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Molecular weight distribution of polysaccharides from edible seaweeds by high-performance size-exclusion chromatography (HPSEC)

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ABSTRACT

Biological properties of polysaccharides from seaweeds are related to their composition and structure. Many factors such as the kind of sugar, type of linkage or sulfate content of algal biopolymers exert an influence in the relationship between structure and function. Besides, the molecular weight (MW) also plays an important role. Thus, a simple, reliable and fast HPSEC method with refractive index detection was developed and optimized for the MW estimation of soluble algal polysaccharides. Chromatogram shape and repeatability of retention time was considerably improved when sodium nitrate was used instead of ultrapure water as mobile phase. Pullulan and dextran standards of different MW were used for method calibration and validation. Also, main polysaccharide standards from brown (alginate, fucoidan, laminaran) and red seaweeds (kappa- and iota-carrageenan) were used for quantification and method precision and accuracy. Relative standard deviation (RSD) of repeatability for retention time, peak areas and inter-day precision was below 0.7%, 2.5% and 2.6%, respectively, which indicated good repeatability and precision. Recoveries (96.3–109.8%) also showed its fairly good accuracy. Regarding linearity, main polysaccharide standards from brown or red seaweeds showed a highly satisfactory correlation coefficient ($r > 0.999$). Moreover, a good sensitivity was shown, with corresponding limits of detection and quantitation in mg/mL of 0.05–0.21 and 0.16–0.31, respectively. The method was applied to the MW estimation of standard algal polysaccharides, as well as to the soluble polysaccharide fractions from the brown seaweed *Saccharina latissima* and the red *Mastocarpus stellatus*, respectively. Although distribution of molecular weight was broad, the good repeatability for retention time provided a good precision in MW estimation of polysaccharides. Water- and alkali-soluble fractions from *S. latissima* ranged from very high (>2400 kDa) to low MW compounds (<6 kDa); this high heterogeneity could be attributable to the complex polysaccharide composition of brown algae. Regarding *M. stellatus*, sulfated galactans followed a descending order of MW (>1400 kDa to <10 kDa), related to the different solubility of carrageenans in red seaweeds. In summary, the method developed allows for the molecular weight analysis of seaweed polysaccharides with very good precision, accuracy, linearity and sensitivity within a short time.

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1. Introduction

Seaweeds are a rich and easily renewable source of structurally and functionally unique polysaccharides, which are increasingly used as natural thickeners, formulation stabilizers, or gelling agents in applications ranging from food industry to pharmaceutics [1]. For brown seaweeds, soluble polysaccharides are alginates, fucoidans and laminarans. For red seaweeds, soluble polysaccharides are sulfated galactans, agar and carrageenans [2,3].

Alginate is the salt of alginic acid, a gelling polyuronide, composed of mannuronic (M) and guluronic (G) acid [4]. Laminarans and fucoidans are considered as the main water-soluble polysaccharides of brown algae. Laminaran is the principal storage polysaccharide of brown seaweeds and is composed of β-glucan [5,6]. Fucoidans, a unique class of sulfated fucans isolated from many brown seaweeds, have not been found in other algae or plants [7]. Their composition varies with the species and the precise structural characteristics have not yet been elucidated, but essentially they always contain fucose and sulfate [8,9], with small proportions of galactose, xylose, mannose and uronic acids [6,10]. Carrageenan is a generic name given to a family of high-molecular-weight

Abbreviations: GPC, gel permeation chromatography; GFC, gel filtration chromatography; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; LOD, limit of detection; LOQ, limit of quantitation; MW, molecular weight; *r*, correlation coefficient; RI, refractive index; RSD, relative standard deviation; SEC, size-exclusion chromatography.

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sulfated polysaccharides isolated from red seaweeds, made up of repeating α-(1,3)-galactose and β-(1,4,3,6)-anhydro-D-galactose alternating units [6,11,12]. Carrageenans are classified in three industrially relevant types (κ-, λ- and ι-carrageenans), which differ in the amount and position of their ester sulfate substitutes and (3,6)-anhydrogalactose content.

These water soluble biopolymers possess important pharmacological activities such as anticoagulant, antioxidant, antiproliferative, antitumoral, anticomplementary, anti-inflammatory, antiviral, antipeptic and antiadhesive activities [13–17]. The relationship between structure and biological activities of algal polysaccharides is not yet clearly established. Usually, water solubility, average molecular weight, chain conformation, and introduction of suitable ionic groups with appropriate degree of substitution can change the bioactivities of polysaccharides [18]. For example, anticoagulant activity of sulfated fucans increases with molecular weight [7,19]. As molecular weight plays an important role on the relationship between structure and function of polysaccharides, it is important to develop a simple but effective method for the molecular weight determination of soluble algal polysaccharides.

Several ways of measuring molecular weight of polysaccharides have been reported in the literature, such as size-exclusion chromatography (SEC), light scattering, sedimentation analysis in analytical ultracentrifugation and intrinsic viscosity [20]. Light scattering and analytical ultracentrifugation methods are reliable and provide an absolute value of molecular mass. The intrinsic viscosity method is easy to operate however it relies on the measurements of viscosity at low sample concentration with consequent difficulties in accurate measurements of low viscosity samples [20]. SEC is the most widely used method for the molecular characterization of polymers in general. The method appeared in the late 1950s and was named gel permeation chromatography (GPC) [21] or gel filtration chromatography (GFC) [22]. It provides information on relative or absolute molecular mass distribution depending on the standards used for calibration. Pullulans and dextrans are the most widely used standards for this purpose. Further breakthroughs in SEC have been the development of online detectors, especially light-scattering detectors [23] and viscometers. Light-scattering detectors yield absolute molecular weights (i.e., no need for a calibration curve) through low-angle laser light scattering, multi-angle laser light scattering, or triple detection [24]. Viscometers enable the determination of molecular weight using a universal calibration curve [24]. And more recently mass spectrometers detectors [24] with the great advantage of providing absolute value of molecular mass without the need for standards or other auxiliary measurements.

The HPSEC system used in this research employs refractive index (RI) detection. This is the most traditional and universal detector for SEC, and has a great advantage for the analysis of polymers since the signal is directly proportional to the polymer concentration (mainly in the case of homopolymers), thus providing an estimation of the molecular weight distribution of the polymer [25]. Moreover, the use of a polymer-based TSK-GEL PW column, more suitable for analyzing water-soluble polymers, gave us the advantage of using aqueous solvents, with fast and easy sample preparation. The column was calibrated with the pullulan and dextran molecular weight standards and commercially available polysaccharide standards of seaweeds, such as, sodium alginate, laminaran, fucoidan and carrageenan were used for the validation of the method. The method was then applied for the molecular weight estimation of soluble polysaccharide fractions from the brown seaweed *Saccharina latissima* and the red *Mastocarpus stellatus*, respectively.

In order to characterize better the relationship between structure and biological activities of algal polysaccharides, our aim was to develop and optimize a simple, reliable and fast HPSEC method with refractive index detection for determining the MW distribution of soluble polysaccharides from brown and red seaweeds.

2. Material and methods

2.1. Reagents and standards

Ultrapure water from Millipore Milli-Q (18.2 MΩ cm, equipped with a Millipack: 0.22 μm filter) was used for the preparation of solutions and mobile phase.

Standards of different molecular mass were used to perform the calibration curves. A Shodex pullulan standard P-82 kit (range of molecular weights in kDa: P-800 = 788, P-400 = 404, P-200 = 212, P-100 = 112, P-50 = 47.3, P-20 = 22.8, P-10 = 11.8, P-5 = 5.9; Showa-Denko, Japan) was obtained from Waters Chromatography, S.A. (Madrid, Spain). Dextran standards: blue dextran-2000, T-500, T-70 and T-10 with molecular mass ranging from 2000 to 10 kDa were obtained from Pharmacia Biotech Europe GmbH (Barcelona, Spain).

Commercial standards of seaweed polysaccharides, fucoidan (from *Fucus vesiculosus* F-5631), sodium alginate (D-7924), laminaran (from *Laminaria digitata* L-9634), carrageenan (type I, C-1013) and iota-carrageenan (type II, C-1138) were obtained from Sigma–Aldrich Chemicals (Alcobendas, Madrid, Spain). Each of these standards was separately injected at 0.5, 1.0 and 2.0 mg/mL (in ultrapure water solution) for the HPSEC analysis.

2.2. Instrument and chromatographic conditions

The HPLC system was equipped with the following instruments: Kontron autosampler 360, Agilent quaternary pump system 1200 Series with online degasser, Agilent differential refractometer 1100 Series (RI detector), Jones chromatography thermostatic oven, Agilent HPLC control unit 1100 Series (console table) and Kontron Data System 450-MT2.

The separation was performed on a TSK-Gel G 5000 PW stainless steel column (300 × 7.5 mm i.d.) with a TSK-Gel PWH guard column (75 × 7.5 mm i.d.) from TosoHaas (Tecknokroma, Barcelona, Spain). The column was eluted isocratically either with ultrapure water or 0.1 M sodium nitrate (vacuum-filtered through 0.45 μm and degassed), at 40 °C with a flow rate of 0.8 mL/min. Standards and polysaccharide fractions were filtered through 0.45 μm (cellulose acetate filters, 25 mm diameter, Análisis Vínicos, Tomelloso, Toledo, Spain) for aqueous samples and injected (50 μL) into the HPLC. Under the conditions used chromatographic runs took less than 15 min.

2.3. Validation of proposed method

2.3.1. Standard curves of polysaccharide for molecular weight estimation

Series of pullulan standards (788, 404, 212, 112, 47.3, 22.8, 11.8 and 5.9 kDa) and dextran standards (2000, 500, 70 and 10 kDa) were used to calibrate the system. Each of these standards was separately injected at 0.5, 1.0 and 2.0 mg/mL (in ultrapure water solution) in triplicate ($n = 9$). A standard calibration curve for the logarithm of the molecular weight versus the HPSEC retention time was obtained for each series of standards.

2.3.2. Standard curves of commercial polysaccharides for quantification

The external standard calibration method was based upon standard solutions of commercial polysaccharides from brown and red seaweeds, namely: alginate, fucoidan, laminaran, kappa-carrageenan and iota-carrageenan. Calibration curves (peak area versus concentration expressed in mg/mL) were prepared at 0.5, 1.0

and 2.0 mg/mL. These solutions were separately injected in triplicate ($n = 9$). The regression curves of each polysaccharide separated according to its molecular weight were obtained. The correlation coefficients, slopes, intercepts and standard deviations of the curves were used to determine the linearity and limits of detection (LOD) and quantitation (LOQ).

2.3.3. Precision

The precision of the method was assessed in terms of repeatability and intermediate (inter-day) precision. Repeatability was expressed as relative standard deviation (RSD) percent of individual measurements of three replicate determinations on the same day and three determinations on three different days. Standard solutions of commercial polysaccharides at 2 mg/mL were used. The RSD (%) values of the results corresponding to retention time and peak area were determined.

2.3.4. Accuracy

To study the reliability and suitability of the HPSEC method, recovery experiments were carried out. The accuracy was determined as percent ratio of the commercial polysaccharide concentration calculated from the calibration line versus nominal polysaccharide concentration at three concentration levels (0.5, 1.0, 2.0 mg/mL) on three replicate measurements. Accuracy was acceptable when the determined concentration (% recovery) reached from 85% to 115% of nominal concentration.

2.4. Algal material

The brown seaweed sweet Kombu [*Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders, formerly *Laminaria saccharina* (Linnaeus) J.V. Lamouroux)] and the red seaweed *Mastocarpus stellatus* (Stackhouse) Guiry were obtained from a local supplier (Porto-Muiños, Cambre, Coruña, Spain). Marine algae were cleaned from epyphytes and sand, washed with tap running water, dried and milled before analysis.

2.5. Sequential extraction of polysaccharide fractions from seaweeds and preparation for HPSEC analysis

The procedure was based on the different solubility of the polysaccharides from seaweeds, as previously reported [26]. Briefly, a dry algal sample (5 g) was extracted with distilled water (500 mL) with constant stirring at 22 °C for 1 h [fraction 1, (F1)]; then the insoluble residue was again extracted with water (500 mL) at 60 °C for 1 h [fraction 2, (F2)], to remove the bulk of water-soluble polysaccharides. The water-insoluble residue was then sequentially extracted with 0.1 M HCl (500 mL) [acid-soluble fraction 3, (F3)] and with 2 M KOH (500 mL) [alkali-soluble fraction 4, (F4)], each treatment at 37 °C for 16 h. After each extraction step a soluble fraction was obtained by centrifugation (12,000 × *g*, 30 min). The final insoluble residue obtained was washed with 2 M HCl until neutral pH, exhaustively dialyzed (dialysis tubing size 9/28.6 mm, molecular weight cut-off 12–14 kDa, Medicell International Ltd., London) against running tap water (7 L/h) at 25 °C for 48 h and then freeze-dried [fraction 5, (F5)]. Thus, by this procedure four polysaccharide soluble fractions (F1–F4) and a final polysaccharide insoluble fraction (F5) were obtained from *S. latissima* and *M. stellatus* (yields: 2352.9 mg and 2947.5 mg, respectively).

The pH of the acid- (F3) and alkali-soluble (F4) fractions was adjusted to 5.5, either with 2 M KOH or with concentrated HCl, as appropriate. Each soluble fraction (F1 to F4) was evaporated to half-volume in a R-114 Buchi vacuum rotatory evaporator with a B-480 Buchi water bath and temperature not exceeding 50 °C. The concentrates were exhaustively dialyzed, against water either for 48 h or until conductivity (μS/cm) of water in the dialysis tank was equal to that of tap running water as measured with a portable conductimeter (Myron L Company, Model EP Meter). The dialyzed solutions were kept at −20 °C.

A portion of soluble fractions (F1 to F4) from *S. latissima* and *M. stellatus* were filtered through 0.45 μm filters just before injection. Average molecular weight of polysaccharide peaks in commercial standards and fractions were calculated by comparison of their retention time with the calibration curves of pullulans and dextrans. Peak areas were utilized for quantitative analysis.

3. Results and discussion

3.1. HPSEC method development

A major topic for method development in aqueous size-exclusion chromatography is mobile phase selection. Specifically, a usual aspect to be solved is the interaction between solvent and column packing. Ionic interactions between the sample and column packing are reduced with addition of salt, 0.1 M $NaNO_3$ is often added as an electrolyte to suppress ion exclusion, and is preferred to NaCl since the latter is corrosive. An addition of salt provides an ionic strength that adds degree of reproducibility to the system [27], especially when analysing unknown samples that can possibly contain ionic species, as alginate and carrageenan polysaccharides of seaweeds. The effect of ionic strength of mobile phase on the elution of alginate has been investigated [20]. Better reproducibility and peak shape is observed when a low pH and high ionic strength buffer is used as mobile phase [20]. In order to achieve the best separation and resolution of peaks related to the ionic interactions between the sample and column packing of ionic species, two different mobile phases were investigated: (1), ultrapure water and (2) 0.1 M $NaNO_3$. Same retention time and peak shape were observed for pullulan and dextran standards either with ultrapure water or 0.1 M $NaNO_3$ as mobile phase.

Main charged polysaccharides from brown and red seaweeds, such as alginates, fucoidans and carrageenans, were analysed in the current study. In this case, the ionic repulsion between the anionic molecules and the sorbent of the stationary phase caused early elution when using ultrapure water. This is due to inter- and intra-molecular ionic repulsions that tend to expand their size [20]. Also chromatograms of abnormal shapes were observed and the repeatability of retention time of the chromatograms was poor with ultrapure water as mobile phase. Fig. 1 shows the chromatograms obtained by using either ultrapure water (Fig. 1A) or 0.1 M $NaNO_3$ (Fig. 1B) as mobile phase for a carrageenan sample. In view of these preliminary results, hereafter 0.1 M $NaNO_3$ was selected as mobile phase and used for the validation of the method. Thereby the HPSEC method was optimized and the separation and resolution of the chromatograms was improved.

3.2. HPSEC method validation

3.2.1. Linearity and sensitivity

The linearity of the method was calibrated using pullulan and dextran standards of different molecular weight. Table 1 shows retention time means and relative response factors of the standards and the calibration curve equations obtained. The calibration curves of pullulans and dextrans were plotted as the molecular weights on a log scale versus the retention time. The absolute response factor of the pullulan P-100 (112 kDa) and the dextran T-500 (500 kDa) were chosen to obtain the relative response factor of pullulans and dextrans, respectively (Table 1). Repeatability of retention time, expressed as mean values ± standard deviation, provided a good precision in the molecular weight estimation. Pullulan and dextran calibration curves showed good linearity

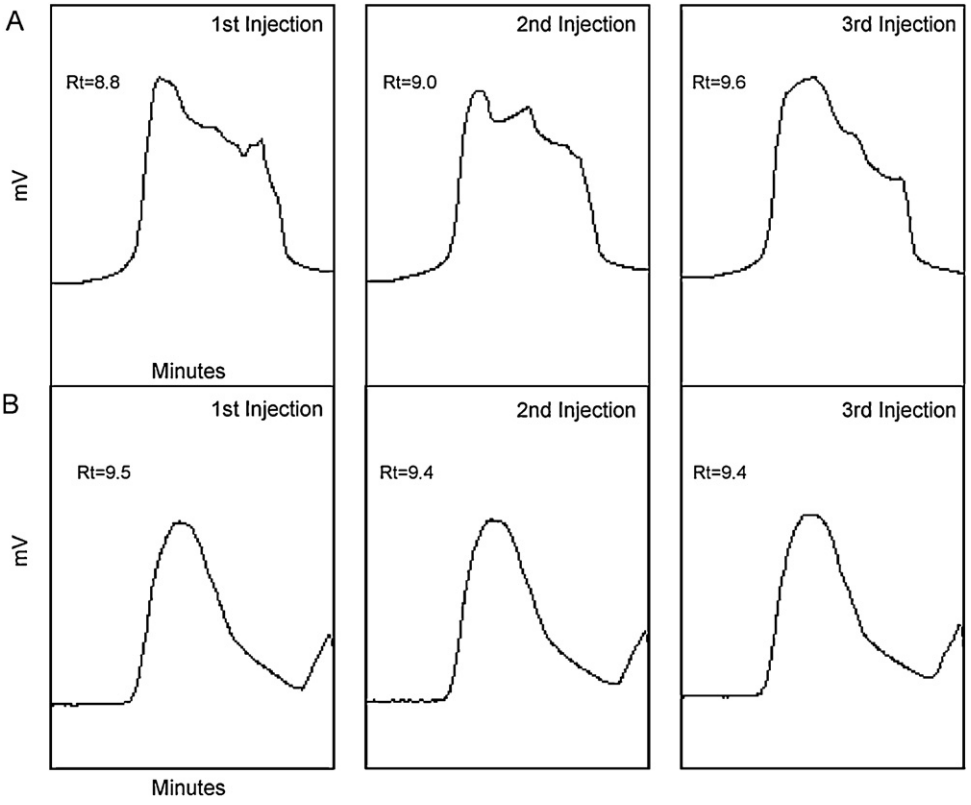

Fig. 1. Comparison of different chromatogram runs obtained from consecutive injections of Kappa-carrageenan standard with ultrapure water (A) or 0.1 M $NaNO_3$ (B) as mobile phase and refractive index (RI) detection.

(coefficient of correlation, $r = 0.9966$ and $r = 0.9933$, respectively) in the range of 5.9–2000 kDa. Although both calibration curves provided a good precision in the MW estimation just the closest one to the retention time of each peak was used for the optimum estimation of the MW.

The linearity of the calibration curve for quantification was evaluated by analyzing various concentrations of commercial brown and red seaweed polysaccharides. The regression curves of each commercial brown or red seaweed polysaccharide showed a satisfactory correlation between concentration (x, in mg/mL) and peak area (y) with $r > 0.999$ within the range in which the curves were established (0.5–2 mg/mL). The results are summarized in Table 2. Also, LOD and LOQ values achieved for the HPSEC method developed are presented in Table 2. The method showed to be sensitive enough for the determination of soluble polysaccharides from seaweed with LOD and LOQ values between 0.05–0.21 mg/mL and 0.16–0.31 mg/mL, respectively.

3.2.2. Precision and accuracy

Main polysaccharide standards from brown (alginate, fucoidan, laminaran) and red seaweeds (kappa- and iota-carrageenan) were used to calculate the precision and accuracy of HPSEC method.

The RSD (%) of repeatability of retention time was below 0.7% and inter-day RSD (%) was below 2.6%, which indicated that the method has both good repeatability and inter-day precision (Table 3). Also assays of repeatability for peak areas were carried

Table 1
Retention time, relative response factors and calibration curve equations of the polysaccharide standards by HPSEC method.

Standard	Grade	MW[a] (kDa)	Retention time (min)[e]	Relative response factor	Calibration curve
Pullulan	P-800	788	9.6 ± 0.0	1.10	$y = -0.46x + 7.333$
	P-400	404	10.2 ± 0.1	1.04	$r = -0.9966$
	P-200	212	10.9 ± 0.0	1.01	
	P-100	112[b]	11.6 ± 0.0	1.00	
	P-50	47	12.4 ± 0.0	1.10	
	P-20	22	13.1 ± 0.0	1.08	
	P-10	11.8	13.6 ± 0.0	1.03	
	P-5	5.9	14.0 ± 0.0	1.01	
Dextran	Blue dextran	2000	8.1 ± 0.2	0.95	$y = -0.392x + 6.512$
	T-500	500[c,d]	11.1 ± 0.0	1.00	$r = -0.9933$
	T-70	70	12.3 ± 0.0	1.04	
	T-10	10	13.8 ± 0.0	1.08	

x = retention time (min); y = log MW; r = correlation coefficient.
[a] MW according to the manufacturer specifications.
[b] Reference standard for response factors of pullulans.
[c] Reference standard for response factors of dextrans.
[d] This standard was not included in the calibration curve.
[e] Data are mean values ± standard deviation with $n = 9$.

Table 2
Linearity, sensitivity, and detection and quantitation limits of polysaccharide standards from seaweeds by HPSEC method.

Seaweed polysaccharide	MW (kDa)	Linearity (r)	Sensitivity[a]		LOD (mg/mL)	LOQ (mg/mL)
			Slope (m)	Intercept (b)		
Alginate	213–277	0.9997	13.85	−0.37	0.07	0.16
Fucoidan	105–117	0.9986	9.87	−0.17	0.10	0.28
Laminaran	7.3–7.6	0.9996	16.27	−2.74	0.21	0.31
Kappa-carrageenan	917–1124	0.9994	9.13	0.44	0.05	0.17
Iota-carrageenan	944–1626	0.9984	9.72	1.15	0.08	0.28

LOD = limit of detection; LOQ = limit of quantitation.
[a] Linear equations: $y = mx + b$; y = area (mV min); x = concentration (mg/mL); range = 0.5–2 mg/mL.

out and RSD (%) values were less than 2.5% (Table 3) showing also a good precision for the analytical method.

Values of recovery percentage for the method ranged between 96.3% and 109.8% (Table 3) showing the fairly good accuracy of the determination.

3.3. Molecular weight distribution of alginate, fucoidan, laminaran and carrageenan

The good precision for retention time of commercial soluble polysaccharides provided a good precision in the molecular weight estimation. Each polysaccharide was injected in triplicate at three concentrations ($n = 9$) and estimation of MW was obtained from their retention time using the calibration curves of pullulan or dextran. Then the MW range was calculated using the formula: MW range = MW mean ± CV (coefficient of variation). The molecular weight distribution in all polysaccharides exhibited a broad distribution. Thus, sodium alginate presented a molecular weight range of 213–277 kDa (Tables 2 and 3). This MW confirms previous results obtained by other authors. High-molecular mass alginic acids extracts from *Macrocystis pyrifera* and *Lessonia nigrescens* have a molecular weight interval of 146–264 kDa and 177–290 kDa, respectively [28].

Commercial fucoidan polysaccharide presented a molecular weight interval of 105–117 kDa (Tables 2 and 3). Some researchers have measured fucoidan's molecular weight at approximately 100 kDa, others have observed a molecular weight range from 638 to 1529 kDa [5], while our group has measured in *Fucus vesiculosus* a major fraction of 1600 kDa [26]. Results by Rupérez et al. [26] were obtained on the same column and chromatographic conditions as currently with ultrapure water as mobile phase, probably causing an early elution of fucoidan sample, due to inter- and intramolecular ionic repulsions, as previously discussed (in Section 3.1).

Laminaran showed a range between 7.3 and 7.6 kDa (Tables 2 and 3). This was a little bit higher than the molecular weight previously reported for laminarans, usually within 3–6 kDa [29]. In the case of carrageenans a higher molecular weight (917–1626 kDa) was obtained (Tables 2 and 3). Commercially available food-grade carrageenans can have a MW distribution ranging from 400 to 600 kDa with a minimum of 100 kDa [30]. Also, some carrageenans can possess a molecular weight of 990 kDa [30].

3.4. Analysis of soluble polysaccharide fractions from seaweeds

3.4.1. Saccharina latissima

The procedure of extraction was based on the different solubility of polysaccharides from brown seaweeds [26]. Solubility of alginates can be influenced by factors such as pH, concentration, ions in solution, the presence of divalent ions and ionic force [18] although solubility of laminarans is influenced by the degree of branching. Thus, highly branched laminaran is soluble in cold water, and can be extracted at 22 °C (F1) whereas lower levels of branching induce solubility only in warm water (60 °C, F2) [26]. Fucoidans were extracted with diluted hydrochloric acid (F3), whereas alginates were extracted with potassium hydroxide (F4).

Retention time and molecular weight values obtained in F1, F2 and F4 are presented in Table 4. The four peaks in F1 and F2 and the triple peak in F4 indicated that soluble fractions contained either four or three components respectively, with different molecular weight eluting between 7.5 and 15 min, approximately. The molecular weight of F3 could not be determined due to the low yields of extraction (0.5% algal dry weight), which concentration was below fucoidan's quantitation limit (data not shown). Peak No. 1 was observed in all fractions with an average molecular weight of 2111–2428 kDa, which corresponded to the lowest area percentage: 5–6 in F1, F2 and 2.5 in F4 (Table 4).

The water soluble fractions (F1 and F2) had high heterogeneity on molecular weight. Peak No. 2 in F1 and F2 presented a molecular weight distribution of 310–433 kDa and peak No. 3 showed a 20–27 kDa molecular weight. Finally, peak No. 4 with a molecular weight distribution between 5 and 5.8 kDa showed the highest concentration (mg/mL). Peaks No. 3 and 4 amounted to more than 50% of total area in the water-soluble fractions. Peak No. 4 could be tentatively identified as laminaran, a low-molecular weight polysaccharide with a MW within 3–6 kDa [29]. Related to peak No. 3, some authors have obtained from various algae, water-soluble polyuronans with a low-molecular weight (30–40 kDa), 5–10 time lower than the typical high-molecular weight alginic acids [28]. According to our previous work, the main polysaccharide found in *S. latissima* is alginate (uronic acids) [3,31], so it is also possible to find polyuronans with a lower molecular weight in the water-soluble fractions (peak No. 3, Table 4). Shevchenko et al. also found a poly(mannuronic acid) with a molecular weight range of 20–50 kDa

Table 3
Repeatability of retention time and peak area, MW estimation and recovery of polysaccharide standards from seaweeds by HPSEC method.

Seaweed polysaccharide	Repeatability ($n = 3$)		Inter-day precision ($n = 9$)		Estimation MW (kDa)	Repeatability ($n = 3$)		Recovery (%)
	Retention time (min)	RSD (%)	Retention time (min)	RSD (%)		Peak area	RSD (%)	
Alginate	10.8 ± 0.0	0.1	10.8 ± 0.1	1.1	213–277	27.4 ± 0.1	0.5	101.1 ± 0.4
Fucoidan	11.4 ± 0.0	0.2	11.4 ± 0.1	0.6	105–117	19.5 ± 0.2	0.9	96.3 ± 2.3
Laminaran	14.0 ± 0.0	0.0	14.0 ± 0.0	0.1	7.3–7.6	29.9 ± 0.1	0.2	101.3 ± 0.2
Kappa-carrageenan	9.4 ± 0.1	0.7	9.4 ± 0.1	1.0	917–1124	18.6 ± 0.1	0.3	109.8 ± 0.3
Iota-carrageenan	9.3 ± 0.0	0.3	9.2 ± 0.2	2.6	944–1626	20.5 ± 0.5	2.5	102.1 ± 1.1

Data are mean values ± standard deviation. Peak area = mV min; RSD = relative standard deviation.

Table 4
Yield (%), repeatability of retention time, MW estimation and amount of soluble polysaccharide fractions from the brown seaweed *S. latissima* by HPSEC method.

Fraction	Yield (%)	Peak No.	Retention time (min)	MW (kDa)	Area account (%)	Amount (mg/mL)
F1	9.6	1	8.1 ± 0.0	2111–2190	5.6 ± 0.4	0.09 ± 0.0
		2	10.4 ± 0.0	338–351	27.9 ± 2.1	0.49 ± 0.1
		3	12.9 ± 0.1	23–27	35.3 ± 2.4	0.28 ± 0.1
		4	14.3 ± 0.0	5.7–5.8	31.5 ± 0.7	0.62 ± 0.0
F2	5.0	1	8.0 ± 0.0	2269–2414	5.9 ± 0.6	0.05 ± 0.0
		2	10.4 ± 0.2	310–433	38.8 ± 4.6	0.23 ± 0.0
		3	13.1 ± 0.1	20–22	29.7 ± 1.6	0.22 ± 0.0
		4	14.4 ± 0.0	5.0–5.1	28.0 ± 8.9	0.30 ± 0.1
F4	25.0	1	8.0 ± 0.0	2228–2428	2.5 ± 0.6	0.04 ± 0.0
		2	12.4 ± 0.1	43–49	62.9 ± 1.1	0.40 ± 0.1
		3	14.0 ± 0.0	7.4–7.9	35.7 ± 1.4	0.11 ± 0.0

Mean value of three determinations ± SD. Yield (%) by gravimetry (g/100 g dry weight); F1, soluble in water at 22 °C; F2, soluble in water at 60 °C; F4, soluble in 2 M KOH at 37 °C.

[29]. As well, peak No. 2 could be related to high-molecular weight alginates (>300 kDa) or to water-soluble fucoidans [26].

The main peak in the alkali-soluble fraction (F4), presented a molecular weight interval of 43–49 kDa (Table 4). This may also correspond to alginates which are the main alkali-soluble polysaccharides in brown seaweeds [26,28]. Also a peak with a molecular weight around 8 kDa was found in this fraction (Table 4).

This alga and its soluble fractions exhibited high heterogeneity on molecular weight, which could be related to the heterogeneous polysaccharide composition (laminaran, fucoidan and alginate) of brown algae, as well as to their extraction process. It is known that molecular weight of laminarans, fucoidans and alginates in brown algae also depend on season of collection [32]. Further research work is necessary to completely characterize polysaccharides in these soluble fractions, in order to fully comprehend the relationships between structure and function. Nevertheless, the proposed method was very useful for determining the molecular weight distribution of soluble polysaccharides in *S. latissima* and could be extended to other brown seaweeds.

3.4.2. *Mastocarpus stellatus*

The same procedure of extraction used in *S. latissima* was applied to this red alga. *M. stellatus* belongs to the Gigartinaceae family, which members are mainly carrageenophytes. According to our previous work, the main polysaccharide in *M. stellatus* is a hybrid kappa-/iota-carrageenan [31], a sulfated galactan [3]. Retention time and molecular weight values estimated in *M. stellatus* fractions are presented in Table 5. Each fraction exhibited a single peak, with a broad molecular weight distribution (Table 5). It is interesting to note that the molecular weight distribution of the soluble polysaccharides in *M. stellatus* goes from the highest to the lowest values through the process of sequential extraction. This behaviour is explained because the polymerization degree of algal galactans varies significantly and is highly dependent on the extraction conditions [33]. Thus, through the process of sequential extraction, the degree of polymerization became lower, giving as a result lower molecular weight distributions. Mild-acid hydrolysis is a commonly used method to obtain low-molecular weight fractions of carrageenans [34]. This was consistent with F3, the acid-soluble fraction that showed a lower average molecular weight than the water-soluble fractions (F1, F2) (Table 5).

Table 5
Yield (%), repeatability of retention time, MW estimation and amount of soluble polysaccharide fractions from the red seaweed *M. stellatus* by HPSEC method.

Fraction	Yield (%)	Retention time (min)	MW (kDa)	Amount (mg/mL)
F1	6.7	8.6 ± 0.1	1248–1425	1.1 ± 0.0
F2	14.4	9.2 ± 0.2	707–983	3.8 ± 0.1
F3	25.5	13.2 ± 0.0	17–134	4.7 ± 0.1
F4	12.1	14.1 ± 0.0	8–10	2.3 ± 0.0

Mean value of three determinations ± SD. Yield (%) by gravimetry (g/100 g dry weight); F1, soluble in water at 22 °C; F2, soluble in water at 60 °C; F3, soluble in 0.1 M HCl at 37 °C; F4, soluble in 2 M KOH at 37 °C.

Commercial carrageenans have a molecular weight distribution ranging from 400 to 600 and 900 kDa. In the case of seaweed samples, the molecular weight distribution also varies from sample to sample, depending upon the sample history, e.g., age of the harvested seaweed, season of harvesting, way of extracting and length of heat treatment [30,33]. This heterogeneity may cause that different molecular weight was obtained by different processes of sequential extraction. As well as for the brown seaweed *S. latissima*, further research work on *M. stellatus* polysaccharides is necessary to correlate their molecular weight distribution to chemical or functional properties. The method could also be extended to the molecular weight estimation of polysaccharides in other red seaweeds.

4. Conclusions

A simple size-exclusion chromatography method for the molecular weight distribution of polysaccharides in seaweeds was proposed. The validation parameters, precision, accuracy, linearity and sensitivity, showed that the HPSEC method was adequate for the analysis of soluble polysaccharides in seaweeds according to their molecular weight. The method was optimized and repeatability of chromatograms was further improved by using 0.1 M $NaNO_3$ as mobile phase. The proposed method was very useful for determining the molecular weight distribution of polysaccharides from seaweeds. Soluble fractions from brown *S. latissima* and red seaweed *M. stellatus* presented high heterogeneity on molecular weight. Also the extraction conditions for carrageenans in *M. stellatus* showed differences in MW of soluble fractions. These preliminary results can provide additional information necessary to correlate the molecular weight of seaweed polysaccharides with their chemical or functional properties. Results of detailed chemical composition and biological activities will be reported elsewhere and should help in acquiring a better understanding of the interplay between chemical structure and functional properties of biopolymers from edible brown and red seaweeds.

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