GREEN EXTRACTION AND CHARACTERISATION OF ULVAN FROM RESIDUAL ULVA RIGIDA ALGA RECOVERED FROM ORBETELLO LAGOON

Nicola Di Fidio[a], Sara Fulignati[a], Martina Vento[b], Laura Pistelli[b], Alberto Pardossi[b], Anna Maria Raspolli Galletti[a]
[a]Department of Chemistry and Industrial Chemistry, University of Pisa, Via G. Moruzzi 13, Pisa, Italy
[b]Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, Pisa, Italy
E-mail: nicola.difidio@unipi.it

ABSTRACT: *Ulva* species have high growth rates across different geo-climatic conditions, thus they can provoke the formation of problematic "green tides", related to the eutrophication phenomena. To solve this problem, a large amount of algal biomass is currently removed, representing a kind of waste often disposed of in landfill or incinerators. However, *Ulva* has a highly exploitable chemical composition and its major product of interest is ulvan. This has promising applications in biomaterial science, nutraceuticals, functional foods and agriculture. In the present study, *Ulva rigida* recovered from the Orbetello lagoon (Tuscany, Italy) was characterised in terms of biomass particle size, chemical composition, elemental analysis, thermogravimetric analysis and infrared spectroscopy. The effect of the main extraction reaction parameters, such as temperature (60–120 °C), pH (1–7), reaction time (30–150 min) and type of heating system (traditional, microwaves and ultrasounds), was investigated to maximise the ulvan extraction yield and purity. Under the optimised extraction conditions under traditional heating (120 °C, pH 7, 90 min, 50 g/L biomass loading), the ulvan yield of 77 wt% with respect to the total sugars content and of 27 wt% with respect to the alga dry weight were achieved. Finally, the extracted ulvan was characterised and its chemical composition confirmed the high purity having a low content of proteins and ash.
Keywords: Algae, *Ulva rigida*, Bio-based economy, Biorefinery, Green Chemistry, Ulvan.

1 INTRODUCTION

In recent years, the replacement of fossil resources with renewable ones has received great interest, especially as regards the production of new valuable bio-products [1]. The necessity of using renewable resources for producing consumer goods is increasing and more and more carbon-neutral products are becoming available on the market, thus avoiding unbalanced Green House Gases (GHG) emissions that are considered responsible for global warming, climate change and other environmental issues [2].

In this context, the exploitation of waste biomasses into added-value bio-chemicals is strongly encouraged [3-5]. Among the waste biomasses, micro- and macroalgae are attracting considerable attention, in particular macroalgae, because they cause eutrophication problems in estuaries and lagoons, due to the drastic reduction of dissolved oxygen during their decomposition. This problematic situation characterises the Orbetello lagoon (Tuscany, Italy), a coastal shallow basin that covers a surface of about 25 km^2 that receives treated urban and land-based fish farms wastewater but is characterised by a low water turnover [6]. In order to solve eutrophication problems, a large amount of algal biomass is currently removed every year from the lagoon (ca. 700-1000 tons per year) through an expensive practice and with consequent environmentally serious disposal problems, thus the algae exploitation could partly resolve this environmental issue. This residual algal biomass is mainly composed of species of green seaweed from the genus *Ulva* that have high growth rates and productivities across different geo-climatic conditions, with a highly exploitable chemical composition [7]. The species of algae collected in Orbetello is *Ulva rigida* which grows spontaneously in that site [8,9]. One of the major bio-products of interest from *Ulva* species is ulvan. This is a cell wall sulphated polysaccharide that contributes from 9 to 36 wt% of the dry biomass and it is mainly composed of sulphated rhamnose, glucuronic acid, glucose and xylose. However, the most abundant sugar in *Ulva rigida* is represented by glucose due to its presence also in the other cell wall polysaccharides, namely cellulose, xyloglucan and glucuronan. Cellulose is composed of glucose; xyloglucan contains both xylose and glucose units, while glucuronan contains glucuronic acid. Overall, these four polysaccharides account for up to 40-50 wt% of the dry biomass.

Ulvan has promising industrial applications in bio-material science (wound dressings, tissue engineering, bio-film prevention, excipients), nutraceuticals (antiviral, antioxidant, anticancer and immunostimulatory), functional foods and agriculture [11]. However, since ulvan is a complex polymer associated with proteins and the other cell-wall components of *Ulva*, the ulvan extraction procedure is a critical point, especially in terms of economic and environmental sustainability, in the perspective of its industrial scale-up. Therefore, the extraction method and the downstream approach need to be optimised in order to maximise the ulvan extraction yield and purity. In particular, the extraction yield of ulvan is affected by several parameters such as properties and particle size of the biomass, temperature, nature of the solvent, biomass loading, pH, extraction time and heating system. Moreover, these parameters can affect the properties of the extracted ulvan in terms of molecular weight, sugar composition and sulphation ratio, all of them influencing the biological properties and possible applications. In particular, the sulphate group in ulvan plays an important role, and the degree of sulphation is usually positively correlated with ulvan bio-activities, especially with its antioxidant activity [11].

Based on these considerations, in the present work, a green and efficient extraction method was developed for the obtaining of almost pure ulvan. In this perspective, *Ulva rigida* was firstly characterised in terms of biomass particle size, chemical composition, elemental analysis (EA), thermogravimetric analysis (TGA), and infrared spectroscopy (ATR-FTIR). Then, the effect of the main reaction parameters, such as temperature (60–120 °C), pH (1–7), reaction time (30–150 min) and type of heating system (traditional, microwaves and ultrasounds), was investigated and optimised in order to maximise the ulvan yield and purity (in terms of minimal protein and ash content). In particular, water was used as the green

solvent and hydrochloric acid (HCl) was tested as the low-cost homogenous catalyst. Moreover, the effect of diverse approaches for purification and drying of extracted ulvan was investigated. In particular, the impact of the dialysis step as well as the comparison between lyophilisation and vacuum drying were investigated. After the optimisation of the extraction process, purification and drying steps, the extracted ulvan was characterised by EA, TGA, ATR-FTIR, and optical microscopy. Its chemical composition was also determined, underlining the low content of proteins and ash, thus confirming that the extracted ulvan could have broad spectrum applications as a nutraceutical in aquaculture and as an antimicrobial agent in plant cultivation [11,12].

2 MATERIAL AND METHODS

2.1 Biomass and materials

Ulva rigida biomass was collected from Orbetello lagoon (latitude 42° 25' 56" N; longitude 11° 9' 39" E, Tuscany, Italy). After the harvesting, the alga was washed with deionized water in order to remove salts, dried and ground in 1.0 mm average-size particles. Then, it was stored in a desiccator up to its use. Unless otherwise specified, all the chemicals of analytical purity grade were provided by Sigma-Aldrich.

2.2 Determination of chemical composition

Analysis of the chemical composition of *Ulva rigida* was performed according to the standard NREL methodologies [13-17]. Briefly, the starting removal of organic extractives was performed using a Soxhlet apparatus, keeping the temperature at 85 °C to obtain a constant reflux of absolute ethanol in the extraction chamber. The extraction process was carried out for 18 h using 2 g of sample and 200 mL of absolute ethanol. After that, for the quantification of the total sugars content and the sugars profile, 0.3 g of the sample was resuspended in 3 mL of H_2SO_4 72 wt% solution. The first hydrolysis was performed at 30 °C for 1 h in a water bath under magnetic stirring. At the end of this reaction, the acid solution was diluted with 84 mL of deionised water up to the H_2SO_4 concentration of 4 wt%. Then, the second acid hydrolysis was performed at 121 °C for 1 h in an autoclave. Finally, the solid residue was filtered on a Whatman glass microfiber filter (grade GF/A), and dried in an oven at 105 °C until constant weight. The liquid fraction was analysed by High Performance Liquid Chromatography (HPLC) for sugars identification and quantification. Each analysis was performed in triplicate. Values represent the mean, n = 3, ± standard deviation.

The ash content of *Ulva rigida* was determined with a muffle furnace model Hulk MSW-Z51 at 550 °C for 8 h [13]. All the determinations were done in triplicate.

2.3 HPLC analysis

The qualitative and quantitative characterisation of sugars (glucose, xylose, arabinose, rhamnose, cellobiose, glucuronic acid) was performed by using HPLC PerkinElmer Flexar Isocratic Platform equipped with a refractive index detector and a Benson 2000-0 BP-OA column (7.8 mm × 300 mm × 10 µm). 20 µL of the micro-filtered sample were analysed at 60 °C employing a 0.5 mM H_2SO_4 solution as mobile-phase with the flow rate of 0.4 mL/min. The determination of all compounds was carried out by the external standard method by using commercial standards. At least three replicates for each concentration of standards and samples were carried out. The reproducibility of the technique was within 3%.

2.4 Ulvan extraction

2.4.1 Traditional heating extraction

For the extraction and quantification of ulvan, 1.5 g of dried alga, 30 mL of deionized water (biomass loading 50 g/L) and eventually HCl were loaded in a pressure tube and heated in an oil bath. At the end of the hydrothermal treatment, the aqueous mixture was cooled down to room temperature and filtered through a ceramic crucible. Extracted ulvan was then precipitated by the addition of 120 mL of absolute ethanol (water/ethanol ratio 1/4 v/v) according to the literature [18]. The resulting suspension was left at 4 °C for 24 h and then centrifuged at 4000 rpm for 5 min. The recovered precipitate (ulvan) was dried in an oven under vacuum at 45 °C until constant weight in order to obtain ulvan as a white powder. The sugars profile of ulvan samples was determined through the standard NREL methodologies, previously described in Section 2.2.

2.4.2 Microwave-assisted extraction

Microwave-assisted extraction was performed by using a monomodal microwave reactor CEM Discover S-class System. For the extraction of ulvan, 1.0 g of dried alga was suspended in 20 mL of deionized water (biomass loading 50 g/L) and heated at 120 °C for 45 min. At the end of the reaction, the vessel was rapidly cooled at room temperature through an external airflow. Then the liquid fraction containing ulvan was recovered by filtration under vacuum on a ceramic crucible and treated as described in Section 2.4.1.

2.4.3. Ultrasound-assisted extraction

Ultrasound-assisted extraction was performed by using an ultrasonicator UP400St. For the extraction of ulvan, 15.0 g of dried alga was suspended in 300 mL of deionized water (biomass loading 50 g/L) and treated for 45 min at 60 °C with a power of 60 W at an amplitude of 100%. At the end of the reaction, the liquid fraction was recovered by filtration under vacuum on a ceramic crucible and treated as described in Section 2.4.1.

2.5 Elemental analysis

Elemental analysis (C, H, N, S) of *Ulva rigida* and extracted ulvan was performed by the automatic analyser Elementar Vario MICRO Cube (Elementar). The elements were quantified by a thermal conductivity detector (TCD). Oxygen content was calculated by difference, according to the following equation:

$$O\ (\%) = 100\ (\%) - C\ (\%) - H\ (\%) - N\ (\%) - S\ (\%)$$

The protein content (PC) of *Ulva rigida* and ulvan samples was determined through the standard equation based on the nitrogen conversion factor reported in the literature for algae [13]:

$$PC\ (\%) = N\ (\%) \times 4.78$$

The amount of sulphate groups (SG) in the extracted ulvan was calculated according to the following equation:

$SG\ (wt\%) = (S\ (\%) / AW_S) \times MW_{SO4}$

where AWs is the atomic weight of sulphur while MW_{SO4} is the molecular weight of sulphate moiety of sulphated sugars.

2.6 Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR was performed by the Perkin–Elmer Spectrum Two spectrophotometer equipped with an attenuated total reflectance apparatus. In all the analyses, the wavenumber ranged from 4000 to 450 cm^{-1} with a resolution of 8 cm^{-1}. 24 scans were acquired for each spectrum.

2.7 Optical microscopy

Optical microscopy monitoring of ulvan was performed by the Stereo Discovery V8Carl Zeiss™ microscope, with a manual 8× zoom.

2.8 Thermogravimetric analysis (TGA)

The TGA of *Ulva rigida* and ulvan was evaluated using a thermogravimetric analyser (TGA Q500, TA Instrument). 10 ± 1 mg of sample was weighed in an alumina crucible and heat-treated through the heating rate of 10 °C/min from 30 to 900 °C under a constant flow (60 mL/min) of N_2 (100% v/v).

3 RESULTS AND DISCUSSION

3.1 Characterisation of *Ulva rigida*

Ulva rigida recovered from the Orbetello lagoon and adopted as starting raw material in the present study was firstly chemically characterised. Its chemical composition (wt% on the dry matter) was reported in Table I.

Table I: Chemical composition of *Ulva rigida* harvested from Orbetello lagoon.

Fraction	wt% on dry matter
Total sugars content	34.8 ± 1.5
Protein	14.8 ± 0.2
Extractives in ethanol	21.8 ± 0.5
Ash	22.0 ± 0.5

The obtained composition agreed with those reported in the literature for the same algal species [19].
The elemental profile was reported in Table II and it is analogous to the common profile reported in the literature for this species [20].

Table II: Elemental analysis of *Ulva rigida*.

Element	wt% on dry matter
C	26.2
H	5.2
N	3.1
S	4.5
O	61.0

The sugars profile of *Ulva rigida* is shown in Table III. The most abundant sugar was represented by glucose due to its presence in the cell wall polysaccharides as well as in the ulvan. The other abundant sugars (rhamnose, xylose and glucuronic acid) constitute the hetero-structure of ulvan [7].

Table III: Chemical profile of total sugars content (TSC) in *Ulva rigida*.

Fraction	wt% on TSC
Glucose	60.3 ± 0.5
Xylose	10.0 ± 0.2
Rhamnose	19.0 ± 0.4
Glucuronic acid	9.2 ± 0.4
Arabinose	1.5 ± 0.1

This sugar profile agreed with those reported in the literature for the same species of green seaweed [20]. In fact, species of *Ulva* contain four cell wall polysaccharides represented by ulvan, cellulose, xyloglucan and glucuronan. Overall, these four polysaccharides account for up to 40 wt% of the dry biomass weight, in agreement with the TSC of around 35 wt% achieved for the samples recovered from the Orbetello lagoon.

The curves of thermogravimetric analysis (TGA) and derivative thermogravimetry (DTG) of *Ulva rigida* were shown in Figure 1.

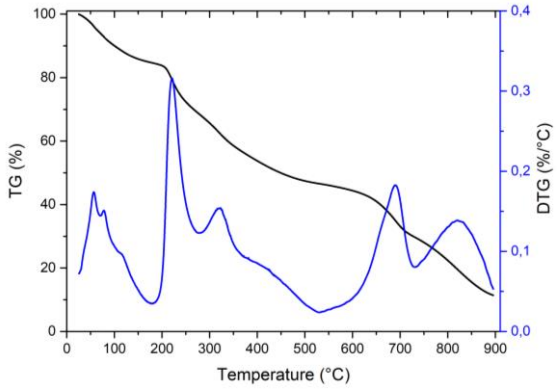

Figure 1: TGA and DTG curves of *Ulva rigida*.

The DTG curve shows four main degradation peaks at: i) 100 °C, corresponding to the evaporation of interstitial water; ii) 220 °C, corresponding to depolymerization and degradation of polysaccharides; iii) 330 °C, due to the degradation of proteins; and iv) 640 °C, due to the carbonization of the degradation products. The inorganic residue at 900 °C accounted for 11.2 wt%.

Figure 2 shows the ATR-FTIR spectrum of *Ulva rigida*. The intense band at 3243 cm^{-1} is due to the stretching of the O–H group in carbohydrates, proteins, lipids (sterols fatty acids), nucleic acids, and chlorophyll [19]. The band at 1630 cm^{-1} is ascribable to the C=O of proteins (amide I band) and to the C=C stretching of chlorophyll a and b (ketone, aldehyde, chelate), while the peak at 1589 cm^{-1} is due to N–H bending and C–N stretching of proteins (amide II band) [19]. The band at 1419 cm^{-1} is related to the C–H bending of aliphatic groups [21]. The most intense peak at 1083 cm^{-1} is due to the C–O–C stretching of sugars of polysaccharides, as well as the peaks at 981 and 1201 cm^{-1} [19]. The band at 842 cm^{-1} is ascribable to the $–SO_4$-binding of sulfated sugar typical of the ulvan structure [22].

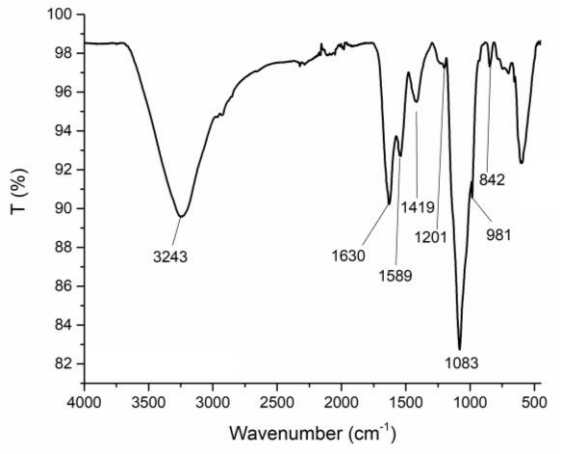

Figure 2: ATR-FTIR spectrum of *Ulva rigida*.

3.2 Optimisation of the extraction protocol of ulvan under traditional heating system

Regarding the experimental procedure for the extraction of ulvan from *Ulva* species, as reported in the literature, the extraction yield is affected by several parameters (properties and particle size of the biomass, extraction temperature and duration, polarity of the solvent, biomass loading, pH and the heating system) [7].

In the present work, the effect of the main parameters, such as temperature, pH and reaction time, was investigated in order to optimise the ulvan extraction yield. In all the tests, the biomass loading was fixed at 50 g/L and the traditional heating system was adopted in the first part of this study, while in the second part of the present work two other heating systems, as microwaves and ultrasounds, have been tested as more sustainable alternatives.

Moreover, regarding the downstream approach, the impact of the dialysis step as well as the comparison between lyophilisation and vacuum drying were investigated. The obtained results underline that the dialysis step was not necessary, and the easier vacuum drying gave comparable results to the lyophilisation step (data not shown). Thus, the experimental setup and results obtained without the dialysis step and drying the extracted ulvan by vacuum were reported in Table IV.

Table IV: Experimental setup (biomass loading 50 g/L, traditional heating system).

Run	T (°C)	pH	t (min)	UY[a] (wt%)	UY[b] (wt%)
1	90	1	90	78.6	27.4
2	90	2	90	45.9	16.0
3	90	7	90	50.9	17.7
4	60	7	90	40.0	13.9
5	120	7	90	76.5	26.6
6	120	1	90	38.2	13.3
7	90	7	30	53.8	18.7
8	90	7	150	57.6	20.0
9	90	1	30	58.1	20.2

UY = ulvan yield.
[a] calculated with respect to total sugars content.
[b] calculated with respect to the alga dry weight.

In runs 1, 2 and 3 the effect of pH was investigated by working at 90 °C and 90 min, namely the average values reported in the literature [7]. At pH=1 the maximum ulvan yield of 78.6 wt% with respect to the total sugar content and 27.4 wt% with respect to the dry weight of biomass were achieved. The increase in the pH to 2 or 7, significantly reduced the ulvan yield to about 50 wt% in both cases. In order to investigate the effect of the temperature on the extraction efficiency, runs 4 and 5 were performed at 60 and 120 °C, respectively, at pH=7. The results evidence that under neutral conditions the increment of temperature allowed the improvement of the ulvan extraction yield up to 76.5 wt% with respect to total sugars content and 26.6 wt% with respect to alga dry weight, working at 120 °C (run 5, Table IV). This is a very interesting result because the achieved yields are similar to those obtained working at 90 °C but in a strong acid solution (run 1, Table IV). On this basis, the result confirmed the possibility to avoid the use of HCl compensating the neutral pH with a higher temperature, thus making the proposed protocol more sustainable from an environmental and applicative point of view. The influence of temperature increasing up to 120 °C was investigated also at pH=1 (run 6, Table IV). In this case, the ulvan yield was only 38.2 wt% with respect to total sugars content due to the very harsh reaction conditions that favour the hydrolysis of polysaccharides. Moreover, the effect of the reaction time was also investigated in runs 7-9. In particular, in runs 7 and 8 reaction times of 30 and 150 min were tested (working at 90 °C, pH=7), respectively. At 30 min, the ulvan yield was about 54 wt%, namely a value similar to that achieved in run 3 performed for 90 min. The increase up to 150 min slightly increased the ulvan yield up to about 58 wt%, thus indicating that the reaction time did not strongly influence the extraction yield working at pH=7. The effect of the reaction time was also investigated at pH=1 and 90 °C (run 9, Table IV). Under these conditions, the ulvan yield was 58.1 wt%, namely lower than the value achieved in run 1, thus highlighting that at pH=1 the reaction time had greater influence than at pH=7.

Based on the obtained results, the optimised extraction conditions under traditional heating system were 120 °C, pH=7 and 90 min, allowing to obtain an ulvan yield of 77 wt% with respect to the total sugars content and of 27 wt% with respect to the alga dry weight.

3.3 Characterisation of extracted ulvan

The ulvan extracted under the best process conditions was observed through optical microscopy and in Figure 3 the obtained picture is reported.

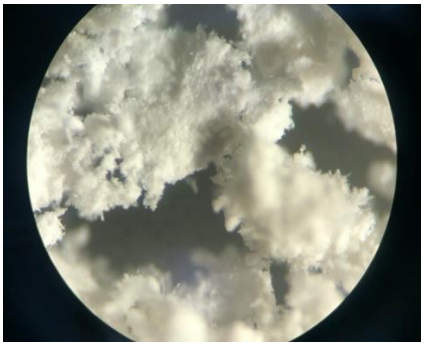

Figure 3: Picture of extracted ulvan by optical microscope.

The white colour was observed also at the micrometric scale, thus highlighting the high purity and quality of the extracted ulvan from the residual biomass.

The high purity of the extracted ulvan is also confirmed by TGA, as shown in Figure 4.

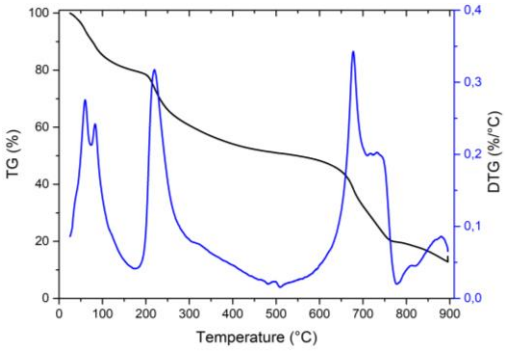

Figure 4: TGA and DTG curves of ulvan extracted under the optimised conditions.

The DTG curve shows three degradation peaks at: i) 100 °C, corresponding to the evaporation of interstitial water; ii) 220 °C, corresponding to depolymerization and degradation of ulvan; iii) 700 °C, due to carbonization of the degradation products. The ash content, namely the inorganic residue at 900 °C, was only 12.6 wt%, slightly lower than the value of around 18 wt% reported in the literature for the ulvan extracted from *Ulva rigida* [7]. The negligible intensity of the peak at about 330 °C, previously identified in the DTG curve of *Ulva* and attributed to the proteins, evidences their low amount in the extracted ulvan. This is also confirmed by the elemental analysis of the ulvan reported in Table V.

Table V: Elemental analysis of ulvan extracted from *Ulva rigida*.

Element	wt% on dry matter
C	21.4
H	4.1
N	1.4
S	9.3
O	63.8

In fact, the elemental composition agreed with that reported in the literature [23,24] and, based on the nitrogen content, the proteins in extracted ulvan amounted to 6.7 wt%. This value was lower than that reported in the literature for the ulvan commonly extracted from *Ulva rigida* (ca. 10 wt%) [7]. Moreover, based on the sulphur content, the amount of sulphate groups was 23.2 wt%.

Table VI reports the complete chemical composition of the ulvan extracted under the optimised conditions, considering also the total sugars content.

Table VI: Chemical profile of ulvan extracted from *Ulva rigida*.

Fraction	wt% on dry matter
Total sugars content	55.3 ± 2.1
Ash	12.6 ± 0.3
Proteins	6.7 ± 0.2
Sulphate groups	27.9 ± 0.3

The extracted ulvan presented a suitable purity and chemical composition, thus finding application as a nutraceutical in aquaculture and as an antimicrobial agent and bio-stimulant in plant cultivation [11,12].

Moreover, the chemical profile of the total sugars of ulvan was determined and reported in Table VII. The chemical profile agreed with the median values reported in the literature for the ulvan extracted from *Ulva rigida* [7].

Table VII: Chemical profile of total sugars content (TSC) of extracted ulvan under the optimised conditions.

Fraction	wt% on TSC
Glucose	56.6 ± 0.6
Xylose	7.2 ± 0.3
Rhamnose	26.0 ± 0.2
Glucuronic acid	10.1 ± 0.4
Arabinose	< 0.1

Lastly, the extracted ulvan was characterised through ATR-FTIR spectroscopy and the recorded spectrum is reported in Figure 5.

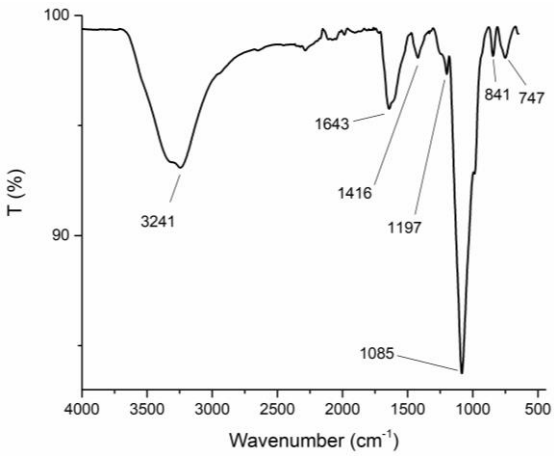

Figure 5: ATR-FTIR spectrum of ulvan from *Ulva rigida*.

The peak at 3241 cm^{-1} is due to the stretching of the O–H group in carbohydrates and proteins. The band at 1643 cm^{-1} is ascribable to the C=O of proteins (amide I band) [19] and it has a lower intensity than in the spectrum of *Ulva* (Figure 2), as consequence of their lower amount in the extracted ulvan. The peak at 1416 cm^{-1} is due to the C–H bending of aliphatic groups [21]. The most intense peak at 1085 cm^{-1}, as well as that at 1197 cm^{-1}, is ascribable to the C–O–C stretching of polysaccharides while the signal at 841 cm^{-1} is ascribable to the $–SO_4$-binding of sulfated sugar. Both these last peaks are typical of the ulvan structure [22].

3.3 Effect of alternative heating systems

The effect of microwaves and ultrasounds was investigated as alternative heating systems. In fact, both of them could improve the efficiency of ulvan extraction thanks to their peculiar working mechanisms. Microwaves are adsorbed by the treated mixture and the electromagnetic energy is converted into the thermal one, which causes the algal cells rupture, thus facilitating the diffusion of polysaccharides into the solvent [25]. On the

other hand, the ultrasounds create high cavitation intensity that leads to the destruction of cell walls. In this way, the penetration of the solvent into the biomass is facilitated, thus enhancing the mass transfer and as a consequence the extraction efficiency [26].

In this work, the extraction of ulvan with microwave heating has been performed under the optimised temperature and pH values previously identified for the traditional heating system (120 °C, pH 7) and with the same alga loading (50 g/L) but the reaction has been prolonged for 45 min, namely half of the optimised time with traditional heating (90 min). Under these conditions, the ulvan extraction yields of 67.8 wt% with respect to the total sugar content and 23.6 wt% with respect to the dry weight of biomass were achieved. These are very interesting results because are similar to those obtained under conventional heating but after only 45 min instead of 90 min (run 5, Table IV), thus confirming the higher extraction efficiency of the microwave heating. Moreover, these results are in agreement with the ulvan extraction yields already reported in the literature starting from other species of *Ulva* performed under microwave irradiation and similar reaction conditions [27,28].

When the ultrasounds were adopted as the heating system, the same alga loading (50 g/L) was employed and the reaction was performed at 60 W at an amplitude of 100% for 45 min reaching a temperature of 60 °C. In this case, the ulvan extraction yields of 62.6 wt% with respect to the total sugar content and 21.8 wt% with respect to the dry weight of biomass were achieved. Remarkably, similar results have been reported in the literature starting from other *Ulva* species but working under acid conditions and higher temperatures [29], whilst when neutral conditions have been adopted the reported ulvan extraction yields were strongly lower [30]. In conclusion, both these alternative heating systems resulted particularly promising and a dedicated optimization of the reaction parameters will be performed in order to further improve the ulvan extraction yield and characterization of the extracted ulvan.

4 CONCLUSIONS

In the present work, the extraction protocol of ulvan from the residual algal biomass *Ulva rigida* harvested from the Orbetello lagoon was optimised. The effect of the main process parameters was investigated and optimised in order to maximise the ulvan extraction yield according to the principles of the Green Chemistry. The highest ulvan yield of ca. 77 wt% with respect to the total sugars content and ca. 27 wt% with respect to the dry weight of biomass was achieved at 120 °C, 90 min, pH 7 and biomass loading of 50 g/L under traditional heating. Under these conditions, the extracted ulvan was characterised by a good purity grade since it contained only 6.7 wt% proteins and 12.6 wt% ash. Moreover, two alternative heating systems, namely microwaves and ultrasounds, were adopted and similar ulvan extraction yields were ascertained after half of time and, in the case of ultrasounds, also at lower temperature (60 °C) than the traditional heating. The last two approaches open the way to further future studies for the optimisation of the main process parameters aiming at increasing the ulvan yield under sustainable extraction conditions.

5 REFERENCES

[1] I. De Bari, D. Cuna, N. Di Fidio, Biorefineries: Biofuels, Biochemicals and Bioproducts, in Biofuels, Biochemicals, and Bioproducts, (Ed. M. R. Riazi, Chiaramonti, D.), CRC Press, Boca Raton, USA, (2017), pag. 533.

[2] N. Singh, R.R. Singhania, P.S. Nigam, C.D. Dong, A.K. Patel, M Puri, Global status of lignocellulosic biorefinery: Challenges and perspectives, (2022), pag. 126415.

[3] S. Fulignati, D. Licursi, N. Di Fidio, C. Antonetti, A.M. Raspolli Galletti, Novel challenges on the catalytic synthesis of 5-hydroxymethylfurfural (HMF) from real feedstocks, (2022), pag. 1664.

[4] N. Di Fidio, F. Minonne, C. Antonetti, A.M. Raspolli Galletti, *Cutaneotrichosporon oleaginosus*: a versatile whole-cell biocatalyst for the production of single-cell oil from agro-industrial wastes, (2021), pag. 1291.

[5] A.M. Raspolli Galletti, R. Lorè, D. Licursi, N. Di Fidio, C. Antonetti, S. Fulignati, Insights on butyl levulinate bio-blendstock: From model sugars to paper mill waste cellulose as feedstocks for a sustainable catalytic butanolysis process. (2023), pag. 114054.

[6] A.M. Raspolli Galletti, C. Antonetti, D. Licursi, L. Mussi, E. Balestri, C. Lardicci, Levulinic Acid Production from the Green Macroalgae *Chaetomorpha linum* and *Valonia aegagropila* Harvested in the Orbetello Lagoon, (2019), pag. 103.

[7] J.T. Kidgell, M. Magnusson, R. de Nys, C.R. Glasson, Ulvan: A systematic review of extraction, composition and function, (2019), pag. 101422.

[8] P. Gennaro, M. Guidotti, E. Funari, S. Porrello, M. Lenzi, Reduction of land based fish-farming impact by phytotreatment pond system in a marginal lagoon area, (2006), pag. 246.

[9] S. Porrello, M. Lenzi, E. Persia, P. Tomassetti, M.G. Finoia, Reduction of aquaculture wastewater eutrophication by phytotreatment ponds system I. Dissolved and particulate nitrogen and phosphorus, (2003), pag. 515.

[10] B. Quemener, M. Lahaye, C. Bobin-Dubigeon, Sugar determination in ulvans by a chemical-enzymatic method coupled to high performance anion exchange chromatography, (19997), pag. 179.

[11] S. Coiai, B. Campanella, R. Paulert, F. Cicogna, E. Bramanti, A. Lazzeri, L. Pistelli, M.B. Coltelli, Rosmarinic Acid and Ulvan from Terrestrial and Marine Sources in Anti-Microbial Bionanosystems and Biomaterials, (2021), pag. 9249.

[12] L. Rossi, C. Bibbiani, J.F. Fierro-Sañudo, C. Maibam, L. Incrocci, A. Pardossi, B. Fronte, Selection of marine fish for integrated multi-trophic aquaponic production in the Mediterranean area using DEXi multi-criteria analysis, (2021), pag. 736402.

[13] L.M.L. Laurens, Summative mass analysis of algal biomass - integration of analytical procedures. Laboratory Analytical Procedure (LAP), NREL, (2013), pag. 275.

[14] B. Hames, R. Ruiz, C. Scarlata, A. Sluiter, J. Sluiter, D. Templeton, Preparation of samples for compositional analysis. Laboratory Analytical Procedure (LAP), NREL/TP-510-42620, (2008), pag. 65.

[15] A. Sluiter, B. Hames, D. Hyman, C. Payne, R. Ruiz,

C. Scarlata, J. Sluiter, D. Templeton, J. Wolfe, Determination of total solids in biomass and total dissolved solids in liquid process samples. NREL/TP-510-42621, (2008), pag. 1.
[16] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedure (LAP), (2008), pag. 1.
[17] A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton, Determination of sugars, byproducts, and degradation products in liquid fraction process samples, Laboratory Analytical Procedure, (2005), pag. 1.
[18] I. Podolean, S. M. Coman, C. Bucur, C. Teodorescu, S. Kikionis, E. Ioannou, V. Roussis, A. Primo, H. Garcia, V.I. Parvulescu, Catalytic transformation of the marine polysaccharide ulvan into rare sugars, tartaric and succinic acids, (2022), pag. 345.
[19] R. Pezoa-Conte, A. Leyton, A. Baccini, M.C. Ravanal, P. Mäki-Arvela, H. Grénman, X. Chunlin, S. Willfor, M.L. Lienqueo, J.P. Mikkola, Aqueous Extraction of the Sulfated Polysaccharide Ulvan from the Green Alga Ulva rigida - Kinetics and Modeling, (2017), pag. 915.
[20] Y. Kumar, A. Tarafdar, D. Kumar, K. Verma, M. Aggarwal, P.C. Badgujar, Evaluation of chemical, functional, spectral, and thermal characteristics of *Sargassum wightii* and *Ulva rigida* from Indian Coast, (2021), pag. 1.
[21] M. Mecozzi, E. Pietrantonio, M. Pietroletti, The roles of carbohydrates, proteins and lipids in the process of aggregation of natural marine organic matter investigated by means of 2D correlation spectroscopy applied to infrared spectra, (2009), pag. 1877.
[22] E. Gómez-Ordóñez, P. Rupérez, FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds, (2011), pag. 1514.
[23] M.I.A. Ibrahim, M.S. Amer, H.A.H. Ibrahim, E.H. Zaghloul, Considerable Production of Ulvan from *Ulva lactuca* with Special Emphasis on Its Antimicrobial and Anti-fouling Properties, (2022), pag. 3097.
[24] R. Pankiewicz, B. Łęska, B. Messyasz, J. Fabrowska, M. Sołoducha, M. Pikosz, First isolation of polysaccharidic ulvans from the cell walls of freshwater algae, (2016), pag. 348.
[25] M. Alboofetileh, M. Razei, M. Tabarsa, M. Rittà, M. Donalisio, F. Mariatti, S.G. You, D. Lembo, G. Cravotto, Effect of different non-conventional extraction methods on the antibacterial and antiviral activity of fucoidans extracted from *Nizamuddinia zanardinii*, (2019), pag. 131.
[26] N. Bayar, T. Bouallegue, M. Achour, M. Kriaa, A. Bougatef, R. Kammoun, Ultrasonic extraction of pectin from *Opuntia ficus* indica cladodes after mucilage removal: optimization of experimental conditions and evaluation of chemical and functional properties, (2017), pag. 275.
[27] J. André, N. Flórez-Fernández, H. Domínguez, M.D. Torres, Microwave-assisted extraction of *Ulva* spp. Including a stage of selective coagulation of ulvan stimulated by a bio-ionic liquid, (2023), pag. 952.
[28] S. Tsubaki, K. Oono, M. Hiraoka, A. Onda, T. Mitani, Microwave-assisted hydrothermal extraction of sulphated polysaccharides from *Ulva* spp. And *Monostroma latissimum*, (2016), pag. 311.
[29] M. Kazemi, M. Fathi, K. Jahanbin, M. Taghdir, S. Abbaszadeh, Optimization of ultrasonic-assisted hot acidic solvent extraction of ulvan from *Ulva intestinalis* of the Persian Gulf: evaluation of structural, techno-functional, and bioactivity properties, (2023), pag. 108837.
[30] F. Rahimi, M. Tabarsa, M. Razaei, Ulvan from green algae *Ulva instestinalis*: optimization of ultrasound-assisted extraction and antioxidant activity, (2016) pag. 2979.

5 ACKNOWLEDGEMENTS

- This research was conducted within the activities of the RTD-A contract (no. 1112/2021, Prot. no. 0165823/2021) co-funded by the University of Pisa in respect of the PON “Ricerca e Innovazione” 2014-2020 (PON R&I FSE-REACT EU), Azione IV.6 “Contratti di ricerca su tematiche Green”.