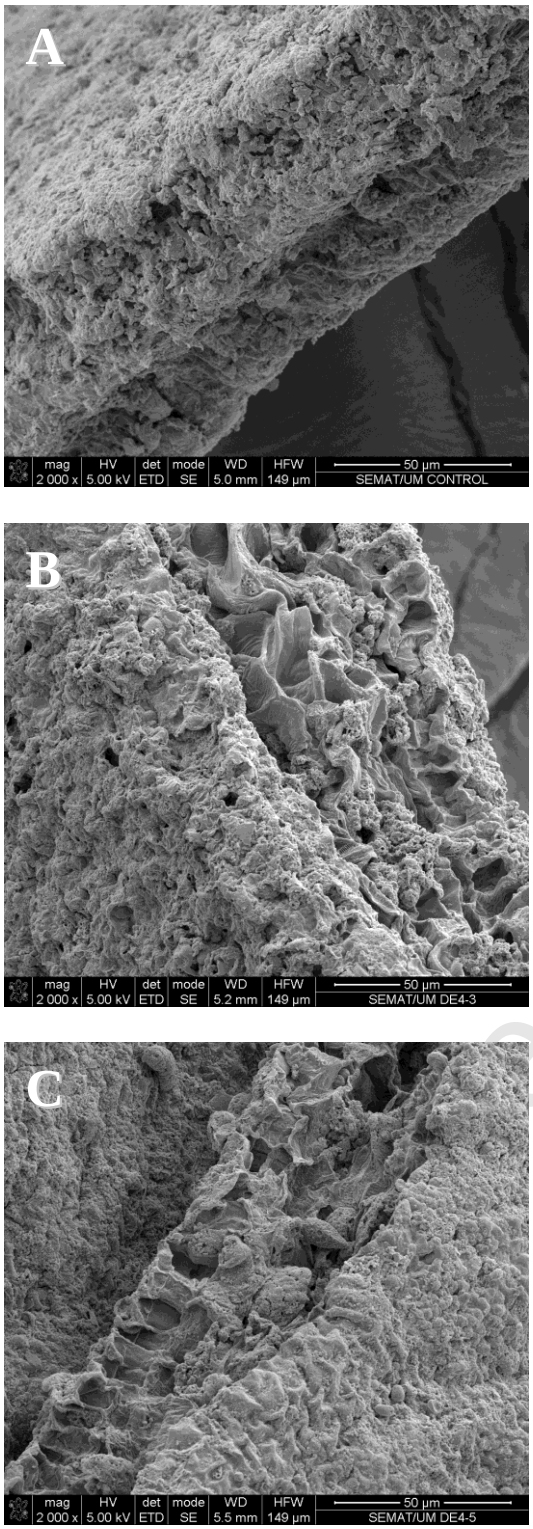

Fig. 3.

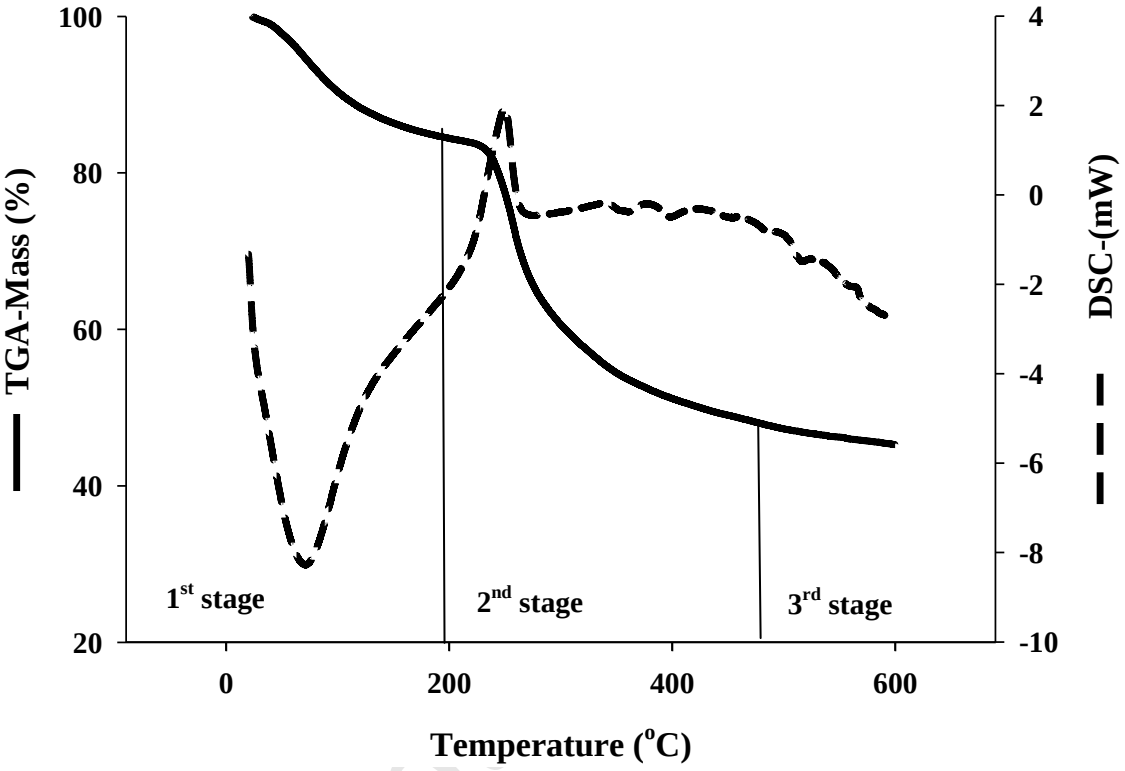

Fig. 4.

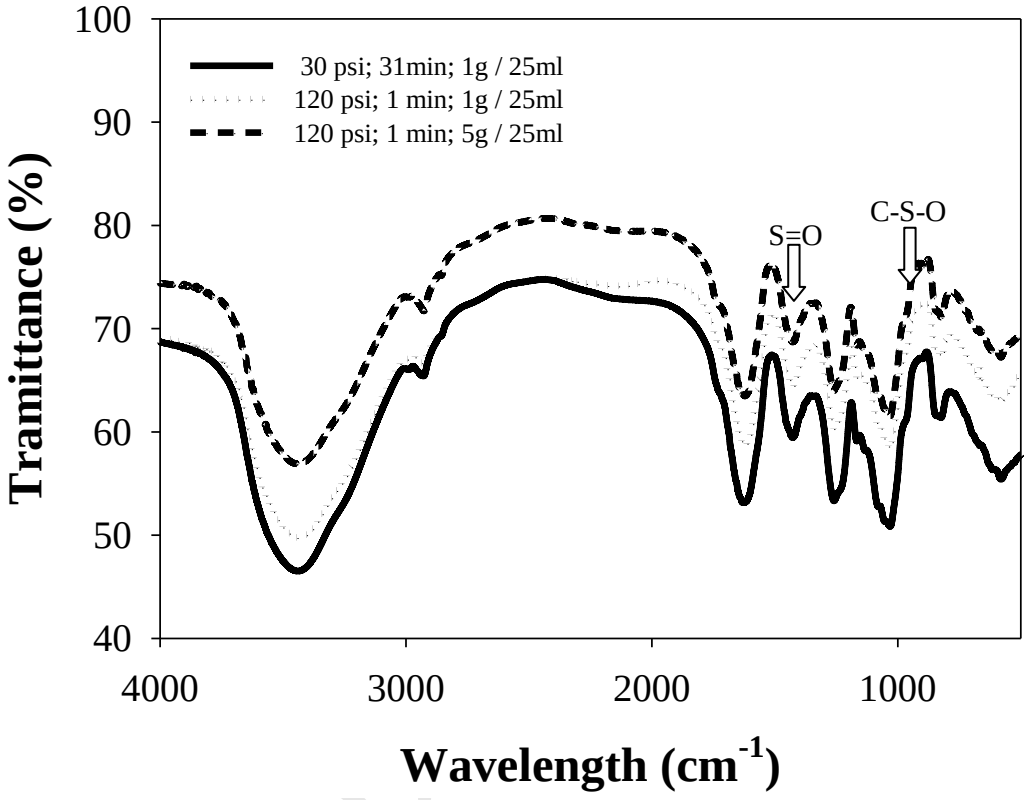

Fig. 5.