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Microwave-assisted extraction of *Ulva* spp. including a stage of selective coagulation of ulvan stimulated by a bio-ionic liquid

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ABSTRACT

Microwave-assisted hydrothermal processing was proposed to recover high valuable compounds with antioxidant and gelling features from *Ulva* spp. green seaweed. The influence of the extraction conditions on the solubles, ulvan fraction and residual solid phase was studied to achieve a global valorization of the seaweed. A particular emphasis was placed on the selective coagulation of ulvan stimulated by a bio-ionic liquid during the extraction process. The achieved outcomes indicated that the selected microwave treatment exhibited a notable impact on the phytochemical properties of the soluble extracts, with the highest values of sulfate and protein content at 160 °C, and the highest antioxidant features at 200 °C. The most prominent molecular weight distributions were also identified for systems hydrothermal treated at 160 °C. The ulvan analyses showed that those extracted after microwave treatment at 160 °C showed the highest yields, molecular weight and the strongest gel features from the rheological point of view. The presence of the chloride chlorine during the extraction process favored the ulvan performance and the enhancement of the corresponding viscoelastic properties.

1. Introduction

Green seaweed from the *Ulva* genus exhibit high growth rates and productivities through different geo-climatic conditions, with highly exploitable biochemical profiles [1]. These species of green macroalgae can be attractive alternatives in several fields, from food supplements to biomedical applications for its health benefits due to the presence of biologically active compounds [2]. *Ulva* spp. has been reported as the most suitable candidates for developing low-fat foods with PUFA-rich nutraceuticals [3]. *Ulva* species are rich in polysaccharides, proteins, minerals, vitamins, dietary fibers, and different functional polyphenols [2]. In addition, recent studies have displayed the potential of *Ulva* species as antibacterial [4], antifungal [5], antioxidant [3], anti-inflammatory [2], anticancer [6] or antiviral [7] agents.

Ulvan is the main cell wall polysaccharide of *Ulva* spp., which is highly hydrophilic, semi-crystalline in nature and represent around 10–40 % dry weight of the seaweed biomass [8]. The backbone of this gelling sulfated polysaccharide is most composed of α- and β-(1,4)-linked monosaccharides that can vary (rhamnose, xylose, glucuronic acid and iduronic acid), with representative repeating disaccharide units [3]. Other minor cell wall polysaccharides of *Ulva* species are cellulose, xyloglucan, and glucuronan, which account for 5 % of the dry weight biomass [8]. The bioactivities of ulvan depend mainly on its molecular weight, monosaccharide composition or the sulfate and glyoxylate content [9], which also drive their rheological features [10], and its extraction yield is highly dependent on the isolation and purification treatments [11]. A remarkable ulvan characteristic is its ability to develop thermo-reversible gels in the presence of calcium ions at a pH around 7.5 [12]. Kidgell et al. [10] found high variability on the viscoelastic properties of ulvans from several *Ulvan* species (*U. australis*, *U. rigida*, *U.* sp. *B*, and *Ulva* spp.) indicating that further understanding of the structure-activity relationships are required. The improvement of the rheological feature of this biodegradable biopolymer can pave way to introduce a new kind of bioink for novel 3D printing applications [12].

The features and bioactivities of ulvans can be modulated by the extraction conditions [13]. Conventional extraction procedures involve the use of organic solvents and longtime treatments with the consequent energy expenditure and environmental pollution [1,10]. Among non-conventional extraction technologies such as ultrasound [14], pulsed electric field, subcritical and supercritical fluid [56], microwave hydrodiffusion and gravidity [58] or microwave assisted extractions

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[15] have been proposed for different seaweeds. Microwave assisted extraction (MAE) technique allows a faster and more selective extraction of compounds using distilled water as solvent [16]. Eco-friendly intensification alternatives should be proposed to enhance the ulvan extraction yields tailoring the required viscoelastic properties. Bio-based ionic liquids could be an interesting possibility to explore, due to their ability to selectively bind with the target polysaccharide and their inherit features such as biodegradability, non-toxicity or biocompatibility [17]. Based on this knowledge, selective precipitation of biopolymers from some agarophytes or carragenophytes macroalgae using bio-based ionic liquids [18] or deep eutectic solvents [17,19] has been explored. However, authors are not aware that precipitation of ulvans using these solvents has been attempted.

The integral valorization of *Ulva* spp. could be a more sustainable approach that contributing to biorefinery processes offers alternatives to global environmental concerns, since the valuable compounds found in *Ulva* and its high productivity promotes its use. In this context, this work is mainly aimed at the extraction of high valuable fractions from *Ulva* spp. green seaweed using a microwave assisted hydrothermal treatment operating under a wide range of processing conditions. The raw material itself, the recovered soluble extracts with bioactive and gelling potential, as well as the residual solid phases were characterized. Special interest was also placed on the impact of the stimulation of the selective coagulation of the gelling fractions using a bio ionic liquid on their rheological features.

2. Materials and methods

2.1. Materials

Dried *Ulva* spp. green seaweed was kindly provided by Porto-Muiños S.L. (A Coruña, Spain). It was stored in a sealed plastic bag protected from light at room temperature until further use. This raw material was micronized with an average particle size <20 μm in a grinder before the extraction process.

2.2. Microwave hydrothermal treatment

The microwave assisted extraction process of *Ulva* spp. green seaweed was carried out on an Anton Paar Microwave reactor Monowave 450 (Austria), supplied with an autosampler MAS 24. Extraction experiments were conducted over the temperature range from 120 to 200 °C, using distilled water as extraction agent with a solid/liquid ratio of 1:30 (w/w) as previously reported for other seaweeds submitted to conventional hydrothermal treatments [20]. Vials containing tested systems were heated up to the desire temperature for 5 min at 850 W and 800 rpm. Then, they were cooled down to 50 °C [21]. Afterwards, the vial content was vacuum filtrated in order to separate the liquid and solid phases. The crude liquid phases were placed in a freezer at −18 °C until further analysis, whereas the solid phases were dried at 40 °C in a convective air oven and stored in dark plastic bags at room temperature for further characterization.

2.3. Characterization of the raw material and solid phase after extraction

Moisture content of *Ulva* spp. and the corresponding solid phases remaining after MAE treatment was determined gravimetrically. Samples (around 1 g) were put in a glass tube placed in a convective air oven at 105 °C for 48 h to ensure constant weight. The ash content was also determined gravimetrically using a furnace muffle furnace at 575 °C for 6 h. The corresponding sulfate content was analyzed following the method developed by Gómez Ordóñez et al. [22]. This characterization was based on ionic chromatography (Metrohm Advanced IC-861, Switzerland), and the column used was a Metrosep A Supp 5-250 (250 × 4 mm). The phase mobile was sodium carbonate/sodium bicarbonate at 3.2 mM and 1 mM, respectively at 0.70 mL/min. The detector used was IC-819. The mineral (Ca, K, Mg, Na) and metal (B, Fe, Cu, As, Cd, Hg and Pb) constituents were determined. Briefly, samples were subjected to an acid digestion on a Marsxpress (CEM) using ash (0.3 g) mixed with nitric acid (10 mL) and hydrogen peroxide (1 mL). Treatment was performed at 1600 W for 15 min, and then heated at 200 °C for 10 min. Then, a SpectrAA-220 Fast Sequential spectrophotometer (Varian, USA) was used to measure the atomic absorption for obtaining Ca, Fe, Cu and Mg content, as well as the atomic emission for Na and K amount. ICP-MS (X Series, Thermo Scientific) was used to determine Cd content.

In order to determine the carbohydrate content, an acid hydrolysis was performed. Dried algae (0.5 g) mixed with 5 mL of sulfuric acid (72 %) were placed in a water bath at 30 °C for 1 h. Subsequently, every tube was diluted with deonized water to reduce the level of sulfuric acid at 4 %, and placed in an autoclave at 121 °C for another hour. In order to remove the acid insoluble residue (AIR) from the liquid fraction, a filtration step through 0.45 μm filter was required, being the filtered liquids evaluated by HPLC (as detailed below).

Protein content was estimated by means of Kjeldahl method using 5.13 as nitrogen conversion factor following the recommendations given by Lourenço et al. [23]. Carbon and hydrogen content were conducted on an elemental analyzer (Thermo Flash EA 1112, Germany). The gas flow was set at 130 mL/min for helium, 100 mL/min for reference and 250 mL/min for oxygen, operating with oxidation and reduction furnace temperatures of 900 and 680 °C, respectively. Multiple analyses were performed for a 6 × 5 mm, 2.0 m column (Cromlab, Spain) with the column temperature set at 50 °C and the chromatogram time at 420 s. Aspartic acid was used as a pattern [15]. Using the nitrogen (N), hydrogen (H) and carbon (C) content obtained experimentally, the higher heating values, HHV, of the solid phases after microwave assisted extraction were estimated according to the following equation [24],

$$\mathrm{HHV\ (kJ/kg)} = 3.55C^2 - 232C - 2230H + 51.2C \bullet H + 131N + 20600 \quad (1)$$

2.4. Characterization of the liquid phase

2.4.1. Total phenolic content

Total phenolic compounds were spectrophotometrically determined employing the Folin method [25]. A standard curve employing gallic acid as pattern was made from 0.009 g/L to 0.09 g/L. In the order, we added 0.25 mL of gallic acid or crude liquid sample, 1.875 mL of distilled water, 0.125 mL of Folin-Ciocalteu's reagent (1:1 Folin/water) and 0.25 mL of Na_2CO_3 at 10 % in the test tube. The content was vortex prior to left in the dark for 1 h before measuring the absorbance at λ = 765 nm.

2.4.2. Trolox equivalent antioxidant capacity

Trolox equivalent antioxidant capacity, TEAC method was selected to estimate the antioxidant activity of the liquid phases [26]. TEAC reagent was diluted with PBS until the mix reaches an absorbance of 0.7 ± 0.02 nm at 734 nm. Then, 1 mL of TEAC reagent was added in the crude liquid samples and control (distilled water) tubes containing in advance 10 μL of Trolox solutions and placed in water bath at 30 °C for 6 min, and the absorbance was run at 734 nm.

2.4.3. Sulfate content

Sulfate content was determined following the method developed by Dodgson [27] known as gelatine-barium chloride method. The crude liquid samples were hydrolyzed with trichloroacetic acid at 4 % (w/v) (Sigma-Aldrich). The Gelatin-$BaCl_2$ reagent was prepared in distilled water, gelatine powder 5 % (Scharlau), after the solution was kept at 4 °C for at least 6 h (or overnight). After this period of time, 5 % $BaCl_2$ (Sigma-Aldrich) was added and mixed obtaining a cloudy solution. A period of incubation of 2–3 h was necessary, after the Gelatin-$BaCl_2$ reagent was ready to use. Samples or distilled water for blank (0.1 mL), TCA solution at 4 % (1.9 mL) and gelatine-$BaCl_2$ reagent (0.5 mL) were introduced in a test tube and mixed using a vortex. An incubation at

room temperature for 15 min was necessary previous to read the absorbance at 500 nm.

2.4.4. Protein content

The Bradford's method was performed at least in triplicate to determine the protein content in the crude liquid phases. [28]. Based on a spectrophotometric measured, 5 mL of Bovine Serum Albumin (BSA) at different concentrations (1 to 16 μg/mL) was mixed to 0.5 mL of Bradford reagent to set the standard curve. The determination for samples was on the same way, 0.5 mL of samples or distilled water for blank, were introduced in a test tube and 0.5 mL of Bradford reagent was added, and mixed using a vortex. In both cases, a period of incubation at room temperature for 35 min was necessary, the absorbance was run 595 nm.

2.4.5. Oligosaccharide content, HPLC

Crude liquid samples obtained by MAE were dialyzed with the aim to remove the salt concentration (Spectra/Por Float-A-Lyzer G2 Dialysis Membrane Tubing, MWCO 0.5 kDa, SpectrumLabs, USA). The content of oligosaccharides from the ulvan samples obtained at different temperatures were determined after quantitative posthydrolysis performed with sulfuric acid at 4 % using an autoclave equipment being the operation parameters: 121 °C and 20 min. The samples obtained were filtered through 0.45 μm membranes. The HPLC measurements were conducted on a 1100 series Hewlett-Packard chromatograph, and an infrared (IR) detector was used. The column used was an Aminex HPX-87H column (300 × 7.8 mm, BioRad, Hercules, CA) being the operation conditions 60 °C and the mobile phase: 0.003 M H_2SO_4, at 0.6 mL/min.

2.4.6. Scanning electronic microscopy

A lyophylized crude liquid samples obtained after MAE were fixed in aluminum stubs and coating with gold using a Emitech K550X equipment (Quorum Technologies, UK). The samples were analyzed by scanning electron microscope equipment (FEI Quanta 200, ThermoFisher Scientific, USA) working at 12.5 kV.

2.5. Characterization of Ulvan fraction

2.5.1. Extraction

Ulvans were extracted using both conventional and alternative methods. The ulvan fraction was conventionally obtained from the raw material thanks to an acid extraction with HCl 0.01 M [29]. Acid solution (200 mL) was added to dried *Ulva* spp. (8 g) and heated at 92 °C for 4 h in a water bath. Then, the sample was cooled at room temperature and centrifuged at 3850 ×*g* for 10 min. The liquid phase was collected and precipitated with ethanol 85 % at the ratio 1:1.5 (v/v) and then left overnight at 4 °C. The sample was centrifuged at 3850 ×*g* for 10 min and the supernatant was collected and stored. The solid phase, the ulvan, was dried in an air oven at 50 °C, collected and stored until further characterization.

Alternatively, ulvan was extracted following an adaptation of the recommendations given by Le et al. [30]. Briefly, 10 mL of the liquid phases obtained after MAE extraction was precipitated with ethanol 85 % at different ratio selected after several preliminary tests (1:1.5 and 1:2.5) in a centrifuge tube and left at 4 °C overnight. Subsequently, samples were centrifuged at 6000 ×*g* for 20 min. The supernatant was colected and the solid phases were dried in an air convective oven at 50 °C. The ulvan fraction was weighed to calculate the biopolymer yield, collected and stored until further analyzes. Additionally, a stage of selective coagulation of ulvan stimulated by a bio-ionic liquid was tried. The microwave liquid phase (10 mL) was mixed to choline chloride at different percentages (1 %, 2.5 %, 5 % and 10 %). Ethanol 85 % (15 mL) was added to precipitate the solution and the sample was left at 4 °C overnight. Then, the sample was centrifuged as aforementioned and the ulvan was dried at 50 °C and weighted.

2.5.2. Molecular weight distribution

The distribution of the molecular weight of the samples obtained by MAE were analyzed by High Performance Size Exclusion Chromatography (HPSEC) using a High Performance Liquid Chromatography equipment with a IR detector (Agilent). A column TSKGel SuperMultipore PW-H (6 × 150 mm) with a TSKGel guard column SuperMP (PW)-H (4.6 × 35 mm), both from Tosoh Bioscience (Germany) was used to evaluate the molar mass distribution. Milli-Q water at 0.6 mL/min was used as mobile phase and the standards were Poly(ethylene oxide) from $2.36 \cdot 10^4$ to $7.86 \cdot 10^5$ g/mol (Tosoh Bioscience, Japan).

2.5.3. Fourier-transform infrared spectroscopy

The lyophilized liquid fractions and ulvan fraction were analyzed using Fourier transform infrared attenuated total reflectance, FTIR-ATR, employing an infrared spectrophotometer (Nicolet 6700, Thermo Scientific, USA). Samples were mixed with KBr, pressed and dehydrated using an infrared lamp for 30 min. FTIR-ATR profiles were recorded between 600 and 1800 nm at 30 scan/min employing the OPUS-2.52 software (Opus Software Limited, UK).

2.5.4. Proton Nuclear Magnetic Resonance

The above liquid fractions and ulvan fraction were analyzed by Proton Nuclear Magnetic Resonance, ^{1}H NMR, conducted on a Bruker ARX400 spectrometer (Bruker BioSpin GmbH, Germany). ^{1}H NMR spectra were achieved using 3-(trimethylsilyl)-L-propane sulfonic acid (Sigma-Aldrich, USA) as internal standard and deuterated water as solvent, operating at 400 MHz and 75 °C.

2.5.5. Rheological properties

Initially, aqueous solutions of recovered ulvans (1 % w/w) were pH adjusted to 7.5 and heated up to 75 °C before adding $CaCl_2$ (7 mM) and H_3BO_3 (33 mM) following the adapted procedure previously reported [10]. Viscoelastic properties (elastic modulus, G′, and viscous modulus, G″) of ulvan from *Ulva* spp. were measured on a MCR302 controlled-stress rheometer (Anton Paar, Germany) with a temperature control Peltier system and a sand blasted plate-plate geometry (25 mm diameter, 1 mm gap). Small-amplitude oscillatory shear tests of gelled matrices were made at 25 °C using a solvent trap over the measuring geometry to prevent water evaporation. Measurements were run from 0.1 to 10 Hz within the linear viscoelastic region (<10 Pa) at 5 Pa.

2.6. Statistical analysis

All above experimental measurements were performed at least in triplicate. Data were statistically assessed using one-factor analysis of variance (ANOVA) employing the PASW Statistics v.22 software (IBM SPSS Statistics, New York, USA). Whenever means exhibited differences with 95 % confidence ($p < 0.05$), a Scheffé post hoc test was tried.

3. Results and discussion

A processing overview of *Ulva* spp. green seaweed using microwave hydrothermal treatment to recover high valuable bioactive and gelling fractions is presented in Fig. 1. An intensification step of selective coagulation of ulvan promoted by a bio-ionic liquid was selected to favor the biopolymer extraction yield and to enhance its rheological features.

3.1. Solid phase features

Proximal composition of *Ulva* spp. and the corresponding solid fractions remaining after microwave assisted extraction are summarized in Table 1. Concerning protein content, *Ulva* spp. exhibited values of this compound around 10 %, which is consistent with those previously reported for this specie [31]. Latter authors found protein values for *Ulva* spp. around 9.3 %, whereas other authors reported higher amounts varying between 15 % [32] and 25 % [33]. Comparing with other green

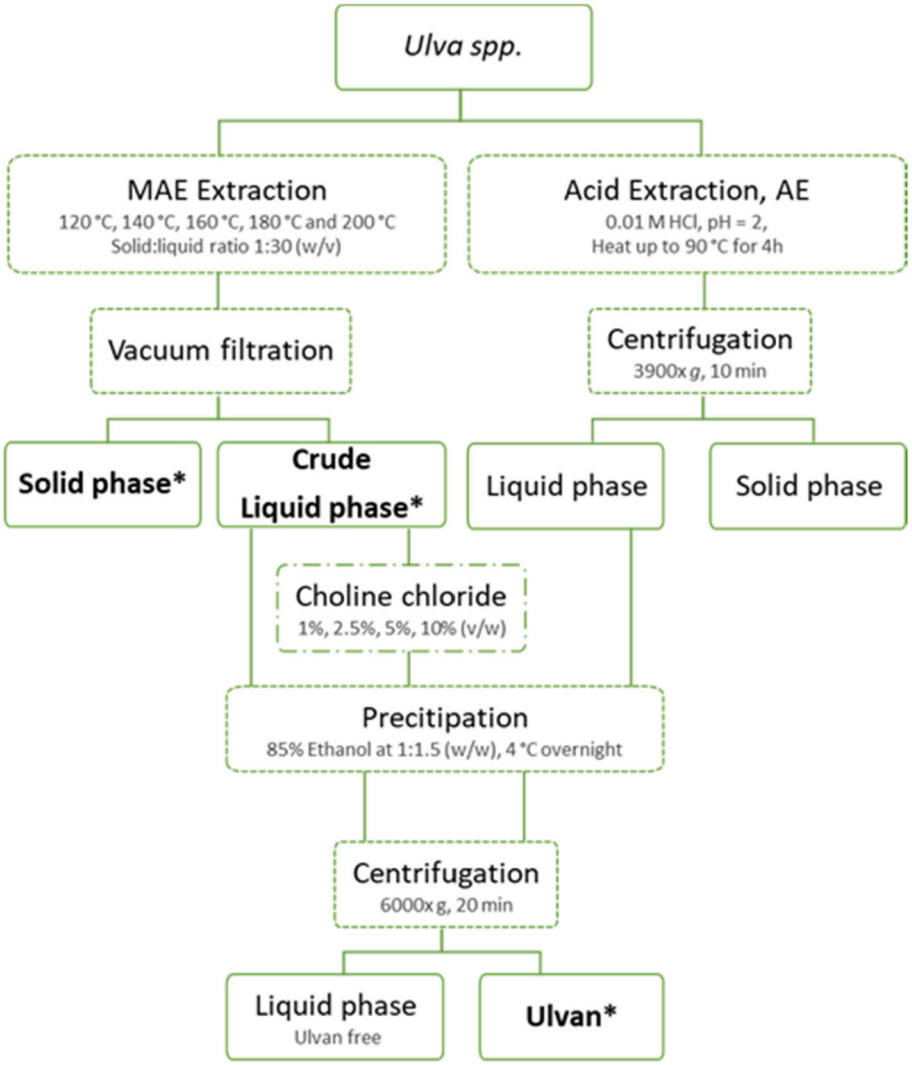

Fig. 1. General scheme of the extraction process for *Ulva* spp. green seaweed. Note here that the characterized fractions were marked with an asterisk.

seaweeds, the protein content ranged from 2.4 for *Ulva pertusa* [34] or 3.4 % for *Halimeda tuna* [35] to 32.3 % for *Ceramium* sp. [36] or 32.7 for *Ulva lactuca* [37]. Ash content is known to be one of the main fractions of green seaweeds, with values varying from 22 to 36 % [38], which is consistent with the results obtained here. Sulfate content identified for tested seaweed was sliglty higher than those previously identified for other green seaweeds, wich ranged between 1.7 and 6.4 % [57]. Carbohydrates of *Ulva* spp. represented around 50 %, being rhamnose, glucose, xylose, glucuronic acid and iduronic acid the major fractions. This is consistent with the behavior previously reported for other Ulva genus, where values between 40 and 70 % were identified [29,39]. *Ulva* spp. exhibited values of acid insoluble residual about 20.21 %. Considerably high mineral values have been identified for tested *Ulva* spp., mainly in terms of magnesium, followed by potassium, sodium and calcium. This is consistent with the results reported for species of the genus *Ulva*, where high concentration of minerals, vitamins and proteins were found, although with notable differences between different species [31].

Table 1 also summarized the chemical composition data relative to the solid phase remaining after microwave-assisted hydrothermal treatment. Protein content increased with increasing processing temperature, whereas sulfate, ash and mineral content decreased with rising of this parameter. Carbohydrate values are in the range of those previously reported, being as expected rhamnose and glucose the major oligosaccharides [29,39]. In general, the rhamnose, fucose, glucuronic acid and iduronic acid content decreased with increasing hydrothermal temperature, whereas glucose and xylose followed the reverse trend. The observed tendencies are consistent with the results previously found for the authors for other solid phases from red [21] and brown [15] seaweeds treated under similar hydrothermal conditions. Microstructural information on the thermal impact of microwave assisted treatment on the residual solid phases is shown in Fig. 2. The surface morphology features of untreated seaweed (Fig. 2a) exhibited entire, compact, regular and smooth microstructure. In all treated solid phases, a shrinkage of the cell wall is observed when compared with untreated samples. This behavior is promoted with increasing operation temperature, being clearly observed in samples treated above 180 °C. These results are consistent with those previously reported for other seaweeds matrices treated at temperatures around 100 °C using different microwave treatments [40].

It should be highlighted that the estimated higher heating value (HHV) of the solid phases rose with increasing processing temperature. Even though, in all cases the magnitude of this parameter is consistent with those found for *Ulva* genus seaweeds (around 10 kJ/kg) [41].

3.2. Liquid phase features

Fig. 3a presents the effect of microwave hydrothermal treatment on

Table 1
Proximal composition of the raw material *Ulva* spp. and the influence of microwave assisted extraction temperature (120 °C, 140 °C, 160 °C, 180 °C and 200 °C) on the composition of the solid fraction and the estimated heating value (HHV).

	Content (%)	*Ulva* spp.	120 °C	140 °C	160 °C	180 °C	200 °C
	Moisture	16.55 ± 0.09	*	*	*	*	*
	Ash	21.21 ± 0.14^{a}	13.70 ± 0.65^{b}	13.76 ± 0.14^{b}	14.33 ± 2.62^{b}	8.83 ± 0.27^{c}	8.28 ± 0.34^{c}
	Protein	10.06 ± 0.31^{e}	12.56 ± 0.12^{d}	14.85 ± 0.36^{c}	18.10 ± 0.28^{b}	17.81 ± 0.27^{b}	21.77 ± 0.70^{a}
	Sulfate content	9.63 ± 0.29^{a}	5.96 ± 0.08^{b}	5.34 ± 0.02^{b}	5.84 ± 0.01^{b}	3.47 ± 0.02^{c}	3.37 ± 0.01^{d}
	Carbon (C)	24.69 ± 0.21^{f}	31.30 ± 0.24^{e}	33.66 ± 0.28^{d}	35.87 ± 0.02^{c}	36.92 ± 0.13^{b}	42.99 ± 0.10^{a}
	Hydrogen (H)	5.39 ± 0.05^{c}	5.66 ± 0.01^{b}	5.74 ± 0.24^{b}	6.01 ± 0.07^{a,b}	6.28 ± 0.08^{a}	6.30 ± 0.17^{a}
	HHV (kJ/kg)	12.08 ± 0.09^{f}	13.59 ± 0.34^{e}	14.28 ± 0.22^{d}	14.95 ± 0.02^{c}	15.20 ± 0.07^{b}	17.56 ± 0.05^{a}
Oligosaccharides (%)	Glucuronic Acid	4.36 ± 0.50^{a}	4.76 ± 0.28^{a}	5.03 ± 0.36^{a}	4.76 ± 0.25^{a}	4.49 ± 0.47^{a}	2.70 ± 0.58^{b}
	Iduronic Acid	6.04 ± 0.33^{a}	5.92 ± 0.12^{a}	5.35 ± 0.22^{b}	4.63 ± 0.13^{c}	2.87 ± 0.16^{d}	1.12 ± 0.11^{e}
	Glucose	14.33 ± 0.94^{b}	5.24 ± 0.05^{d}	4.62 ± 0.14^{e}	4.05 ± 0.18^{f}	6.11 ± 0.31^{c}	25.21 ± 0.27^{a}
	Xylose	5.56 ± 0.42^{e}	8.67 ± 0.24^{b}	8.09 ± 0.11^{c}	7.34 ± 0.10^{d}	8.08 ± 0.18^{c}	12.79 ± 0.21^{a}
	Rhamnose	15.16 ± 1.22^{f}	37.44 ± 0.25^{a}	35.31 ± 0.08^{b}	33.18 ± 0.27^{c}	32.02 ± 0.16^{d}	25.55 ± 0.09^{e}
	Fucose	1.07 ± 0.07^{b}	1.47 ± 0.05^{a}	1.38 ± 0.07^{a}	1.19 ± 0.04^{b}	0.87 ± 0.13^{c}	0.82 ± 0.08^{c}
Macroelements (mg/kg)	Magnesium (Mg)	19,838	14,348	13,594	12,068	9808	5406
	Potassium (K)	10,934	4405	4571	4216	3379	2510
	Sodium (Na)	6689	3192	3175	3015	2332	1727
	Calcium (C)	3480	3225	3889	3111	2568	2510
	Iron (Fe)	332.2	463.0	495.8	647.3	788.1	1289.6
	Boron (B)	54.4	24.4	21.2	21.6	17.7	15.8
Microelements (μg/kg)	Copper (Cu)	2104.5	3261.7	3928.7	5226.9	6450.8	10,841.6
	Arsenic (As)	2325.1	2172.1	2521.2	3206.1	3623.0	5280.5
	Lead (Pb)	711.2	1075.8	1293.0	1855.9	2314.1	2284.0
	Cadmium (Cd)	88.9	110.5	126.7	156.1	169.3	114.9
	Mercury (Hg)	11.6	15.3	15.4	19.2	21.8	32.6

Data are given as mean ± standard deviation, except for micro- and macro- elements where standard deviations were <5 % in all cases. Data values in a row with different superscript letters are statically different ($\rho \leq 0.05$).

* Moisture content of the residual solids was in all cases below 10 %.

the sulfate and protein content determined in the recovered crude liquid phases and expressed as g/100 g extract. Maximum values of both parameters were identified in the crude soluble extracts obtained at 160 °C and they drastically dropped above that temperature. Note here that the sulfate values presented in Fig. 3a expressed as g/100 g raw material corresponded with values varying between 9.1 and 12.1 %. These results agree with those reported for the production of sulfated polysaccharides from *Ulva* genus seaweeds such as *Ulva meridionalis* and *Ulva ohnoi*, where hydrothermal processing at 160 °C also led to the highest yields corresponding with the highest solubilization rates [42]. Latter authors also found relatively low protein content, varying between 2.3 and 5.9 %. Similarly, protein content between 1.95 and 3.15 % was identified for *Ulva prolifera* [43]. Both authors related these magnitudes with a higher purity of the extracted polysaccharides.

The antioxidant features of the crude soluble extracts were also notably dependent on the thermal conditions of the microwave processing of *Ulva* spp. (Fig. 3b and c). Total phenolic content and antioxidant activity drastically increased with increasing temperature, achieving the maximum values at 200 °C. A parallel trend was observed for DPPH radical scavenging measurements. The highest scavenging effect was identified at the most severe processing conditions, the rest of the temperatures led to inhabitation percentages lower than 50 %. This behavior was previously reported for *Ulva prolifera* treated under milder hydrothermal conditions [43], exhibiting the highest antioxidant activity at the highest tested temperature (150 °C). Xu et al. [44] also demonstrated that degraded polysaccharides from *Ulva prolifera* featured stronger DPPH radical scavenging ability than non-degraded ones.

Fig. 3d shows the influence of microwave processing temperature on the oligosaccharide fraction of the corresponding crude soluble extracts. The main differences observed in the oligosaccharides content were observed above 180 °C. The most enhanced increase was identified for glucose at 200 °C, being the major oligosaccharide at the highest tested temperature. Glucose and xylose content increased with increasing microwave temperature, whereas rhamnose and glucuronic acid decreased. Tsubaki et al. [42] suggested that the glucose rise with increasing processing temperature indicates promoting of starch solubilization at higher temperature. These authors also stated that the extracted polysaccharides from *Ulva species* are almost stable until 160 °C starting to degrade at 180 °C. Total oligosaccharide content between 31.3 % and 84 % were found for hydrothermal treated *Ulva prolifera* over the temperature range of 150 and 90 °C [43].

High pressure size exclusion chromatography spectra of crude soluble extracts from microwave hydrothermal treatment as well as the ulvans extracted after conventional and alternative treatments are displayed in Fig. 4. In all cases, a single peak was identified independently on the processing temperature. In general, the observed peak was shifted to lower average molecular weight with increasing microwave temperature (Fig. 4a). A relevant peak was detected above 786,000 g/mol for soluble extracts from *Ulva* spp. hydrothermal treated at the mildest processing conditions (120 and 140 °C), indicating the influence of the extraction temperature on the decrease of molecular weight. Bands around 580,000 and 277,000 g/mol were identified for those from seaweeds treated at 160 °C and around 107,000 and 580,000 g/mol for samples processed at 180 and 200 °C, respectively. These tendencies are consistent with those previously identified for other *Ulva* genus hydrothermally treated [42]. Latter authors found that the molecular weight distribution of extracts from *Ulva meridionalis* did not change between 100 and 140 °C, starting to decrease above 160 °C. The observed trends suggested that the increase in processing temperature favor the hydrolysis of the ether bonds on the polymeric chain leading to the drop of the polysaccharide molecular weight [43].

The corresponding Fourier transform infrared spectroscopy spectra of above crude soluble extracts are collected in Fig. 4d. Similar profiles with characteristic infrared spectra of green seaweed extracts were identified in all cases [45]. All profiles exhibited strong bands at around 1090 and 1630 cm^{-1}, and weak ones around 850 and 1430 cm^{-1}. The strongest signal at about 1090 cm^{-1} was attributed to C—O stretching from two main sugars, rhamnose and glucuronic acid. The signal at 1630 cm^{-1} was linked to the two carboxylate groups (C═O) indicating an asymmetric stretching band of uronic acid and a weaker symmetric stretching band at 1430 cm^{-1} [46]. The signals at 850 cm^{-1} corresponded the C—O—S bending vibration of the sulfate ester in the axial position [29], decreasing the magnitude of the band for soluble extracts

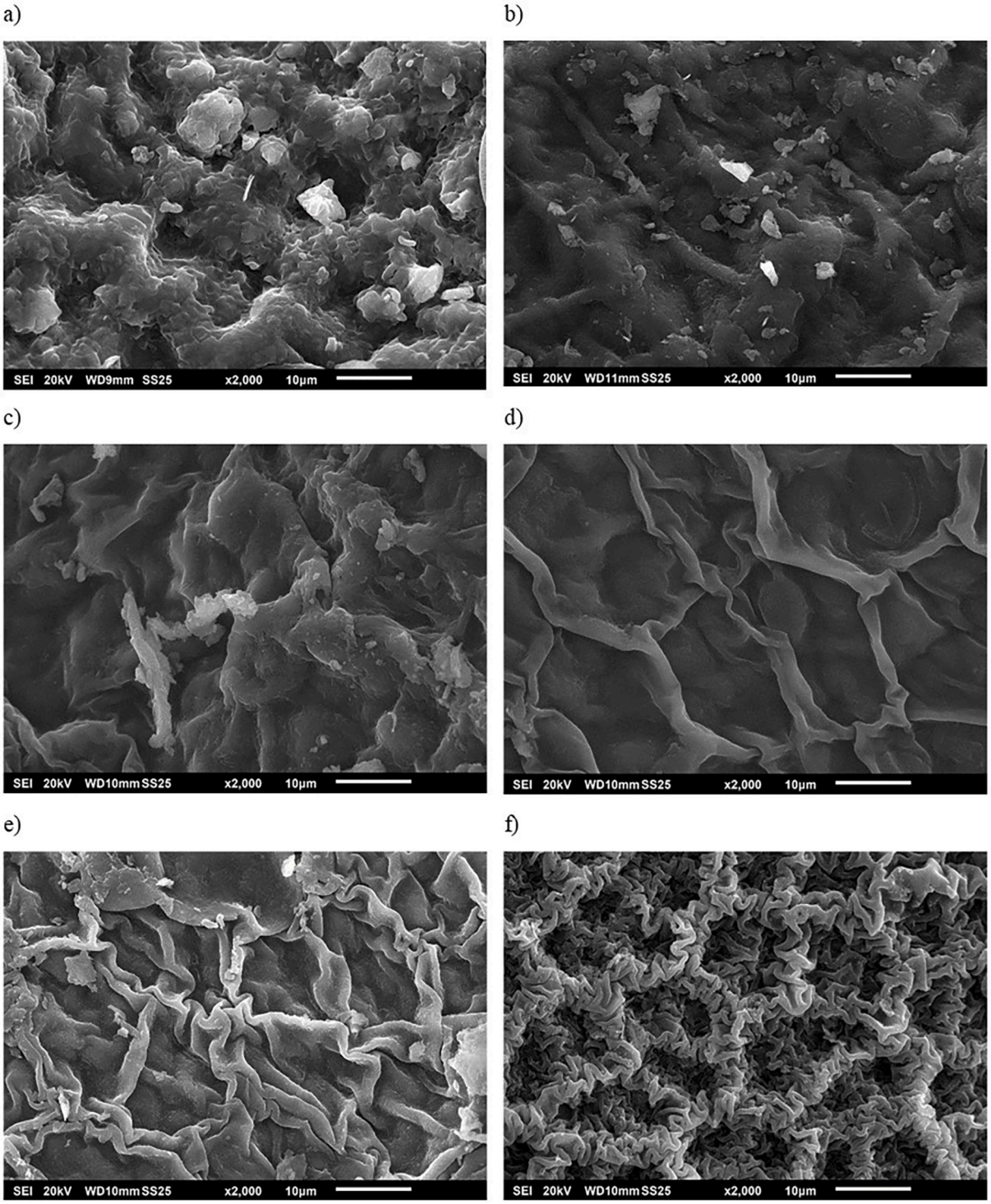

Fig. 2. SEM micrographs showing *Ulva* spp. raw material (a) and the corresponding solid phases after microwave-assisted hydrothermal treatment at (b) 120 °C, (c) 140 °C, (d) 160 °C, (e) 180 °C and (f) 200 °C.

from seaweeds treated at 200 °C.

3.3. Ulvan fraction

The yield of ulvan extracted from *Ulvan* spp. using the conventional acid method was around 30 % (Table 2). Lower extraction yields of ulvan for other Ulva species as *Ulva lactuca* (around 18 %) using acid extraction were previously reported and dropped to 11 % when water extraction was performed [29]. Here, comparable yields (around 30 %) were identified for ulvans isolated from the microwave hydrothermal liquid phases using in the precipitation 1.5 volumes of ethanol, independently of the microwave processing thermal conditions. Higher ethanol volumes involved lower biopolymer yields, which suggests that there is a maximum amount of ethanol from which the medium is saturated without promoting more biopolymer extraction [1]. Even though, Le et al. [30] found ulvan yields around 25.23 % for *Ulva pertusa* processed by microwave hydrothermal treatment, higher biopolymer yield (around 35 %) was identified for samples microwave treated at

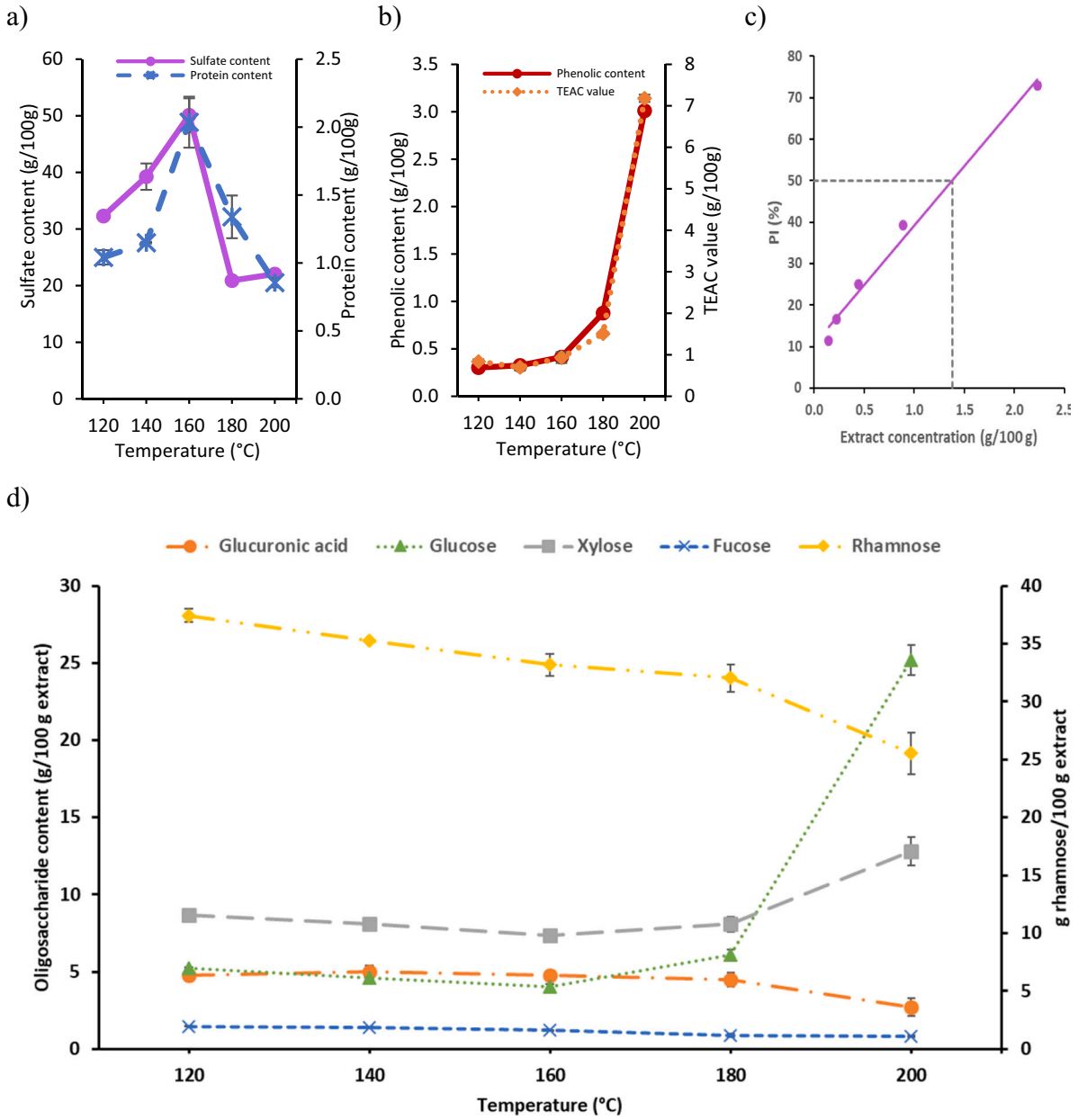

Fig. 3. Influence of the microwave assisted extraction temperature on (a) sulfate and protein content, (b) phenolic content and TEAC value, (c) DPPH and (d) oligosaccharide content in the crude liquid phase. Note here that panel c corresponds to sample hydrothermally treated at 200 °C.

600 W for longer time (45 min). It should be noted that the presence of the choline chloride favored the ulvan yield, with values around 32 % for tested *Ulva* spp. Das et al. [17] also found a polysaccharide yield increase using deep eutectic solvents during extraction treatment of other marine biopolymers as k-carrageenan from *Kappaphycus alvarezii* red seaweeds.

3.3.1. Structural characteristics

Fig. 4 displays the molecular weight distribution of ulvans extracted from microwave liquid phases processed in the (b) absence and (c) presence of choline chloride. For comparative purposes, the ulvan isolated after conventional acid extraction was also displayed. HPSEC profiles indicated that most of the tested ulvans presented two peaks, except those extracted after the most severe microwave thermal conditions or conventional acid extraction, where only a single peak in the region of lower molecular weights was identified. Ulvan extracted under acid conditions exhibited consistent results with those reported in a previous work [10], where an acidic extraction with sulfuric acid was employed with different *Ulva* species to extract the ulvan. The presence at different molecular weights of two peaks for ulvan from *Ulva meridionalis* (400 and 800 kDa) and one peak for *Ulva ohnoi* (2–3 kDa) was previously reported [42]. Other authors [47,48] indicated that drastic extractions as the acid ones triggers the degradation of high molecular weight fractions into low molecular weight compounds, favoring a disruption of the biopolymer chains. Microwave thermal processing conditions notably impacted the molecular weight distribution of the recovered ulvans, both in the absence and presence of choline chloride. Similar profiles were observed in both cases, although ulvans extracted in the presence of the bio-ionic liquid exhibited the peak of higher molecular weights shifted to the left. In both systems, the most prominent profile was identified for ulvans from *Ulva* spp. microwave hydrothermal treated at 160 °C. The broad molecular weight distributions of ulvan can be linked to the self-aggregation behavior of this biopolymer in aqueous solution, as previously suggested for ulvans from

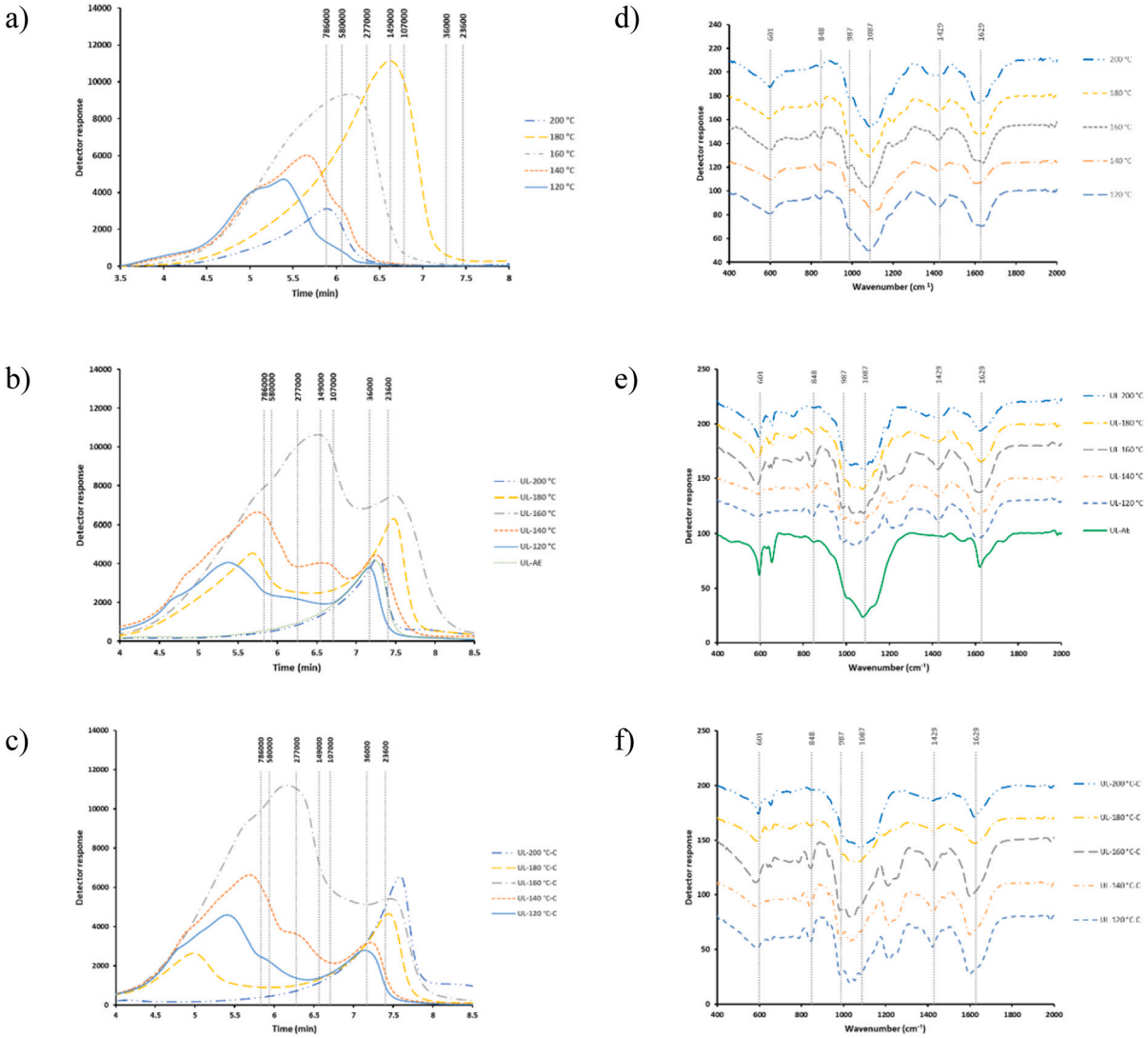

Fig. 4. High-performance size exclusion chromatography profiles from (a) microwave hydrothermal extraction at different temperatures and the ulvan fraction of these liquors obtained in the (b) absence and (c) presence of choline chloride, as well as the corresponding (d, e, f) Fourier transform infrared spectra, respectively.

Table 2
Extraction yield (%) of ulvan from the green seaweed *Ulva* spp. at different temperatures. Conventional acid extraction and MAE hydrothermal extraction precipitated with ethanol (1:1.5, 1:2.5), and in the presence of choline chloride (ChCl) at 1 %.

Yield (%)	Seaweed	120 °C	140 °C	160 °C	180 °C	200 °C
Acid extraction	30.36 ± 0.31	–	–	–	–	–
$MAE_{[liquid\ phase/EtOH,\ 1:1.5]}$		30.48 ± 0.33^{b}	30.46 ± 0.22^{b}	30.66 ± 0.22^{b}	30.70 ± 0.13^{b}	30.66 ± 0.32^{b}
$MAE_{[liquid\ phase\ /EtOH,\ 1:2.5]}$		23.74 ± 0.32^{d}	25.42 ± 0.25^{d}	24.13 ± 0.41^{d}	23.99 ± 0.24^{d}	23.90 ± 0.54^{d}
$MAE_{[liquid\ phase/EtOH,\ 1:1.5,\ ChCl]}$		32.52 ± 0.10^{a}	32.10 ± 0.15^{a}	32.16 ± 0.21^{a}	31.97 ± 0.12^{a}	31.88 ± 0.15^{a}
$MAE_{[liquid\ phase/EtOH,\ 1:2.5,\ ChCl]}$		24.71 ± 0.15^{c}	26.32 ± 0.11^{c}	25.85 ± 0.18^{c}	25.24 ± 0.05^{c}	25.28 ± 0.10^{c}

Data are given as mean ± standard deviation. Data values in a column with different superscript letters are statically different ($\rho \leq 0.05$).

Ulva lactuca, which will be a key factor for their rheological properties [47].

Fig. 4 shows the FTIR spectra of above ulvan samples recovered in the (e) absence and (f) presence of the tested bio-ionic liquid. Similar profiles were observed in all cases independently of the hydrothermal treatment. The main differences were identified with ulvan from acid extraction where a more prominent band of the C—O stretching from two main sugars was noticed at 1090 cm^{-1}. For ulvans from *Ulva* spp. microwave treated below 160 °C, also these signals and that noticed at 1630 cm^{-1} suggesting an asymmetric stretching band of uronic acid, was more pronounced in the presence of the bio-ionic liquid. FTIR spectra of *Ulva* spp. determined here were consistent with those structural analyses previously reported for ulvans from a number *Ulva* species such as *Ulva intestinalis* and *Ulva rigida*, or a commercial one from *Ulva armoricana* [45]. Latter authors also found that ulvans are complex biopolymers exhibiting a band at 1250 cm^{-1} corresponding to S═O stretching vibrations. This signal was also observed here for ulvans from *Ulva* spp. microwave hydrothermal treated below 160 °C.

Fig. 5 presents the impact of the extraction conditions on the corresponding ^{1}H NMR spectra of aforementioned ulvans. In all cases, the peaks of the ring protons were observed over the range of 3.25 to 4.25 ppm and a signal around 1.25 ppm linked to the methyl proton of the α-L-rhamnosyl residues was also detected as previously reported for

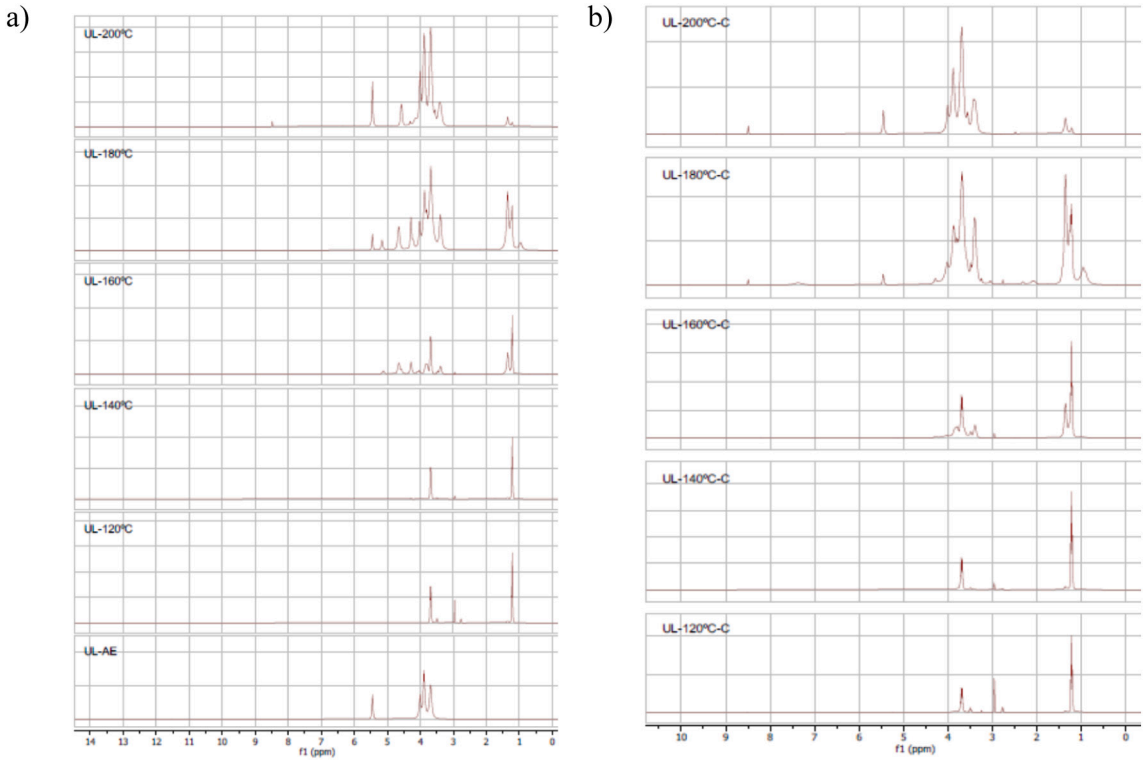

Fig. 5. Proton nuclear magnetic resonance spectra for ulvans from microwave hydrothermal liquid phases in the (a) absence and (b) presence of bio-ionic liquid. Ulvan from acid extraction (AE) is also included in plot (a) with comparative purposes.

ulvans from *Ulva fasciata* [49–51]. Latter authors also identified the *O*-acetyl group in some ulvans at 2.49 ppm, suggesting it as a part of the polysaccharide structure or impurities. This signal was not found here for ulvans from *Ulva* spp. In general, the main identified signals were more intensified for ulvans extracted in the presence of bio-ionic liquid. Sari-Chmayssem et al. [52] explained the complexity of these profiles splitting different minor signals over the range of 3.25 to 5.25 ppm, which were related to the hybrid nature of these biopolymers, rich in rhamnose and uronic acids, and containing small amounts of glucose or xylose. The structural complexity of ulvans extracted from blade (*Ulva ohnoi*) and filamentous (*Ulva tepida* and *Ulva prolifera*) Ulva species was also corroborated by Glasson et al. [53].

3.3.2. *Rheological characteristics*

The effect of extraction conditions on the viscoelastic features of the recovered ulvans is shown in Fig. 6. The biopolymer directly isolated from the seaweed under conventional acid conditions is also presented with comparative purposes. All gelled matrices featured characteristic gel profiles with the elastic modulus, G′, larger than the viscous modulus, G″, over the tested angular frequency range, and both viscoelastic moduli almost frequency invariant as reported elsewhere [54]. Ulvans from conventional acid extraction showed lower elastic and viscous moduli values when compared with those from hydrothermal treatments (Fig. 6a). This rheological behavior suggests that the conventional acid extraction conditions could be too harsh for the recovery of a carbohydrate biopolymer α- and β-(1,4)-linked monosaccharides, as it may induce the hydrolysis of the α- or β-(1,4)-linkage in the ulvan and consequently the drop on the viscoelastic features. Those biopolymers obtained after microwave processing at 160 °C exhibited the highest values (around 3.5 fold) of both moduli. Even though, the magnitude of both moduli indicated weak gel properties, as previously reported [42]. The viscoelastic profiles observed for the ulvan extracted after the microwave assisted treatment at the highest tested temperatures (i.e. 180 and 200 °C) suggested that these hydrothermal treatments can involve a thermal scission of the α- or β-(1,4)-linkage, decreasing the average molecular weights and consequently the viscoelastic strength of the gelled matrices.

The strength of the gels was markedly improved for ulvans extracted in the presence of the bio-ionic liquid (Fig. 6b). The values of the viscous and elastic moduli of these samples were doubled when compared with those in the absence of choline chloride, and around 7-fold higher than those of the ulvans from the conventional acid method. This behavior involved a notably improvement of the viscoelastic properties of ulvans regarding to those found in the literature from either conventional or hydrothermal treatments (G′: around 20 Pa, G″, around: 1 Pa) at the same biopolymer content [42,52]. Note here that proposed gels did not present water release after two cold storage weeks.

Fig. 6c presents an overview of the viscoelastic characteristics, in term of $G'_{0(1Hz)}$, of all the ulvans studied. It can be clearly observed a maximum of the viscoelastic features at 160 °C, more prominent in the presence of the bionic liquid as aforementioned. Sari-Chmayssem et al. [52] suggested that the elastic modulus of ulvans gels from *Ulva linza* is directly proportional to their molar weight well as to the quantity of rhamnose, glucuronic acid, or sulfate, which was also corroborated here for *Ulva* spp. Kidgell et al. [10] reported a high variability in the physicochemical and consequently mechanical properties of ulvans from different *Ulva* sources. Those from blade species featured lower molecular weight (190–254 kDa) and elastic modulus (G′: 0.1–6.6 Pa) than filamentous ones with higher values (260–406 kDa, G′: 22.7–74.2 Pa). Morelli et al. [55] proposed ulvan based thermosensitive gels (>3 %) for biomedical applications, since they found solutions at 25 °C that were converted in weak gels at 34 °C and the development of stable gels at 37 °C. These outcomes again show the complexity of these biopolymers, and the necessity of further insight on the interplay between chemical, structural and thermorheological behavior.

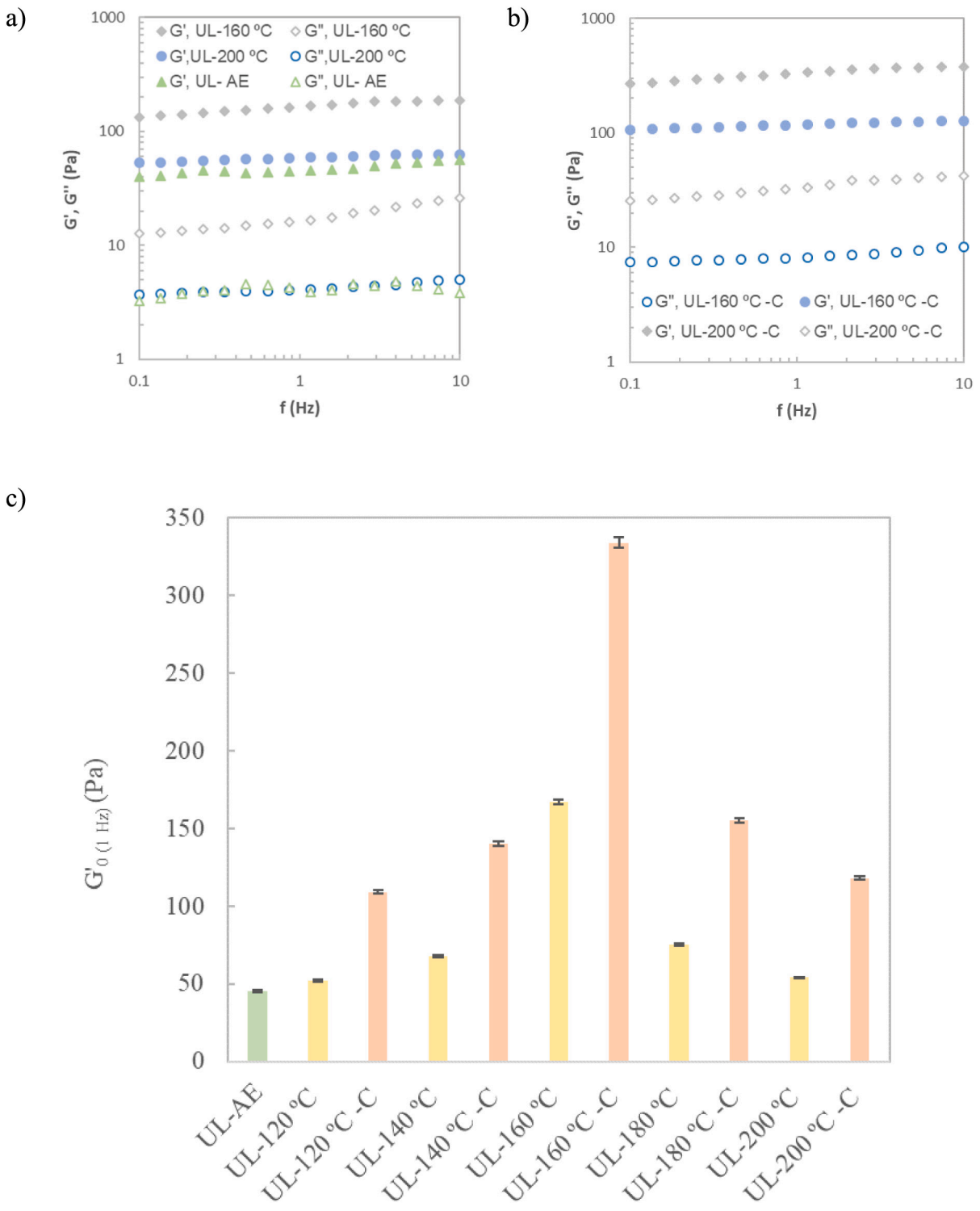

Fig. 6. Viscoelastic properties of representative ulvans extracted after microwave hydrothermal treatment from liquid phases in the (a) absence and (b) presence of bio-ionic liquid. For comparative purposes, ulvan from acid extraction (AE) is also included in plot (a). The global effect of extraction conditions on the viscoelastic features of all extracted ulvans is presented in plot (c).

4. Conclusions

To conclude, microwave-assisted hydrothermal processing of *Ulvan* spp. green seaweed can be an attractive alternative to extract a palette of soluble extracts with antioxidant features with potential interest in the growing market of healthy natural extracts for food, cosmetic or nutraceutical applications. A novelty stage of selective coagulation of ulvan stimulated by a bio-ionic liquid led to biopolymers with enhanced viscoelastic characteristics, with the consequent advantage from the industrial point of view. Following a biorefinery framework, the phytochemical potential of the solid residues could be also further exploited. In general, the obtained outcomes involve a global valorization of this green alga that could be applied to other macroalgae with feasible food and non-food uses.

CRediT authorship contribution statement

Conceptualization, H.D. and M.D.T; methodology, N.F.F., M.D.T and H.D.; investigation, J.A., N.F.F, and M.D.T; data curation, J.A., N.F.F. and M.D.T.; writing - original draft preparation, N.F.F., M.D.T. and H.D.; writing - review and editing, N.F.F., M.D.T. and H.D.; funding acquisition, M.D.T. and H.D.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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