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Conventional *vs.* Innovative Protocols for the Extraction of Polysaccharides from Macroalgae

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Abstract: Macroalgae are one of the most environmentally friendly resources, and their industrial by-products should also be sustainable. Algal polysaccharides represent valuable products, and the definition of new eco-sustainable extraction processes, ensuring a safe and high-quality product, is a new goal in the context of reducing the carbon footprint. The aim of the present work was to determine the influence of the extraction methodology on the properties and structure of the polysaccharides, comparing conventional and innovative microwave-assisted methods. We focused on extraction times, yield, chemical composition and, finally, biological activities of raw polymers from three macroalgal species of *Chlorophyta*, *Rhodophyta* and *Phaeophyceae*. The main objective was to design a sustainable process in terms of energy and time savings, with the aim of developing subsequent application at the industrial level. Extraction efficacy was likely dependent on the physico-chemical polysaccharide properties, while the use of the microwave did not affect their chemical structure. Obtained results indicate that the innovative method could be used as an alternative to the conventional one to achieve emulsifiers and bacterial antiadhesives for several applications. Natural populations of invasive algae were used rather than cultivated species in order to propose the valorization of unwanted biomasses, which are commonly treated as waste, converting them into a prized resource.

Keywords: alginate; antibiofilm assay; carragenans; emulsifying activity; DNA barcoding; Fourier transform infrared spectroscopy-ATR; gas chromatography with flame ionization detection; gel permeation chromatography; invasive macroalgal biomasses; ulvans

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1. Introduction

The polyphyletic group of marine macroalgae, due to their diversity, produces various chemical components. Among them, structural polysaccharides contained in their cell wall are the most valuable and are exploited commercially worldwide [1,2]. Nowadays, the world export of seaweeds amounts to more than USD 2.65 billion (more than 98 countries), of which USD 1.74 billion originate from seaweed hydrocolloids [3]. Macroalgae, and their derivate products, come from many commercial sectors, such as agriculture, feed, food, cosmetics and pharmaceutics, and many species are under investigation in the search for novel bioactive compounds [4–7]. Extraction of phycocolloids is one of the most profitable markets of macroalgae, which are used as thickeners, gelling agents and stabilizers. Thanks

to their structural and morphological versatility, they find applications in various industrial sectors not just as a food additive, but also in pharmaceutical and cosmetics products [8,9].

Polysaccharides useful for human affairs are mainly extracted from taxa of *Rhodophyta* and *Phaeophyceae*, but lately also *Chlorophyta*, which has entered fully into the market [10].

Species from *Rhodophyta* contain sulfated galactans, namely agars and carrageenans, a group of well-studied molecules with great variability [11,12]. Similarly, species of *Phaeophyceae* contain alginate, heteropolymers of guluronic and mannuronic acids, whose chemical characteristics vary by taxa, anatomical parts of thalli and extraction steps that result in acid and derivate salts [13]. Recently, other sulfated polysaccharides have been extracted from *Chlorophyta*, promising molecules used in nutraceutical, pharmaceutical and cosmetical applications and as antibiofilm agents [14].

Cultivated seaweeds are traditionally used to obtain phycocolloids, such as species of *Gracilariaceae* and *Gelidiaceae* for agar, species of *Solieriaceae* for carragenans and species of *Laminariales* for alginates [15]. However, the cultivation of macroalgae has a cost in terms of skilled personnel, equipment and facilities (including laboratories), which affects the retail price. Seaweed biomasses collected from anthropized coastal environments, where they thrive prosperously, obstructing human activities and, in part, also other organisms, might be dredged and used as an additional source of phycocolloids, given the pressing demand of the market [16]. Such populations have the added value of being able to resolve issues of disposal of unwanted biomasses, converting them into profitable products.

A variety of protocols have been proposed for obtaining raw polysaccharides, mainly by conventional chemical extractions [17]. In the last decade, novel methods have been tested, including extractions based on the use of enzymes, ultrasounds, microwaves and supercritical fluids [18]. Among them, microwave-assisted extraction has several advantages, namely a shorter processing time, a lower amount of solvent and a higher quality of derivate products [19,20].

The present research aimed to compare conventional and innovative polysaccharide extraction methods, focusing on microwave-assisted extraction, from different macroalgal species. Natural populations of the invasive *Ulva ohnoi* (*Chlorophyta*), *Agardhiella subulata* (*Rhodophyta*), both from the brackish lake of Ganzirri, in the Oriented Natural Reserve of Cape Peloro (Messina, Italy) and *Sargassum muticum* (*Phaeophyceae*), from the lagoon of Venice (Italy), were used to obtain ulvans, carrageenans and sodium alginate, respectively. Compared parameters were extraction times, polysaccharide yield and chemical composition. Obtained raw extracts were finally tested for their biological activities.

2. Materials and Methods

2.1. Sample Collection and DNA Barcoding

Samples of *Ulva ohnoi* (*Chlorophyta*) and *Agardhiella subulata* (*Rhodophyta*) were collected from the brackish lake of Ganzirri, in the Oriented Natural Reserve of Cape Peloro (Messina, Italy). Samples of *Sargassum muticum* (*Phaeophyceae*, *Ochrophyta*) were collected from the lagoon of Venice (Italy). Species names and *phylum* attributions are in accordance with algaebase.org [21].

After collection, samples were immediately washed with seawater to remove possible debris, adhered sand particles and associated organisms, transported to the laboratory in plastic bags at low temperature and washed with tap water to remove surface salt. Thalli were oven-dried at 40 °C for 48 h and stored in silica gel at room temperature until polysaccharide extractions.

From each fresh sample, a voucher was exsiccated on an herbarium sheet and a portion of thallus was subsampled, manually cleaned of epiphytes, dried in silica gel and stored at −20 °C for molecular identification.

Taxonomic identifications were performed through DNA barcoding methods, according to Miladi et al. [22] and Manghisi et al. [23]. Selected barcodes were COI-5P for *A. subulata* and *S. muticum* and *tuf* A for *U. ohnoi* [24]. Sequencing reactions were performed by an external company (Macrogen Europe, Amsterdam, The Netherlands). Forward and re-

verse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd., South Brisbane, QLD, Australia), and species attributions were performed by the identification engine in BOLD Systems (www.boldsystems.org, accessed on 6 March 2022).

Voucher specimens are housed in the Phycological Lab Herbarium (PhL, http://sweetgum.nybg.org/science/ih/herbarium-details/?irn=253162 (accessed on 8 March 2022). Collection information, voucher IDs, barcode identification numbers (BINs) and BOLD process IDs (PID) are listed in Table 1.

Table 1. List of the algal samples used in this study.

Phylum (Class)	Species	Collection Information	Voucher ID	BIN/PID
Chlorophyta (Ulvophyceae)	*Ulva ohnoi* M.Hiraoka et S.Shimada	Lake Ganzirri, Messina, Italy (38°15′28.8″ N 15°36′29.3″ E), 17 July 2020	PhL-APP028	GRAPP015-17
Rhodophyta (Florideophyceae)	*Agardhiella subulata* (C. Agardh) Kraft et M.J. Wynne	Lake Ganzirri, Messina, Italy (38°15′31.0″ N 15°36′49.5″ E), 30 June 2011	PhL-APP046	BOLD: AAC0053
Ochrophyta (Phaeophyceae)	*Sargassum muticum* (Yendo) Fensholt	Lagoon of Venice, Italy (45°25′42.6″ N 12°19′50.7″ E), 9 July 2020	PhL-APP031	BOLD: AAO5681

2.2. Extraction Protocols

Crude polysaccharides were obtained from each collected taxon with two different protocols implemented in parallel, a conventional protocol (CP) and an innovative protocol (IP), both modified from the literature [14,25–28], as detailed in Tables 2–4.

Table 2. Polysaccharide extraction protocols for *Ulva ohnoi*.

Conventional Protocol	Innovative Protocol
Incubate in distilled water at 70 °C for 3 h in convection oven.	Incubate in distilled water at 140 W for 10 min (70 °C) in microwave oven; repeat once, waiting until the mixture reaches 30 °C before the second incubation.
Use hot filtration with a cheese cloth to remove residual thalli.	
Precipitate in a volume of 94% ethanol at 25 °C for 24 h.	
Filtrate and centrifugate at 4000× *g*, 25 °C for 15 min.	
Dry precipitate at 40 °C for 48 h in convection oven.	

Table 3. Polysaccharide extraction protocols for *Agardhiella subulata*.

Conventional Protocol	Innovative Protocol
Soak in NaOH (2 M) at 70 °C for 2 h in convection oven.	
Filtrate with a cheese cloth and wash thalli in tap water to remove the solvent [(1)].	
Incubate in distilled water at 70 °C for 24 h in convection oven.	Incubate in distilled water at 140 W for 10 min (70 °C) in microwave oven; repeat thrice, waiting until the mixture reaches 30 °C before each incubation.
Hot filtrate with a cheese cloth to remove residual thalli.	
Precipitate in a volume of 94% ethanol at 25 °C for 24 h.	
Filtrate and centrifugate at 4000× *g*, 25 °C for 15 min.	
Dry precipitate at 40 °C for 48 h in convection oven.	

[(1)] Pretreated thalli can be dried at 40 °C for 48 h in convection oven and stored in silica gel at room temperature before subsequent extraction steps.

Table 4. Polysaccharide extraction protocols for *Sargassum muticum*.

Conventional Protocol	Innovative Protocol
Soak in HCl (0.01 M) at 25 °C for 24 h.	
Filtrate with a cheese cloth and wash thalli in tap water to remove the solvent [(1)].	
Incubate in 3% Na_2CO_3 at 70 °C for 24 h in convection oven.	Incubate in 3% Na_2CO_3 at 140 W for 10 min (70 °C) in microwave oven; repeat once, waiting until the mixture reaches 30 °C before the second incubation.
Use hot filtration with a cheese cloth to remove residual thalli.	
Precipitate in a volume of 94% ethanol at 25 °C for 24 h.	
Filtrate and centrifugate at 4000× *g*, 25 °C for 15 min.	
Dry precipitate at 40 °C for 48 h in convection oven.	

[(1)] Pretreated thalli can be dried at 40 °C for 48 h in convection oven and stored in silica gel at room temperature before subsequent extraction steps.

2.3. Chemical Characterization of Algal Extracts

The polysaccharide crude extracts obtained with CP and IP from each collected taxon were chemically analyzed. Determination of the main functional groups was performed by Fourier transform infrared (FTIR) spectroscopy, and molecular weights were determined by gel permeation chromatography (GPC). Monosaccharide composition was performed for polysaccharide crude extracts from *U. ohnoi* by gas chromatography with flame ionization detection (GC-FID) analysis.

2.3.1. Determination of the Functional Groups

The FTIR spectra of the dried extracted were determined with Nicolet iS 5 Thermo Scientific equipped with iD5 ATR (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The spectra were scanned at room temperature in the range of 4000–600 cm^{-1}, with a resolution of 4 cm^{-1} using a diamond crystal. For each sample, the measurement was performed in triplicate to obtain an average spectrum.

2.3.2. Determination of Molecular Weights

The molecular weight of the extracts was measured by gel permeation chromatography (Agilent GPC PL-GPC 220, Agilent Technologies, Inc., Santa Clara, CA, USA) with a refractive index detector (cell volume 8 µL, wavelength 890 nm).

The following conditions were used for each polysaccharide extract from *U. ohnoi* and *S. muticum*: 0.1 M sodium nitrate was used as a mobile phase at a flow rate of 1 mL min^{-1}, the temperature of the column was 30 °C and the injection volume of the aqueous solution containing the extract (3 mg mL^{-1}) was 100 µL [14].

For each polysaccharide extract from *A. subulata*, the same conditions were used with slight modifications, as follows: 0.05 M sodium nitrate as a mobile phase, column temperature set at 35 °C.

The calibration was performed using six pullulan standards with molecular weight in the range of 350–700 kDa.

2.3.3. Monosaccharide Composition

The assessment of the monosaccharide content in each polysaccharide extract from *U. ohnoi* was performed using gas chromatography with flame ionization detection (GC-FID) analysis (Agilent technologies HP 6890, Agilent Technologies, Inc., Santa Clara, CA, USA). Before the analysis, the extracts were subjected to acid methanolysis following the reported protocol [14]. In brief, 10 mg of each dried extract was soaked in 2 mL of 2 M HCl obtained by dilution of a commercial solution of 3 M HCl with anhydrous methanol (>99%). The methanolysis was carried out for 4 h at 100 °C. As the HCl concentration and the reaction time are crucial parameters affecting the release of the monosaccharides and their

stability, the methanolysis kinetics was investigated. For this purpose, two concentrations were used, 2 M HCl and 3 M HCl. Concentrations higher than 3 M were not used as they would result in monosaccharide degradation. The reaction was carried out for 4, 8, 12, 24, 48 and 72 h at 100 °C in triplicate, to find out optimal conditions to obtain the maximum release of polysaccharides with minimal degradation.

Following the methanolysis, the residual HCl was neutralized with pyridine (>99%). Sorbitol and methanol solution (0.1 mg mL^{-1}) were added, and the solvent was removed with rotavapor. The dried product was dissolved in a mixture made of 150 mL of pyridine, 150 mL of bis (trimethylsilyl)-trifluoroacetamide and 50 mL of chlorotrimethylsilane and kept for 30 min at 80 °C under stirring.

The quantitative determination of monosaccharides was performed by gas chromatography with FID detector (GC-FID). Detector temperature was set at 290 °C, injector temperature at 250 °C, and the program was set as follows: raise to 100 °C, from 100 °C to 180 °C at 4 °C min^{-1} and from 180 °C to 290 °C at 8 °C min^{-1}.

The external calibration was conducted using Rha, Xyl, GlcA, Glc and IdoA. All standards had purity >99%.

2.4. Microbiological Tests

2.4.1. Antibacterial Activity

The antibacterial activity of crude extracts against *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 was evaluated by the standard disk diffusion method (Kirby Bauer test), as accepted by the Clinical and Laboratory Standards Institute (CLSI) [29].

Pseudomonas aeruginosa and *S. aureus* were grown overnight on lysogeny agar (LA) or tryptic soy agar (TSA) plates, respectively, and incubated at 37 °C for 24 h, and colonies from each strain were suspended in 3 mL of 0.9% NaCl solution ($OD_{600\,nm}$ = 0.1). Aliquots of each suspension (100 µL) were inoculated onto plates of Muller Hinton agar (Oxoid Holdings ltd, Hampshire, UK) in triplicate.

Each algal extract (20 mg) was dissolved in 1000 µL of sterile distilled water, and 20 µL of each solution was applied to sterile filter paper disks (6 mm in diameter, Oxoid Holdings Ltd., Hampshire, UK). After evaporation, the disks, each containing 400 µg of extract, were placed onto inoculated plates. Plates were incubated overnight at 37 °C. The diameter of inhibition zones was measured, and means and standard deviations (SD) (n = 3) were calculated.

2.4.2. Antibiofilm Assay

The antibiofilm activity of algal extracts against *P. aeruginosa* and *S. aureus* was carried out in 96-well polystyrene microplates (Falcon®, Fisher Scientific, Milan, Italy), as previously reported by [30]. Overnight cultures (180 µL) of *P. aeruginosa* grown in Luria–Bertani (LB) or *S. aureus* in tryptic soy broth (TSB) (6 replicates) ($OD_{600\,nm}$ = 0.1 equivalent to 1.5×10^8 bacteria m L^{-1}) were poured in the microwells, and each crude polysaccharide extract (20 µL), diluted in phosphate-buffered saline (PBS, Sigma Aldrich, St. Louis, MI, USA) at 400 µg mL^{-1} or 20 µL of PBS used as a control, were added to each well. Microplates were incubated at 37 °C for 48 h (for *P. aeruginosa*) or 24 h (for *S. aureus*), without shaking. Nonadherent bacteria were removed by washing the samples 5 times with distilled water. Biofilms were stained with 0.1% (w/v) crystal violet solution for 20 min. Excess stain was removed by aspiration, and then the plates were washed 5 times and air-dried for 45 min. The stained biofilms were solubilized with absolute ethanol, and the biofilm mass was spectrophotometrically determined ($OD_{585\,nm}$) by the level of the crystal violet present in the de-staining solution, using a microtiter plate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA).

The reduction in biofilm formation of each strain was expressed as antibiofilm activity (%) by applying the following formula:

$$(OD_{control} - OD_{sample}/OD_{control}) \times 100.$$

2.4.3. Emulsifying Activity

The emulsifying activity of crude algal extracts was evaluated according to the method described by Mata et al. [31]. Each crude lyophilized extract was dissolved in 2 mL of distilled water (0.05%, *w*/*v*), mixed with an equal volume of kerosene in a glass tube (5 cm high and 1 cm in diameter) and stirred at high speed in the vortex for 2 min. The emulsion and aqueous layers were measured after 24 h, and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture, and multiplying by 100 [32].

2.4.4. Coating Assay for Polystyrene Surfaces

The ability of the extracts to modify the hydrophobic surface properties was investigated by the coating assay. Briefly, a volume of 20 µL of each extract diluted in PBS was transferred to the center of a 24-well polystyrene microtiter plate (Falcon no. 353047) [33]. To allow the complete evaporation of the solutions the plates, were incubated for 30 min at 37 °C. Aliquots (1 mL) of diluted overnight bacterial culture (containing 10^5 CFU mL^{-1}) in LB for *P. aeruginosa* and TSB for *S. aureus* were poured into each well of microplate. After incubation at 37 °C for 18 h in static conditions, the wells were emptied gently, washed with distilled water and stained with 1 mL of 0.1% crystal violet solution. To remove the excess crystal violet, wells were rinsed with distilled water and air-dried.

2.4.5. Statistical Analysis

Statistical analysis was performed using two-way ANOVA. Differences were considered statistically significant at a *p*-value of <0.01 or 0.05.

3. Results and Discussion

Polysaccharide yields and processing time of both the conventional protocol (CP) and innovative protocol (IP) for the extractions of *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum* are shown in Table 5.

Table 5. Polysaccharide yields and processing time of extractions from *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum* for both conventional protocol (CP) and innovative protocol (IP). The yields are expressed as the average ± SD (*n* = 3).

Extract	Yield (g)	Time (h)
Ulva ohnoi CP	4.52 ± 0.72	3
Ulva ohnoi IP	13.20 ± 0.88	0.33
Sargassum muticum CP	38.38 ± 0.47	24
Sargassum muticum IP	33.67 ± 1.11	0.33
Agardhiella subulata CP	24.13 ± 0.25	24
Agardhiella subulata IP	28.50 ± 0.41	0.66

Innovative protocol extractions gave higher yields from *U. ohnoi* and *A. subulata*, but lower values from *S. muticum*, showing that the extraction efficacy is likely dependent on polysaccharide type. However, with the notable situation of *U. ohnoi* polysaccharides, whose yield almost tripled in IP extraction, the yields of both *A. subulata* and *S. muticum* polymers were comparable between the two tested methods.

Results of chemical analyses and microbiological tests are presented below.

3.1. Characterization of Extracted Polysaccharides

3.1.1. Determination of the Functional Groups

The chemical structure of the extracted polysaccharides is directly related to their chemical and biological properties, and any change could cause either a favorable or an adverse variation. As a consequence, the effects of the extraction procedure were evaluated by FTIR-ATR spectroscopy by verifying the presence of characteristic peaks of expected polysaccharide types in each extract.

The FTIR-ATR spectra are presented in Figure 1.

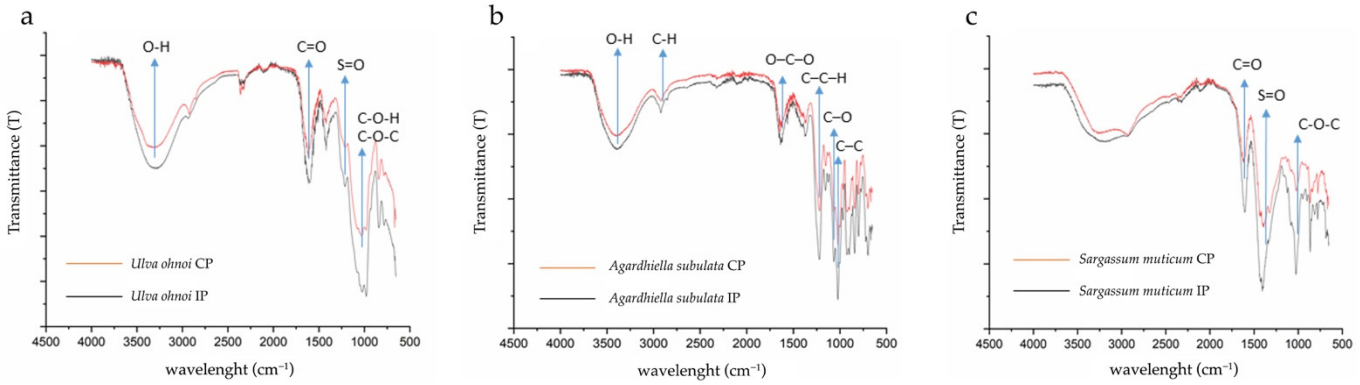

Figure 1. FTIR-ATR spectra of polysaccharide extracts (**a**) from *Ulva ohnoi*; (**b**) from *Agardhiella subulata*; (**c**) from *Sargassum muticum*. The black curves refer to conventional protocol (CP), while red curves refer to innovative protocol (IP).

Both spectra of *U. ohnoi* extracts (Figure 1a) confirm the structure of ulvans, in agreement with data reported in the literature [14,34]. The peak at 1653 cm^{-1} refers to the asymmetric stretching of the C=O while the band at 1248 cm^{-1} is related to the S=O. The peaks at 846 cm^{-1} and 800 cm^{-1} are related to sugar rings. The region around 1200–1000 cm^{-1} includes stretching vibration of C-OH groups and the C-O-C vibrations. The peak at 1050 cm^{-1} is related to the stretching of the two main monosaccharides in *U. ohnoi*, rhamnose (Rha) and guluronic acid (GlcA).

In the spectra related to *Agardhiella subulata* extracts, the peaks confirm the presence of carrageenan (Figure 1b). In fact, as reported [14], the peak at 1410 cm^{-1} is ascribed to the sulfate ester while that at 1020 cm^{-1} to the glycosidic bridge C-O-C. In the ranges of 920–940 cm^{-1} and 830–850 cm^{-1}, the peaks correspond to 3,6 anhydrogalactose and galactose-4-sulfate, respectively.

For both extracts of *S. muticum*, the presence of alginate is confirmed (Figure 1c). The large band centered around 3400 cm^{-1} is ascribed to O-H stretching vibrations while at 2900 cm^{-1} the C-H stretching vibrations are recognizable, and the asymmetric stretching of carboxylate O-C-O vibration is visible at 1620 cm^{-1}, as reported in the literature [35]. The peak at 1420 cm^{-1} might be assigned to C-OH deformation vibration with the contribution of O-C-O symmetric stretching vibration of the carboxylate group. Minor bands at 1300, 1120 and 1090 cm^{-1} are related to the C-C-H and O-C-H deformation, C-O stretching and C-O and C-C stretching vibrations, respectively, in the sugar ring. The peak at 950 cm^{-1} can be appointed to the C-O stretching vibration of uronic acid residues, and that at 880 cm^{-1} can be assigned to the deformation vibration of the C1-H in β-mannuronic acid residues. The band at 815 cm^{-1} is also ascribed to the mannuronic acid residues [36,37].

For all tested species, both CP and IP spectrum pairs overlap without substantial differences in the position and number of peaks. However, hypochromic effects were observed at a magnitude that varied with the functional group involved in all IP extracts. The obtained FTIR-ATR spectra suggest that the use of the microwave, at the frequency, duration and number of cycles tested, did not affect the chemical structure of the polysaccharide extracted.

3.1.2. Determination of Molecular Weights

The molecular weight (MW) is a key parameter as it influences the chemical, biological and physical properties of the extracted polysaccharides. In addition, it is highly sensitive to the extraction procedures and its evaluation is fundamental to understanding the occurrence of the degradation process during extraction.

The MWs of the polysaccharides extracted with both the CP and IP are presented in Table 6.

Table 6. Molecular weight (MW) and refractive index (RI) of polysaccharides extracted by both conventional (CP) and innovative (IP) protocols. The measurements were performed in triplicate, and the data are expressed as average (SD up to 10%).

Extract	MW_1 (kDa)	RI_1 (mV)	MW_2 (kDa)	RI_2 (mV)	MW_3 (kDa)	RI_3 (mV)
Ulva ohnoi CP	56	8	-	-	-	-
Ulva ohnoi IP	53	6	-	-	-	-
Agardhiella subulata CP	489	41	69	15	-	-
Agardhiella subulata IP	320	27	35	11	10	6
Sargassum muticum CP	157	12	21	7	-	-
Sargassum muticum IP	83	9	-	-	-	-

The acquired values show that an effect of the microwave during the extraction procedure could occur. In the case of *U. ohnoi* specimens, a monomodal distribution centered at 56 kDa (CP) and 53 kDa (IP) was observed.

Conversely, in the case of carrageenan, the CP-derived *A. subulata* extract, a bimodal distribution was observed at the major peak corresponding to MW 400–560 kDa, while the minor one represents a fragment between 55 and 110 kDa. In the IP-derived *A. subulata* extract, the main peak has a lower molecular weight, between 248 and 389 kDa, while the minor peak is between 20 and 50 kDa. In addition, a small peak is present in the range from 5 to 16 kDa, suggesting the presence of fragments.

In the case of alginate, in the *S. muticum* CP extract, a bimodal distribution was observed with the major peak corresponding to MW 110–205 kDa, while the minor one represents fragments between 10 and 35 kDa. In contrast, in the *S. muticum* IP extract, a monomodal distribution was observed with the peak shifting towards a lower molecular weight with a narrow distribution in the range of 60–95 kDa. The data presented in Table 6 suggest that a reduction in the molecular weight takes place when microwaves are applied during extraction.

3.1.3. Monosaccharide Composition

The composition of polysaccharides extracted by both conventional (CP) and innovative (IP) protocols is presented in Table 7. The data display that the whole content of monosaccharides obtained from *U. ohnoi* via methanolysis (2 M HCl and 4 h) was approximately 25%, which is in accordance, and in some cases higher, than values reported in the literature [38]. Rha and GlcA were the main residues, reaching 30% and 45%, respectively, in the CP extract, and 27% and 48%, respectively, in the IP extract.

The data in Table 8 confirm Rha and GlcA as the main monosaccharides, with a content of 28% and 52%, respectively, in the CP extract, and of 29% and 56%, respectively, in the IP extract.

Rha and GlcA represent the main monosaccharides; however, when increasing the HCl concentration from 2 M to 3 M, the total number of released monosaccharides increased by 33% and 42% with the CP and IP, respectively. This finding reflects the trend reported in previously published work, in which the same experimental conditions were used for the release of monosaccharide units from ulvan [38].

Monosaccharide content released by methanolysis over time at different acid concentrations, expressed as percentages of the dried weight of the extracts, is presented in Figure 2.

Table 7. Monosaccharide composition of the polysaccharides extracted by both conventional (CP) and innovative (IP) protocols from *Ulva ohnoi* (expressed as percentage of dry weight of the extract) after acid methanolysis using 2 M HCl/MeOH, for 4 h at 100 °C. The data are expressed as the average ± SD (n = 3).

		***Ulva ohnoi* CP**	***Ulva ohnoi* IP**
Monosaccharide	Rha	7.5 ± 0.2	7.1 ± 0.3
	GlcA	11.3 ± 0.7	12.7 ± 0.9
	IdoA	2.2 ± 0.1	1.8 ± 0.1
	Glc	1.9 ± 0.1	1.5 ± 0.1
	Xyl	2.1 ± 0.1	2.9 ± 0.2
Disaccharide	Rha-GlcA	2.3 ± 0.1	1.4 ± 0.4
	Total	27.3 ± 1.5	27.4 ± 2

Table 8. Monosaccharide composition of the polysaccharides extracted by both conventional (CP) and innovative (IP) protocols from *Ulva ohnoi* (expressed as percentage of dry weight of the extract) after acid methanolysis using 3 M HCl/MeOH, for 4 h at 100 °C. The data are expressed as the average ± SD (n = 3).

		***Ulva ohnoi* CP**	***Ulva ohnoi* IP**
Monosaccharide	Rha	10.4 ± 0.8	11.3 ± 0.5
	GlcA	19.1 ± 1.1	21.7 ± 1.1
	IdoA	2.7 ± 0.1	2.4 ± 0.1
	Glc	1.9 ± 0.3	1.5 ± 0.1
	Xyl	2.3 ± 0.1	2.1 ± 0.1
Disaccharide	Rha-GlcA	-	-
	Total	36.4 ± 2.4	39.0 ± 1.9

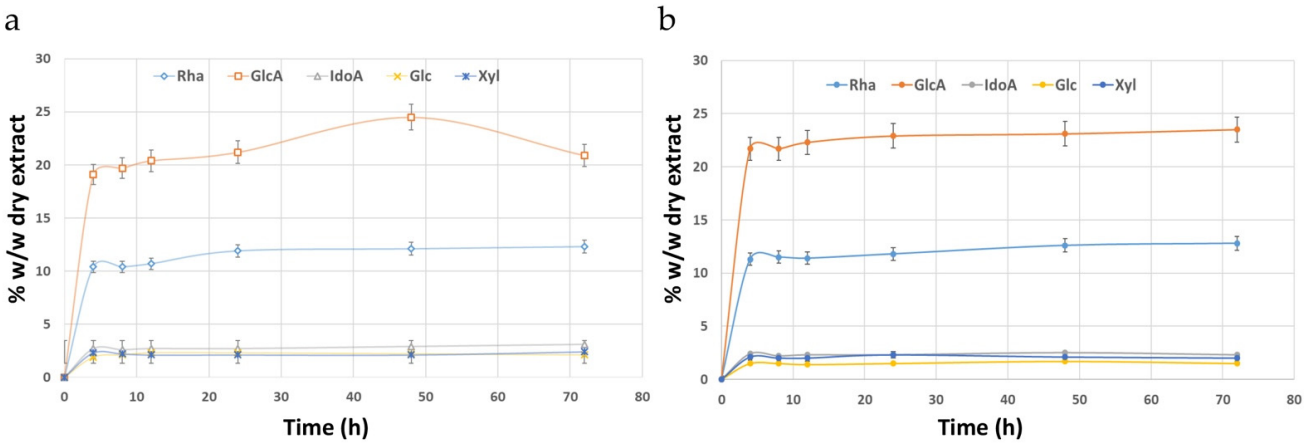

Figure 2. Methanolysis kinetics expressed as number of monosaccharides released over time using (**a**) 2 M HCl and (**b**) 3 M HCl. The data are reported as the average ± SD (n = 3).

With 2 M and 3 M HCl, the total release of monosaccharides after 4 h of reaction was the lowest recorded. As reported by Costa et al. [38], the trend could be related to the degradation of some residues, in particular GlcA, which occurs at the initial stage

of the reaction due to its low stability in acidic environments. This statement is also supported by published studies claiming that HCl is mainly consumed in the first 12 h of the methanolysis [39,40]. As reported by [41], in the first 4 h of the reaction, more acid-sensitive monosaccharides are released, and may further undergo degradation, while starting from 6 h into methanolysis, the break-up of more resistive and protected linkages takes place with an increment in the number of monosaccharides released. It can be seen that the increase in the content of the two main monosaccharides (Rha and GlcA) is linked to the reduction in the Rha-GlcA disaccharide due to the cleavage of the aldo-glucuronosyl linkage in the ulvanobiuronic acid [42].

A higher number of released monosaccharides was observed for a reaction time greater than 24 h in 3 M HCl. Specifically, a noticeable increase could be observed in GlcA and Rha residues while a minor one could be seen in IdoA, Glc and Xyl units. The optimum condition in which the highest release of monosaccharides takes place was established at 48 h in both extracts.

Methanolysis in 2 M HCl/MeOH for 4 h at 100 °C, performed for each polysaccharide extract from *U. ohnoi*, represents an optimal compromise for the effective cleavage of chemical links between the monosaccharide units and prevents degradation after release, as reported by Kidgell et al. [43].

3.2. Microbiological Tests

Obtained raw extracts were finally tested for their biological activities.

3.2.1. Antibacterial Activity

None of the algal extracts showed antibacterial activity at the concentration of 400 μg mL^{-1}, confirming the results previously reported [14,26].

3.2.2. Antibiofilm Activity

The effects of algal extracts at concentrations of 400 μg mL^{-1} on the biofilm formation are reported in Figure 3.

Neither extract (CP and IP) from *U. ohnoi* possessed any relevant antibiofilm activity, either against *Pseudomonas aeruginosa* ATCC 27853 or *Staphylococcus aureus* ATCC 29213 (Figure 3).

The *A. subulata* IP extract reduced the *P. aeruginosa* biofilm by 56%, whereas the *A. subulata* CP extract reduced the *P. aeruginosa* biofilm by 50% (Figure 3a). Neither *A. subulata* extracts, processed with different methodologies, showed a significant difference. The *Sargassum muticum* CP extract strongly reduced the *P. aeruginosa* biofilm (53%) while its IP counterpart exerted a lower effect (38%).

The *Staphylococcus aureus* biofilm was mainly reduced (45%) by the *A. subulata* CP extract (Figure 3b), while the *A. subulata* IP extract exhibited a lower antibiofilm activity (36%) against *S. aureus*. On the contrary, the *S. muticum* IP extract possessed a greater antibiofilm activity (34%) than that obtained with CP extraction (18%).

3.2.3. Emulsification Activity

The emulsifying activity of the crude extracts from *U. ohnoi*, *A. subulata* and *S. muticum* is reported in Table 9.

All the algal extracts possessed emulsifying activity, while at different levels.

Although emulsification indices (E_{24}) of both *U. ohnoi* extracts were lesser than those of extracts of other algae, they exhibited a significant difference between CP and IP methods.

Emulsifying activity of polysaccharides extracted from *A. subulata* and *S. muticum* was higher than that of Triton X, with higher values observed for IP extracts than CP extracts, and the *S. muticum* IP extract had the highest.

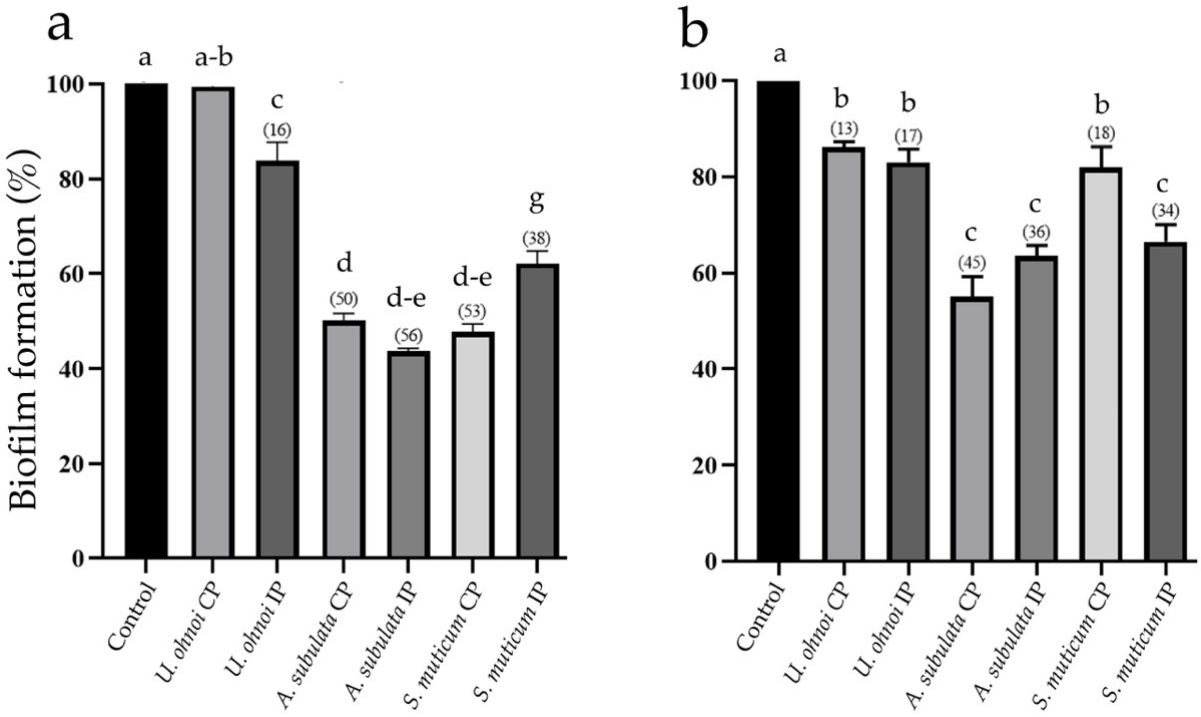

Figure 3. Biofilm formation (%) of (**a**) *Pseudomonas aeruginosa* ATCC 27853 and (**b**) *Staphylococcus aureus* ATCC 29213 in the absence (control, C) or in the presence of the crude extract from *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum* (400 µg mL^{-1} *w*/*v*) obtained using the traditional (CP) and innovative (IP) extraction methods. Data represent mean ± SD for six replicates (*n* = 6). The lowercase letters above the bars denote groups that were found to be significantly different after ANOVA followed by Tukey test. In brackets are data on biofilm reduction as a percentage.

Table 9. Emulsification index (E_{24}) of crude extracts, both from conventional (CP) and innovative (IP) methods, from *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum*. Triton X was used as a positive control at the concentration of 0.05% *w*/*v*. The data are expressed as the average ± SD (*n* = 3). The letters denote groups that were found to be significantly different after ANOVA followed by Tukey test.

Extract	E_{24}
Ulva ohnoi CP	22.5 ± 0.6 ^{a}
Ulva ohnoi IP	38.1 ± 0.5 ^{b}
Agardhiella subulata CP	72.5 ± 0.2 ^{c}
Agardhiella subulata IP	75.0 ± 0.3 ^{c}
Sargassum muticum CP	73.0 ± 0.4 ^{c}
Sargassum muticum IP	89.5 ± 0.9 ^{d}
TritonX	70.5 ± 0.6 ^{c}

3.2.4. Coating Assay

The ability of the extracts to inhibit the early adhesion to polystyrene surfaces of *P. aeruginosa* and *S. aureus* after 18 h is reported in Table 10.

All extracts showed at least a moderate ability to inhibit the adhesion of *P. aeruginosa* and *S. aureus* to polystyrene. An exception was represented by *U. ohnoi*, whose CP extract showed no activity against adhesion of both microorganisms, while the IP extract had a moderate activity only against *P. aeruginosa* adhesion.

Full inhibition of bacterial cell adhesion was observed only for *A. subulata* and *S. muticum* CP extracts against *P. aeruginosa*. More investigations are necessary to elucidate the rationale behind such variations and whether they could be related to polymer fragmentation due to microwaves.

Table 10. Ability to inhibit the adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to poly-styrene surfaces precoated with polysaccharides from *Ulva ohnoi*, *Agardhiella subulata* and *Sargasssum muticum* (400 μg mL^{-1} *w*/*v*) extracted using both traditional (CP) and innovative (IP) methods. Uncoated polystyrene surfaces were used as control (C).

Extract	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Ulva ohnoi CP	–	–
Ulva ohnoi IP	+	–
Agardhiella subulata CP	+++	+
Agardhiella subulata IP	+	+
Sargassum muticum CP	+++	+
Sargassum muticum IP	+	+
Control	–	–

(–) = negative inhibition of adhesion to polystyrene. (+) = moderate inhibition of adhesion to polystyrene. (+++) = inhibition of adhesion to polystyrene.

4. Conclusions

The main goal of the present work was to elucidate the influence of the extraction methodology on the chemical composition and structure of polysaccharides from different macroalgae. Possible alterations in the chemical structure in terms of molecular weight and the monosaccharide composition, specifically for the lesser-known ulvans, were investigated. All of these characteristics define the chemical, physical and, therefore, biological properties of biopolymers.

Polysaccharide extraction from various sources is the most important task in the investigation and application of bioactive polysaccharides, and the chosen protocol may significantly influence the final product. The present experimental plan was meant to compare conventional (CP) and innovative (IP) microwave-assisted polysaccharide extraction protocols, focusing on extraction times, polysaccharide yield, chemical composition and, finally, biological activities of raw extracts. The main objective was to design a sustainable process in terms of energy and time savings.

Under the tested conditions, extraction efficacy was likely dependent on the physicochemical polysaccharide properties. Nevertheless, even if *Agardhiella subulata* and *Sargassum muticum* gave comparable yields of isolated polymers in both tested methods, it is noteworthy that the obtained polysaccharides almost tripled in IP extraction from *Ulva ohnoi*.

As proved by spectroscopy analyses (FTIR-ATR), the use of microwaves, under the tested settings, did not affect the chemical structure of the polysaccharides extracted, even if hypochromic effects were observed in all IP extracts. However, gel permeation chromatography (GPC) allowed us to hypothesize that microwaves in the IP could produce a reduction in the molecular weight of extracts from *A. subulata* and *S. muticum* (bimodal distribution), but not from *U. ohnoi* (monomodal distribution). This hypothesis confirms the literature data, which some authors use to posit that microwave treatment could result in a dishomogeneous distribution of temperature with local increases in the extraction mix [44,45].

To evaluate any differences in biological properties of crude extracts obtained from the two protocols, we tested their antibacterial and antibiofilm activities against *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213, our model microorganisms. Without any antibacterial activity, all the extracts possessed antibiofilm activity against *P. aeruginosa* and *S. aureus*, at different levels. The antiadhesive and emulsi-

fying properties of raw extracts could inhibit the bacterial biofilm formation, likely thanks to their ability to modify the surface properties. Comparing the CP and the IP, the highest differences were observed in the emulsification activity, with the IP extracts from *S. muticum* and *U. ohnoi* being more active than their CP counterparts, suggesting that modifications in their structural composition might occur in extraction processing.

Obtained results indicate that the innovative method of extraction could be used as an alternative to the conventional one to achieve bacterial antiadhesives and emulsifiers in different applications. Nevertheless, considering the large number of variables implicated, such as the algal taxon and the polysaccharide type, more investigations are needed to clarify the entire extraction process.

Finally, we chose to carry out the entire experimentation using natural populations of invasive algae rather than cultivated species in order to propose the valorization of unwanted biomasses, which are commonly treated as a waste, converting them into a prized resource and setting the framework for a sustainable process.

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