

Article

Application of Ultrasound-Assisted Extraction and Non-Thermal Plasma for *Fucus virsoides* and *Cystoseira barbata* Polysaccharides Pre-Treatment and Extraction

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Abstract: Brown algae *Fucus virsoides* and *Cystoseira barbata* are an abundant source of sulfated polysaccharide fucoidan, which has shown a wide range of biological activities. These activities are significantly dependent on the fucoidan chemical composition, which is closely linked with the applied extraction technique and process parameters. In order to overcome the drawbacks of lengthy conventional extraction (CE), advanced extraction techniques, such as ultrasound-assisted extraction (UAE) and non-thermal plasma (NTP), were applied. Furthermore, this study also investigated the efficiency of different solvents as well as UAE and NTP as 5 min pre-treatments prior to CE as a more effective course of cell wall breakage and, consequently, a higher polysaccharide yield (%PS). Apart from %PS, the effect of this procedure on the chemical composition and antioxidant capacity of the extracted polysaccharides was also monitored. When comparing the extraction solvent, the application of 0.1 M H₂SO₄, instead of H₂O, resulted in a three-fold higher %PS, a higher sulfate group, and a lower fucose content. Application of CE resulted in higher %PS, uronic acids, and fucose content as well as oxygen radical absorbance capacity (ORAC) and DPPH values, while the average molecular weight (M_w), sulfate group, and glucose content were lower during CE when compared to 30 min of UAE and NTP treatment. Application of UAE and NTP as 5 min pre-treatments decreased fucose content, while %PS and sulfate content were similar to values obtained when using CE.

Keywords: ultrasound-assisted extraction; non-thermal plasma; polysaccharides; extraction; brown algae; advanced extraction techniques; fucoidan; antioxidant capacity



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1. Introduction

Seaweeds or marine macroalgae are an excellent source of nutrients and bioactive molecules with a broad range of applications in the food, pharmaceutical, cosmetics, and textile industries. Among the nutrients and bioactive molecules present in macroalgae (polysaccharides, proteins, peptides, amino acids, polyphenols, pigments, vitamins, minerals, etc.), polysaccharides (PS) are the most prominent with respect to possible commercial application. According to the latest worldwide statistics on aquaculture compiled by the FAO in 2018, total production of aquatic algae was 32.4 million tons with a total sale value of USD 13.3 billion [1].

Fucus virsoides is an endemic species in the Adriatic Sea, and it is the only species of the *Fucus* genus to occur in the Adriatic Sea [2]. It has a perennial flat dark brown thallus that is dichotomously branched, flattened, and with a distinct midrib [3]. *Cystoseira barbata* is an endemic species of the Mediterranean Sea, growing mostly in the coastal area of the

northern Adriatic and most abundantly during April and May [4]. It is a perennial, large, strongly branched, flexible, brown alga that can grow up to 2 m.

F. virsoides and *C. barbata* are potentially a good source of PS—fucoidan, laminarin, and alginate. Fucoidan is sulfated PS with high fucose content interconnected by α -(1,3) glycoside bonds or by alternating α -(1,3) and α -(1,4) bonds (and very rarely α -(1,2) bonds). Apart from fucose, it contains lower amounts of other monosaccharides including glucose, galactose, mannose, xylose, rhamnose, and uronic acids [2]. It is found in the fibrillar tissue of the cell wall and the intercellular space of brown algae, and it has been established that it possesses a wide range of positive effects such as antioxidant, anti-inflammatory, and antitumor [5–7], which give it great potential for use in a wide range of functional food, cosmeceutical, and pharmaceutical products [8]. Biological functions of fucoidan are closely correlated with its physicochemical properties [9], namely, sulfate group content, sulfate group position, molecular weight, types and ratios of constituent monosaccharides, and features of glycosidic bonds. However, these physicochemical properties are influenced by algal species, location, harvesting season [10], extraction techniques, and different extraction conditions (e.g., time, temperature, solvent, pH, particle size, pressure, agitation speed, and sample-to-solvent ratio) [6,9,11].

Compared to the currently employed conventional extraction (CE) techniques, advanced techniques have advantages of shorter extraction time; lower temperature; reduced energy, cost, and organic solvents consumption [12]. As it was previously summarized [8], microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), and enzyme-assisted extraction (EAE) have been successfully used for brown algae PS extraction. Although these extraction techniques have different principles of operation, their common goal is breakdown of brown algae cell walls, where most of the bioactive molecules, including fucoidan, are stored [13].

UAE is recognized as an effective extraction technique based on the application of high-frequency sound waves (>20 kHz), and it has been studied for the extraction of bioactive compounds from various plant and algal matrices [14–17]. The ultrasonication technique applies physical forces in the extraction of molecules generated by acoustic cavitation, such as shear, shockwaves, microjets, and acoustic streaming [18], which results in rapid formation and collapse of cavitation bubbles within irradiated liquid medium, leading to intense stress and irreversible chain splitting [19].

Recently, the potential of cold or non-thermal plasma (NTP) in isolation of bioactive molecules has also been explored. NTP is generated by the application of an electric or electromagnetic field to a gas. This accelerates the free electrons and ionizes the gas atoms and molecules releasing more free electrons, provoking new ionizations and producing molecular dissociations [20]. Therefore, the plasma is constituted of molecules and atoms in an excited state, positive and negative ions, free radicals, electrons, UV radiation, and reactive oxygen and nitrogen species such as ozone, hydroxyl radicals, superoxide, atomic oxygen, singlet oxygen, nitric oxide, or nitrogen dioxide [20]. These reactive species could damage cell structure, rapidly and easily diffuse into the cells, and provoke damage of lipids, proteins, nucleic acids, and carbohydrates [20]. In the food industry, NTP is mostly used for food preservation and maintenance of food safety due to the fact of its effectiveness in microbial inactivation. However, some studies also reported its application in phenolics extraction from tomato pomace [21], essential oil extraction [22–25], lipid extraction from microalgae *Nannochloropsis gaditana* [26], and as a pre-extraction procedure prior to Soxhlet extraction from coffee grounds [27]. To the best of our knowledge, there are no reported studies on the use of NTP for algal PS extraction.

Since physicochemical properties of algal PS are influenced by various parameters, and the aim of this research was to study the effect of different solvents (i.e., H₂O, 0.1 M HCl, and 0.1 M H₂SO₄) and extraction techniques (i.e., CE, UAE, and NTP) for their extraction from brown algae *F. virsoides* and *C. barbata* and to compare them in terms of yield, chemical composition, and antioxidant capacity. Furthermore, the research goal was to investigate if application of UAE and NTP as a pre-treatment prior to CE would

cause more cell wall breakage and, consequently, higher yield as well as how it would affect chemical composition of the extracted PS. Overall, this study aimed to determine if the application of advanced extraction techniques could successfully extract algal PS but with a reduced time and lower temperature, which is economically and ecologically more beneficial.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents used in this research were of analytical grade. Gelatin, L-cysteine, sodium tetraborate, sulfamic acid, chloroform, potassium hydroxide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 1-phenyl-3-methyl-5-pyrazolone (PMP) were purchased from Acros Organics (Geel, Belgium). Fucoïdan from *Fucus vesiculosus*, phenol, *m*-hydroxydiphenyl, D-(+)-glucose, L-(−)-fucose, D-(+)-mannose, D-(−)-fructose, D-galacturonic acid, L-rhamnose, arabinose, trimethylamine, ammonium acetate, and 2,2′-azobis(2-methylpropionamide) dihydrochloride (AAPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol, acetone, potassium sulfate, and sodium carbonate were obtained from Gram-mol Ltd. (Zagreb, Croatia); Folin–Ciocalteu reagent, acetonitrile, trichloroacetic acid (TCA), and silicone oil from Fisher Scientific (Leicestershire, UK); absolute ethanol, hexane, and ethyl acetate from Carlo Erba Reagents (Cornaredo, Italy). Fluorescein sodium salt was purchased from Honeywell Riedel-de-Haën (Bucharest, Romania); barium chloride from abcr GmbH (Karlsruhe, Germany); sodium hydroxide from Lach-Ner (Zagreb, Croatia); D-(−)-ribose from TCI (Portland, OR, USA); sulfuric acid from Scharlab S.L. (Barcelona, Spain); hydrochloric acid (HCl) from TKI Hrastnik (Hrastnik, Slovenia).

2.2. Algal Material and Preliminary Treatments

In February 2020, from the coastal region of Zadar (Croatia), marine biologist Donat Petricioli harvested and identified brown algae *Fucus virsoides* (44°12′02″ N; 15°28′51″ E) and *Cystoseira barbata* (44°12′42″ N; 15°09′23″ E) (Figure 1). Freshly harvested algae were washed, frozen at −60 °C (ScanCool SCL210P, Labogene ApS, Lillerød, Denmark), and freeze-dried (CoolSafe lyophilizer, 55-9 PRO, Labogene, Lillerød, Denmark) for 24 h. The freeze-dried algae were milled with an electric grinder WSG30E/K (Waring Commercial, Stamford, CT, USA), and the powder was stored at −4 °C for one week before the extraction process.

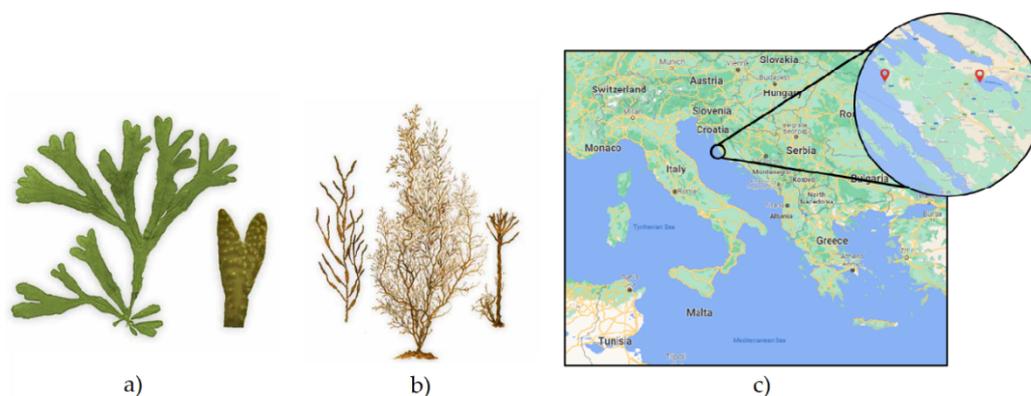


Figure 1. (a) *Fucus virsoides*; (b) *Cystoseira barbata* (c); location of seaweed collection.

2.3. Extraction of PS

Prior to extraction, a pre-treatment process was performed with continuous stirring in two steps under previously established conditions [28]: 18 h at room temperature with acetone followed by 4 h at 70 °C with 96% ethanol. After centrifugation, filtration, and

air-drying, pre-treated dried algae (1 g) were extracted with H₂O, 0.1 M HCl, or 0.1 M H₂SO₄ (30 mL) by the following procedures (Figure 2):

- CE for 3 h at 80 °C under constant stirring (400 rpm) [28];
- UAE with an ultrasonic homogenizer UP200HT (200 W, 26 kHz) (Hielscher, Teltow, Germany), titanium sonotrode Ø14 mm (154 mm²) for 30 min at 100% amplitude, and a cold water bath was used to keep the temperature at approximately 25 °C;
- Five minutes of UAE treatment followed by CE for 3 h at 80 °C under constant stirring (400 rpm);
- Hybrid NTP with a pulse high-voltage generator HVG60/1 PL (Impel, Croatia) for 30 min; electrical voltage—30 kV; frequency—60 Hz; electric current—10 mA; distance between electrodes—1 cm; gas—argon at 1 L h⁻¹;
- Five minutes NTP treatment followed by CE for 3 h at 80 °C under constant stirring (400 rpm).

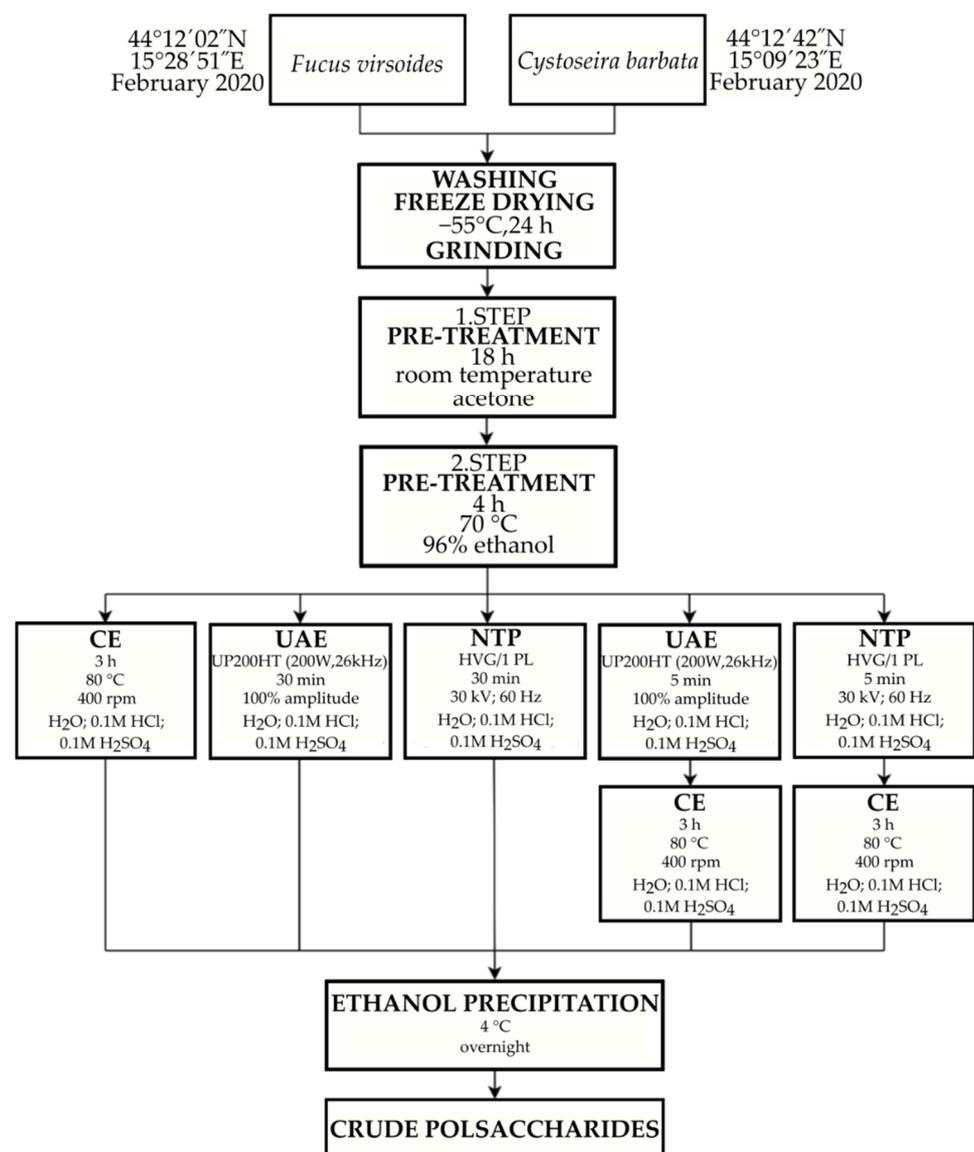


Figure 2. Schematic diagram of the experiment.

Afterwards, a double volume of absolute ethanol (60 mL) was added to the filtrates in order to precipitate PS. The mixture was kept overnight at +4 °C after which it was centrifuged (5500 rpm, 30 min), dried for 48 h at room temperature, and milled in a mortar

and pestle to a fine powder. Dried samples were stored at $-4\text{ }^{\circ}\text{C}$. PS extraction yield (%PS) was calculated according to Equation (1), where WP is the weight obtained after ethanol precipitation, and WA is the algae weight used in each experiment.

$$\% \text{ PS} = \frac{\text{WP}}{\text{WA}} \times 100 \quad (1)$$

2.4. Chemical Composition of PS

The concentration of L-fucose units in algal PS was determined according to the colorimetric assay with L-cysteine, using L-fucose as the standard [29]. Sulfate group content was determined by the turbidimetric barium chloride–gelatin method, with potassium sulfate as the standard, after PS hydrolysis (1 M HCl, $105\text{ }^{\circ}\text{C}$, 5 h) [30]. Total sugar content in PS was measured by the colorimetric phenol–sulfuric acid method with glucose as the standard [31]. Uronic acid concentration was determined through modified sulfamate/m-hydroxydiphenyl colorimetric method with D-galacturonic acid as the standard [32].

2.5. HPLC Analysis of PS Monosaccharide Composition

HPLC analysis of PS monosaccharide composition was performed according to the previously described method [33] with some modifications. The PS sample (100.0 mg) was hydrolyzed in 1 M H_2SO_4 (2.0 mL) and incubated in an oil bath at $110\text{ }^{\circ}\text{C}$ for 4 h. After cooling to a room temperature, 2 M sodium hydroxide was used to neutralize (pH 7) the reaction mixture. An internal standard solution (2 mL) was added, shaken well, diluted to 10 mL, and filtered.

A mixture of filtered hydrolyzed PS solution (or monosaccharide standards) (100 μL), 0.5 M methanolic solution of PMP (100 μL), and 0.3 M aqueous sodium hydroxide (100 μL) was incubated in water bath for 30 min at $70\text{ }^{\circ}\text{C}$. Afterwards, the reaction mixture was cooled to a room temperature and neutralized (pH 7) with 0.3 M hydrochloric acid. Chloroform (1 mL) was added to the solution, shaken well on vortex mixer and centrifuged (5000 rpm, 10 min). The chloroform layer was discarded, while the aqueous layer was extracted two more times with chloroform, and the final aqueous layer was analyzed by HPLC.

The internal standard solution was ribose ($\sim 1\text{ mmol}$) (dissolved in water and diluted to 50 mL). Five known concentrations of the following monosaccharides standards (mixed with internal standard) were prepared by successive dilutions from stock solutions and injected into the instrument: arabinose (0.25–1.75 mg mL^{-1}), glucose (0.25–2 mg mL^{-1}), fucose (0.1875–1.5 mg mL^{-1}), galacturonic acid (0.25–1.75 mg mL^{-1}), rhamnose (0.25–1.75 mg mL^{-1}), fructose (0.25–1.75 mg mL^{-1}), and mannose (0.25–1.75 mg mL^{-1}). Calibration curves were plotted (Table 1) as the ratio of the peak areas of the standard monosaccharide and the internal ribose standard vs. concentration.

Table 1. Calibration curves for PMP sugars.

PMP Sugar	Standard Curve	R^2
fucose	$y = 0.3549x - 0.0712$	0.9940
glucose	$y = 2.8327x - 0.6781$	0.9925
arabinose	$y = 1.2784x - 0.2645$	0.9948
galacturonic acid	$y = 2.3517x - 0.6414$	0.9951
fructose	$y = 0.1633x - 0.0453$	0.9925
rhamnose	$y = 0.0706x + 0.0064$	0.9961

An HPLC Agilent Infinity 1260 system (Agilent Technologies, Santa Clara, CA, USA) equipped with UV/Vis and DAD, an automatic injector, ChemStation software, and a Zorbax Eclipse XDB-C18 column ($4.5 \times 250\text{ mm}$, 5 μm) (Agilent Technologies, Santa Clara, CA, USA) were used for monosaccharide analysis. A mixture of 0.4% trimethylamine in 20 mmol L^{-1} ammonium acetate buffer solution (pH 6.30 with acetic acid) and acetonitrile

(9:1) was used as solvent A, while a mixture of 0.4% triethylamine in 20 mmol L⁻¹ ammonium acetate buffer solution (pH 6.30 with acetic acid) and acetonitrile (4:6) was used as solvent B. Chromatographic separation was achieved with the following gradient: 0–9 min, 10–14% B; 9–30 min, 64% B; 30–35 min, 64% B; and 35–37 min, 10% B. The mobile phase flow rate was set at 1 mL min⁻¹, column temperature at 25 °C, injection volume at 10 µL, and the chromatograms were monitored at 245 nm. All experiments were carried out in duplicate.

2.6. Determination of Molecular Properties

High-performance size exclusion chromatography with refraction index detector (HPSEC-RID) was used to assess molecular properties (average molecular weight (M_w), the number average molecular weight (M_n), and the polydispersity index (PDI)) of *F. virsoides* and *C. barbata* PS extracted with CE, UAE, and NTP with H₂SO₄. The 1260 Infinity II LC system (Agilent Technologies) consisted of a quaternary gradient pump G7111B, an autosampler G4767A, a multicolumn thermostat G7116A, and a refraction index detector G7162A. HPSEC analysis was performed using PL aqua gel–OH guard column (8 µm, 50 × 7.5 mm; Agilent Technologies) and PL aqua gel–OH MIXED-M column (8 µm, 300 × 7.5 mm; Agilent Technologies). Column temperature was 30 °C, RID temperature was 40 °C, mobile phase was H₂O, flow rate was 0.5 mL min⁻¹, and injection volume was 50 µL. Pullulan standards (Agilent Technologies), with M_w in a range from 180 to 700 kDa, were used to construct a molecular weight calibration curve and OpenLAB CDS ChemStation Edition (Agilent Technologies) was used for data collection and processing.

2.7. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was used to measure the antioxidant capacity in *F. virsoides* and *C. barbata* PS extracts obtained with CE, UAE, and NTP with H₂SO₄ [34]. An automated plate reader (BMG LABTECH, Offenburg, Germany) with 96-well plates was used, and data were analyzed by MARS 2.0 software. Fluorescein solution, AAPH, and different Trolox dilutions were prepared in 75 µM phosphate buffer (pH 7.4). After dissolving dry PS in ddH₂O (4 mg mL⁻¹) and filtration, samples were added in a 96-well black plate containing a fluorescein solution (70.3 nM). The plate was incubated at 37 °C for 30 min, and after the first three cycles (representing the baseline signal) to initiate the peroxyl radical generation, AAPH (240 mM) was injected into each well. Different Trolox dilutions (3.12–103.99 µM) were used as the reference standard. During a total measurement time of 120 min, fluorescence intensity (emission at 528 nm and excitation at 485 nm) was observed every 90 s.

2.8. DPPH Radical Scavenging Assay

Spectrophotometric assay was used to measure the ability of the extracts to scavenge DPPH radical. Polysaccharide solution (1 mg mL⁻¹) (1.5 mL) was mixed with DPPH solution (0.2 mM in 70% ethanol) (1.5 mL), shaken on a vortex mixer, and kept at room temperature, in dark, for 30 min. Absorbance decrease was measured at 517 nm in duplicate. The free radical scavenging activity was calculated according to Equation (2):

$$\text{Scavenging effect (\%)} = \frac{A-C}{A} \times 100 \quad (2)$$

where A is the control absorbance (DPPH without sample), and C is the sample absorbance.

2.9. Statistical Analysis

All extractions and measurements, apart from molecular properties determination, were performed in duplicate. The results were analyzed for statistical significance at $p \leq 0.05$, using the STATISTICA 8.0 software (StatSoft Inc., Tulsa, OK, USA). Continuous variables were analyzed by multifactor analysis of variance (ANOVA) and marginal means were compared with Tukey's HSD multiple comparison tests. Dependent variables were:

%PS, sulfate group content (%), and fucose content (%), while independent variables were: (a) seaweed species (*C. barbata* and *F. virsoides*), (b) solvent (H₂O, 0.1M HCl and 0.1 M H₂SO₄), and (c) treatment (CE, 30 min UAE, 5 min UAE + CE, 30 min NTP and 5 min NTP + CE). For comparison of different extraction techniques (CE, UAE, and NTP—independent variable) one-way ANOVA with Tukey's HSD multiple comparison tests were used, where the dependent variables were %PS, total sugar, fucose, sulfate group, uronic acid content, monosaccharide composition, M_w, M_n, and PDI. The design matrix for the experiment and the regression model for each response were calculated as follows [35]:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (3)$$

where Y is the predicted response; β_0 is the fixed response; β_i , β_{ii} , and β_{ij} are the linear, quadratic, and interaction coefficients; X_i and X_j are independent factors.

3. Results and Discussion

3.1. Influence of Algal Species, Solvent, and Extraction Technique on PS Yield

PS from *F. virsoides* and *C. barbata* were extracted using CE as well as advanced techniques: UAE and NTP. Furthermore, UAE and NTP were also applied as 5 min pre-treatments prior to CE. The use of three different extraction solvents (i.e., H₂O, 0.1 M HCl, and 0.1 M H₂SO₄) was also examined on yield and chemical composition of the extracted PS. It is necessary to emphasize that all extracts analyzed in this study were crude (not purified) extracts; thus, it was more accurate to report them as %PS rather than fucoidan yield.

Influence of algae species, extraction solvent, and extraction treatment on polysaccharide yield (%PS), sulfate group, and fucose content of the extracted polysaccharides is shown in Table 2 and regression model equations in Table 3. All studied parameters had a significant ($p \leq 0.05$) influence on %PS. Average crude %PS from *F. virsoides* was 9.75% and from *C. barbata* 8.86%. Although both of these seaweeds are order *Fucales*, PS% highly depended on seaweed species, and within the same species it is also influenced by several other factors, such as extraction procedure and solvent, harvest season, and geographic location as well as the maturity of the plant [36,37]. Usov et al. [38] reported that %PS from 25 different brown seaweed species ranged between 0.4% and 20.4%, while in the study of Turan et al. [39] they constituted up to 25–30%. The only commercially available fucoidan is prepared from *Fucus vesiculosus* [7] which is, along with *Fucus evanescens*, especially attractive as the PS source due to the fact of their wide growth area, growth in shallow waters, and large amounts of alginic acid, fucoidan, and mannitol [38]. On the other hand, *F. virsoides* is a species of brown alga endemic to the Adriatic Sea, and it has not been used for PS extraction so far. When comparing the obtained results with the literature [28], PS% of the *Fucus* genus seaweed have been reported to be as high as 21.5% for *F. distichus* [40] and as low as 1.40% for *F. vesiculosus* [41]. The %PS of *C. barbata* obtained in this research was slightly higher than other similar studies performed on different seaweed from *Cystoseira* genus, where it ranged from 2.8% for *C. crinite* [42] to 5.45% for *C. barbata* [43].

A remarkable significant difference ($p \leq 0.05$) in %PS between different solvents was observed. The highest %PS (15.45%) obtained with 0.1 M H₂SO₄ was more than two-fold higher in comparison with %PS obtained with H₂O (5.67%) and 0.1 M HCl (6.80%). PS extraction was probably facilitated due to the cell wall hydrolysis, which is caused by acid application [44]. Similarly, use of H₂O gave more than a two-fold lower %PS than when 1 M HCl was used in research of brown seaweed *Sargassum fusiforme* by Liu et al. [44]. Since %PS obtained by 0.1 M H₂SO₄ with pH 0.7 was much higher than %PS obtained by 0.1 M HCl with pH 1, it appeared that lowering the pH increased the PS% [45]. Similar findings were reported by Ptak et al. [45], who achieved marginally better fucoidan and laminarin yield with 100 mM HCl (pH 2) then with 10 mM H₂SO₄ (pH 4) for *Fucus* seaweed harvested in France. However, they reported opposite observations for the *Fucus* seaweed harvested

in Germany. Considering these results, H₂SO₄ was chosen as optimal solvent and further used for comparison of the extraction techniques.

Table 2. Influence of the algae species, extraction solvent, and extraction treatment on polysaccharide yield (%PS), sulfate group, and fucose content of the extracted polysaccharides.

	N	% PS	Sulfate Group (%)	Fucose (%)
Algae		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
<i>F. virsoides</i>	30	9.75 ± 0.04 ^b	41.98 ± 0.38 ^a	31.68 ± 0.20 ^b
<i>C. barbata</i>	30	8.86 ± 0.04 ^a	53.60 ± 0.38 ^b	11.19 ± 0.20 ^a
Solvent		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
H ₂ O	20	5.67 ± 0.05 ^a	32.50 ± 0.47 ^a	21.33 ± 0.24 ^b
0.1 M HCl	20	6.80 ± 0.05 ^b	38.02 ± 0.47 ^b	25.97 ± 0.24 ^c
0.1 M H ₂ SO ₄	20	15.45 ± 0.05 ^c	72.86 ± 0.47 ^c	17.00 ± 0.24 ^a
Treatment		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
CE	12	11.74 ± 0.06 ^e	42.74 ± 0.61 ^a	32.41 ± 0.31 ^e
UAE 5 min + CE	12	11.41 ± 0.06 ^d	47.92 ± 0.61 ^b	25.73 ± 0.31 ^c
UAE 30 min	12	6.72 ± 0.06 ^a	44.32 ± 0.61 ^a	27.29 ± 0.31 ^d
NTP 5 min + CE	12	9.39 ± 0.06 ^c	42.12 ± 0.61 ^a	11.96 ± 0.31 ^b
NTP 30 min	12	7.29 ± 0.06 ^b	61.87 ± 0.61 ^c	9.76 ± 0.31 ^a
Algae; solvent		$p \leq 0.05^*$	$p = 0.07$	$p \leq 0.05^*$
<i>F. virsoides</i> ; H ₂ O	10	7.18 ± 0.06 ^c	27.44 ± 0.67 ^a	35.41 ± 0.34 ^d
<i>F. virsoides</i> ; 0.1 M HCl	10	7.72 ± 0.06 ^d	32.33 ± 0.67 ^b	39.34 ± 0.34 ^e
<i>F. virsoides</i> ; 0.1 M H ₂ SO ₄	10	14.35 ± 0.06 ^e	66.19 ± 0.67 ^e	20.29 ± 0.34 ^c
<i>C. barbata</i> ; H ₂ O	10	4.16 ± 0.06 ^a	37.56 ± 0.67 ^c	7.25 ± 0.34 ^a
<i>C. barbata</i> ; 0.1 M HCl	10	5.87 ± 0.06 ^b	43.71 ± 0.67 ^d	12.61 ± 0.34 ^b
<i>C. barbata</i> ; 0.1 M H ₂ SO ₄	10	16.56 ± 0.06 ^f	79.54 ± 0.67 ^f	13.71 ± 0.34 ^b
Algae; treatment		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
<i>F. virsoides</i> ; CE	6	12.64 ± 0.08 ^h	28.43 ± 0.86 ^a	45.27 ± 0.44 ^f
<i>F. virsoides</i> ; UAE 5 min + CE	6	13.27 ± 0.08 ⁱ	45.09 ± 0.86 ^{c,d}	40.30 ± 0.44 ^e
<i>F. virsoides</i> ; UAE 30 min	6	7.57 ± 0.08 ^c	41.76 ± 0.86 ^c	38.60 ± 0.44 ^e
<i>F. virsoides</i> ; NTP 5 min + CE	6	10.17 ± 0.08 ^f	33.66 ± 0.86 ^b	19.22 ± 0.44 ^d
<i>F. virsoides</i> ; NTP 30 min	6	5.11 ± 0.08 ^a	60.98 ± 0.86 ^{f,g}	14.97 ± 0.44 ^c
<i>C. barbata</i> ; CE	6	10.85 ± 0.08 ^g	57.04 ± 0.86 ^f	19.54 ± 0.44 ^d
<i>C. barbata</i> ; UAE 5 min + CE	6	9.54 ± 0.08 ^e	50.75 ± 0.86 ^e	11.16 ± 0.44 ^b
<i>C. barbata</i> ; UAE 30 min	6	5.86 ± 0.08 ^b	46.88 ± 0.86 ^{d,e}	15.98 ± 0.44 ^c
<i>C. barbata</i> ; NTP 5 min + CE	6	8.60 ± 0.08 ^d	50.58 ± 0.86 ^e	4.71 ± 0.44 ^a
<i>C. barbata</i> ; NTP 30 min	6	9.47 ± 0.08 ^e	62.76 ± 0.86 ^g	4.54 ± 0.44 ^a

CE = conventional extraction, UAE = ultrasound-assisted extraction, and NTP = non-thermal plasma. Results are expressed as the mean ± SE. * Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$.

As it can be seen in Table 2, none of the advanced treatments, alone or in combination with CE, resulted in a yield higher than the one achieved with 3 h long CE (11.74%). Application of UAE and NTP for 30 min, without CE, gave significantly lower ($p \leq 0.05$) %PS, i.e., 6.72% and 7.29%, respectively. UAE and NTP as 5 min pre-treatments in combination with

CE resulted in %PS of 11.41% and 9.39%, respectively. These values were higher than those obtained with treatments alone but still lower than the one obtained when using CE. These results indicate that either too short of an extraction time or the lack of higher temperatures could be the reason for lower efficiency of UAE and NTP in PS extraction when compared to CE. This finding is supported by Okolie et al. [13], who obtained *Ascophyllum nodosum* %PS of 4.56% by UAE (35 min, amplitude 40%) and 11.9% by CE (70 °C for 3 × 3 h). On the contrary, in research by Hanjabam et al. [46], the UAE method (30 min, 50% amplitude) gave higher *Sargassum wightii* %PS (14.61 g 100 g⁻¹) than CE (10.59 g 100 g⁻¹; 85 °C for 2 h). Moreover, in research by Kadam et al. [47], laminarin yield of *L. hyperborea* and *A. nodosum* extracted by 15 min UAE treatment was 15.02–91.76% higher, depending on the solvent.

Table 3. Regression model equations and determination coefficients (R^2) for polysaccharide yield (%PS), sulfate group, and fucose content.

	Regression Model	R^2
%PS	$y = 32.81 - 10.46X_1 - 14.02X_2 + 3.76X_2^2 - 4.86X_3 + 0.27X_3^2 + 2.62X_1X_2 + 1.45X_1X_3 - 0.02X_2X_3$	0.775
Sulfate group (%)	$y = 51.34 + 21.11X_1 - 51.47X_2 + 14.66X_2^2 - 10.55X_3 + 2.18X_3^2 + 1.61X_1X_2 - 4.24X_1X_3 + 3.53X_2X_3$	0.795
Fucose (%)	$y = 103.44 - 55.64X_1 + 7.30X_2 - 6.81X_2^2 - 10.34X_3 - 0.57X_3^2 + 10.79X_1X_2 + 4.52X_1X_3 + 0.53X_2X_3$	0.761

X_1 = algae species (1 = *F. virsoides*; 2 = *C. barbata*), X_2 = solvent (1 = H₂O; 2 = 0.1 M HCl; 3 = 0.1 M H₂SO₄), and X_3 = extraction technique (1 = CE; 2 = UAE 5 min + CE; 3 = UAE; 4 = NTP 5 min + CE; 5 = NTP).

3.2. Influence of Algal Species, Solvent, and Extraction Technique on PS Chemical Composition

As already mentioned, algal PS chemical composition is a key factor in determination of their biological activities; however, it is highly influenced by various parameters. The influence of algal species (*F. virsoides*, *C. barbata*), solvent (H₂O, 0.1 M HCl, 0.1 M H₂SO₄) and extraction method (CE, 30 min UAE, 30 min NTP, 5 min UAE + CE, 5 min NTP + CE) on fucose and sulfate group content is shown in Table 1. All studied parameters had a statistically significant ($p < 0.05$) influence on fucose and sulfate group content.

Sulfate group content is particularly important for biological activities, such as anti-HIV [48], anticoagulant [49], and antioxidant [50], and on levels below 20% it leads to a complete loss of antiproliferative and anticoagulant activity [51]. The average sulfate group content of *F. virsoides* obtained in this research was 41.98%, which is slightly above the range (9–40.3%) reported for the *Fucus* genus [40,41,51–54]. The average *C. barbata* sulfate group content obtained in this research was 53.60%, which is higher than the range (14.65–22.3%) reported for the *Cystoseira* genus in other studies [43,55–57]. The lower amount of sulfate groups found in *F. virsoides* when compared to *C. barbata* indicates the presence of higher amounts of non-sulfated fucose in the linear part of the PS or the lower amount of branching zones that contain sulfated fucose [41]. Acid induces the sulfate ester cleavage so more sulfate groups can be liberated [58,59], which explains the significantly ($p \leq 0.05$) higher sulfate content that was obtained by acid extractions in comparison to water extraction. However, Liu et al. [44] and Saravana et al. [60] reported opposite results for *Sargassum fusiforme* and *Saccharina japonica*, respectively. Average sulfate content obtained by 0.1 M H₂SO₄ was almost two-fold higher than the one obtained by 0.1 M HCl. This is in agreement with previous findings that diluted HCl tends to yield low sulfate fucoidan, while diluted H₂SO₄ tends to yield high sulfate fucoidan as it may interfere with sulfate analysis due to the sulfate group in the sulfuric acid [58,59]. Along with seaweed species and harvesting season, extraction technique is one of the most important factors that influences the sulfate group content [46]. For both examined algae, 30 min NTP treatment resulted in significantly ($p \leq 0.05$) higher sulfate group content. CE gave the lowest sulfate group content in *F. virsoides*, while in *C. barbata* the sulfate group content obtained by CE

was lower than 30 min NPT but higher than any other studied treatment. Okolie et al. [13] reported lower sulfate content in UAE extraction in comparison with CE, while Hanjabam et al. [46] found similar sulfate content obtained with UAE and CE.

Studies have reported that fucose content varies among seaweeds species, extraction techniques [37], extraction conditions [46], harvest time [51,61], plant part [38], and maturity of the seaweed [37], and it was suggested that the higher amount of fucose may contribute to the higher cytotoxic activity of algal PS [62]. The average fucose content obtained in this research was 31.68% and 11.19% for *F. virsoides* and *C. barbata*, respectively. These values are comparable with values reported by other authors [28]. Contrary to the sulfate group content, *C. barbata* fucose content was significantly lower than in *F. virsoides*, which indicates higher degree of sulphation for each fucose in *C. barbata*. The highest fucose content was obtained when using 0.1 M HCl followed by H₂O and 0.1 M H₂SO₄. Higher fucose content, in fucoidan from sporophytes of *Splachnidium rugosum*, was also achieved by 0.2 M HCl than 1% H₂SO₄ [63], and the use of acid (0.03 M HCl) instead of water resulted in a 6% increase in the amount of fucose [64]. However, acid might cause unwanted degradation of functional groups attached to the fucoidan backbone which can result in the failure of fucose detection [58]. In present study, fucose content was significantly ($p \leq 0.05$) higher in extracts obtained by CE than any of the UAE and NTP treatments due to the destructive effect of ultrasound on the structure of fucose [46]. A similar trend of higher fucose content obtained by CE than UAE was previously reported [46], as well as similar fucose content obtained with UAE and conventional hot water extraction [13,65]. Both of the NTP treatments lead to lower fucose content than ones obtained with UAE treatments, suggesting that the effect of OH radicals formed with NTP on glycosidic bonds [66] is inferior in comparison with cavitation effect of ultrasound.

Fucoidan antioxidant and anticoagulant bioactivity [67–69] has been positively correlated with sulfate:fucose (or total sugar) ratio, and it was noted that anticoagulant activity remarkably decreased when the ratio was below 1 [69], while fucoidan with ratio below 0.3 had no anticoagulant activity [68]. The average sulfate:fucose ratio obtained in this research was 1.33 (*F. virsoides*) and 4.79 (*C. barbata*). Previous studies reported that for *F. serratus*, the sulfate:fucose ratio ranged between 0.73 and 1.5 [51,70,71], for *F. vesiculosus* between 1.1 and 2.5 [51,70,72], for *A. nodosum* between 1.1 and 2.7 [51,72,73], for *C. sedoides* between 0.3 and 0.9 [42,58], for *C. compressa* it was 0.27 [42], and for *C. crinite* 0.36 [42]. The highest reported sulfate:fucose ratio was 5.2 for *Ecklonia maxima* [58] fucoidan extracted with 0.15 M HCl. The same *E. maxima* fucoidan but extracted with water had sulfate:fucose ratio of 0.7. Similar to the results of this research, where extracts obtained with 0.1 M H₂SO₄ had a much higher sulfate:fucose ratio than water extracts, *Laminaria pallida* and *Splachnidium rugosum* fucoidan extracted with acid had a significantly higher sulfate:fucose ratio [60]. PS obtained with CE had the lowest sulfate:fucose ratio, followed by UAE treatments, while in NTP treatments this ratio was much higher. As it was previously discussed, this higher ratio was the result of higher sulfate group and lower fucose content obtained by NTP treatments. Hanjabam et al. [46] reported that *Sargassum wightii* fucoidan had average sulfate:fucose ratio of 0.67 with hot water extraction and 0.72 with UAE.

3.3. Comparison of CE, UAE, and NTP Treatment

As it was mentioned earlier, H₂SO₄ was chosen as the optimal solvent and used for further comparison of 3 h long CE with 30 min UAE and NTP treatments, in terms of yield, chemical composition, molecular properties, and antioxidative capacity. The results are shown in Tables 3 and 4 and Figure 2. CE was considerably longer and performed on higher temperatures than UAE and NTP, which could explain the higher %PS, lower amount of sulfate groups, and higher proportion of uronic acids (Table 4) as described by several studies [74–76]. For *F. virsoides*, %PS obtained by UAE and NTP was respectively two- and three-fold lower than in CE. It seems as if the UAE and NTP treatments were too short or the temperature was too low for higher PS extraction, since PS extraction was generally a lengthy process. *F. virsoides* and *C. barbata* %PS obtained with CE were 18.53% and 16.47%,

while their respective predicted values (from regression model equations) were 18.82% and 17.65%.

Table 4. Polysaccharide yield (%PS) and chemical composition of *Fucus virsoides* and *Cystoseira barbata* polysaccharides obtained with CE, UAE and NTP.

		% PS	Total Sugars (mg g ⁻¹)	Fucose (%)	Sulfate Group (%)	Uronic Acid (%)
		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
<i>F. virsoides</i>	CE	18.53 ± 0.00 ^e	20.17 ± 0.00 ^e	41.54 ± 0.01 ^f	28.46 ± 0.01 ^a	20.06 ± 0.00 ^e
	UAE	12.14 ± 0.28 ^c	11.58 ± 0.58 ^d	14.75 ± 0.12 ^c	83.37 ± 0.20 ^b	1.77 ± 0.35 ^b
	NTP	6.10 ± 0.20 ^a	22.95 ± 0.24 ^f	9.49 ± 0.16 ^b	88.31 ± 5.56 ^b	3.68 ± 0.25 ^c
<i>C. barbata</i>	CE	16.47 ± 0.18 ^d	6.34 ± 0.18 ^b	22.53 ± 0.14 ^d	35.53 ± 0.80 ^a	15.72 ± 0.34 ^d
	UAE	11.80 ± 0.07 ^c	1.19 ± 0.12 ^a	31.70 ± 0.12 ^e	90.44 ± 1.40 ^b	1.16 ± 0.02 ^{a,b}
	NTP	9.64 ± 0.15 ^b	10.120.28 ^c	2.83 ± 0.08 ^a	88.86 ± 1.73 ^b	0.46 ± 0.04 ^a

CE = conventional extraction, UAE = ultrasound-assisted extraction, and NTP = non-thermal plasma. Results are expressed as the mean ± SD. * Statistically significant variable at $p \leq 0.05$. Values with different letters within a column are statistically different at $p \leq 0.05$.

Even though longer extraction time and higher temperatures can lead to fucose chain degradation [37] and consequently lower fucose content [75,77], CE which is considerably longer and performed at higher temperature than UAE and NTP resulted with higher fucose content. Possible explanation could be that the bond between fucoses was disrupted due to the intensive treatment of these advanced techniques. On the contrary, sulfate group content was the lowest in extracts obtained by CE indicating that their bonds were disrupted by long extraction at high temperature rather than cavitation or partially ionized gas. Some research showed that lower uronic acids content is more desirable property of algal PSs since higher uronic acids content shows lower anticoagulant [78] and anticomplement activity [79]. Therefore, UAE and NTP showed an advantage in comparison with CE since significant reduction of uronic acids can be observed in those extracts.

The monosaccharide compositions of CE, UAE, and NTP extracted PSs from *F. virsoides* and *C. barbata* are given in Table 4. Generally, in all extracted PSs, L-fucose was the predominant monosaccharide, which was expected and in accordance with most of the previously published studies [65,67,73,80,81]. Other detected monosaccharides were glucose, galacturonic acid (oxidized form of D-galactose), and arabinose, while mannose, rhamnose, and fructose were not detected. Our results showed that the monosaccharides ratio varied according to the algal species and extraction method used, which is in accordance with literature data [81]. For both algae, it can be observed that PS extracted with CE had higher fucose and lower glucose content than PS extracted with UAE and NTP. In both algae, PS extracted with UAE had similar concentration of fucose, glucose and galacturonic acid.

HPSEC was used to analyze molecular properties of the PS extracts, and the results of the weight average molecular weight (M_w), the number average molecular weight (M_n), and the polydispersity index (PDI) are presented in Table 5. M_w is the molecular size of the sample, while M_n represents an average molecular weight of all polymer chains within a sample [82]. M_w is more influenced by high molecular weight chains, whereas M_n is more influenced by the lower molecular weight chains [82]. PDI is the ratio between M_w and M_n , and it measures the heterogeneity of molecular weight distributions of polymers, where larger differences between M_w and M_n (larger PDI) indicate a more heterogeneous molecular weight distribution [82].

Table 5. Monosaccharide composition and molecular properties of *Fucus virsoides* and *Cystoseira barbata* polysaccharides obtained with CE, UAE and NTP.

		Monosaccharide Composition (%)				Molecular Properties		
		Glucose	Fucose	Galacturonic Acid	Arabinose	M _w (kDa)	M _n (kDa)	PDI (M _w /M _n)
<i>F. virsoides</i>		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
	CE	18.65 ± 0.24 ^a	44.83 ± 0.45 ^c	19.48 ± 0.26 ^b	17.04 ± 0.25 ^a	693.43 ^b	264.42 ^b	2.62 ^{a,b}
	UAE	31.47 ± 0.32 ^b	36.30 ± 0.34 ^a	32.23 ± 0.31 ^d	n.d.	1133.78 ^e	500.16 ^e	2.27 ^{a,b}
	NTP	46.20 ± 0.27 ^c	39.83 ± 0.41 ^b	13.97 ± 0.14 ^a	n.d.	16.38 ^a	16.38 ^a	1.00 ^a
<i>C. barbata</i>	CE	n.d.	100 ^e	n.d.	n.d.	766.00 ^c	322.87 ^c	2.37 ^{a,b}
	UAE	31.04 ± 0.29 ^b	35.96 ± 0.33 ^a	33.00 ± 0.22 ^d	n.d.	1651.22 ^f	616.65 ^f	2.68 ^b
	NTP	19.15 ± 0.18 ^a	51.08 ± 0.23 ^d	29.76 ± 0.25 ^c	n.d.	930.83 ^d	492.50 ^d	1.89 ^{a,b}

M_w = weight average molecular weight, M_n = number average molecular weight, PDI = polydispersity index, CE = conventional extraction, UAE = ultrasound-assisted extraction, and NTP = non-thermal plasma. Results are expressed as the mean ± SD. * Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$.

The M_w of CE, UAE, and NTP extracted PSs ranged from 16.38 to 1133.78 kDa for *F. virsoides* and 766 to 1651.22 kDa for *C. barbata*. Obtained values were within the range of reported fucoidan M_w values, 1.4–1323 kDa [41,83]. Algal species, growth conditions, and extraction methodology [82] are some of the factors influencing algal PS molecular weight, therefore explaining the slight difference when comparing measured values with literature ones. According to their molecular weight, fucoidans can be classified as low-molecular-weight fucoidans (<10 kDa), medium-molecular-weight fucoidans (10–10,000 kDa), and high-molecular-weight fucoidans (>10,000 kDa) [84]. For both algae, the highest M_w was achieved in samples obtained by UAE, while the lowest M_w in *C. barbata* was achieved in samples obtained by CE and *F. virsoides* by NTP. Likewise, *N. zanardinii* M_w obtained by UAE was higher (1021 kDa) than one obtained with CE (823 kDa) [81], while M_w of *A. nodosum* fucoidan extracted by CE (40.2 kDa) was significantly lower when compared to samples obtained by UAE (121.1 kDa) [13]. PS was polydispersed with PDIs higher than one unlike natural polymers, such as proteins, which are usually monodispersed with a PDI of approximately one. The PDI of PS extracted in this study ranged from 1 to 2.68, and it was within the 1–6.2 range of reported fucoidan PDI values [56,81]. For *C. barbata*, PDI was higher in the samples obtained by UAE, indicating larger degradation during the extraction process, while for *F. virsoides*, the results were the opposite. Likewise, PDI was lower in *N. zanardinii* fucoidan extracted with UAE (1.27) in comparison with CE (1.56) [81].

3.4. Antioxidant Capacity

The antioxidant capacity of *F. virsoides* and *C. barbata* PS extracts obtained with CE, UAE and NTP, with H₂SO₄ as a solvent was assessed with ORAC and DPPH assays. Obtained results are presented in Figure 3. PS from *F. virsoides* obtained by CE had the highest ORAC value of 37.14 μmol TE g⁻¹, while UAE and NTP extracts had ORAC values of 19.97 and 20.21 μmol TE g⁻¹, respectively. However, the ORAC value was not significantly different among extraction techniques for *C. barbata*. For both algae, the highest DPPH value was determined in CE compared to both of the advanced techniques. Despite the fact that antioxidant capacity of fucoidan has been previously proved, the relationship between antioxidant capacity and chemical structure remained unknown. It is recognized that antioxidant capacity is not influenced only by one factor, but rather a combination of few physicochemical characteristics such as sulfate group content, uronic acid content, protein content, and molecular weight [44]. For this reason, correlation between physicochemical characteristics (i.e., sulfate group, uronic acid, M_w, and PDI) and antioxidant measurements (i.e., ORAC and DPPH) were evaluated. For *F. virsoides* only uronic acid content had strong positive correlation ($r > 0.8$) with ORAC and DPPH values. For *C. barbata*, uronic acid content showed strong positive ($r > 0.8$) and M_w strong negative ($r < -0.8$) correlation with DPPH values. Literature data showed that there was no correlation of ORAC values with the total sulfate content and M_w of brown algae *Lessonia vadosa* and red algae *Gigartina skottsbergii* and *Schizymenia binderi* [85].

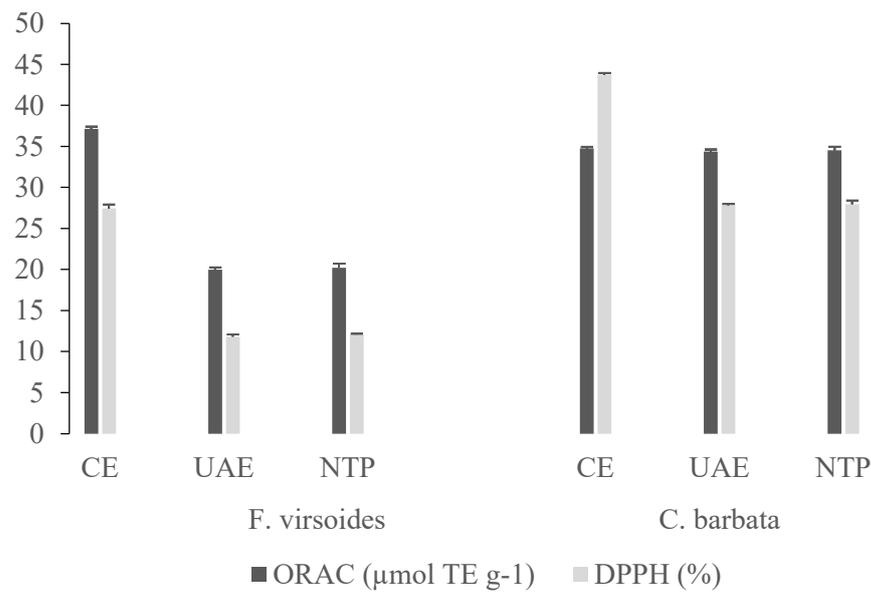


Figure 3. Antioxidant capacity of *F. virsoides* and *C. barbata* polysaccharides obtained by CE, UAE, and NTP as determined by ORAC and DPPH assays.

4. Conclusions

The results of present study showed that the advanced extraction techniques, UAE and NTP, have a great potential for *F. virsoides* and *C. barbata* PS extraction, even though higher %PS was achieved with CE, most likely due to the longer extraction time and higher temperatures. However, a significant time reduction, from 3 h to 30 min, and application of lower temperatures, are beneficial from ecological and economic points of view. Furthermore, both of these advanced techniques resulted in higher amounts of sulfate groups, lower proportions of uronic acids, and higher M_w , which are more desirable properties in terms of PS biological activity. UAE and NTP as 5 min pre-treatments followed by CE did not cause more cell wall breakage, as they gave more or less comparable %PS to that of CE alone.

Diverse biological activities previously attributed to brown algae fucoidan makes them interesting for use in processed and functional food, pharmaceutical, and chemical industries; thus, all these additional improvements in their extraction method could be extremely beneficial from an industrial point of view.

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