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Influence of the extraction–purification conditions on final properties of alginates obtained from brown algae (*Macrocystis pyrifera*)

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ABSTRACT

In this work, three methods (ethanol, HCl, and $CaCl_2$ routes) of sodium alginate extraction–purification from brown seaweeds (*Macrocystis pyrifera*) were used in order to study the influence of process conditions on final properties of the polymer. In the $CaCl_2$ route, was found that the precipitation step in presence of calcium ions followed by proton-exchange in acid medium clearly gives alginates with the lowest molecular weight and poor mechanical properties. It is well known that the acid treatment degrade the ether bonds on the polymeric chain. Ethanol route displayed the best performance, where the highest yield and rheological properties were attained with the lowest number of steps. Although the polymer I.1 showed a molar mass and polydispersity index (M_w/M_n) similar to those of commercial sample, its mechanical properties were lower. This performance is related to the higher content of guluronic acid in the commercial alginate, which promotes a more successful calcium chelation. Moreover, the employment of pH 4 in the acid pre-treatment improved the yield of the ethanol route, avoiding the ether linkage hydrolysis. Therefore, samples I.2 and I.3 displayed a higher M_w and a narrower distribution of molecular weights than commercial sample, which gave a higher viscosity and better viscoelastic properties.

1. Introduction

Although the early state of biosynthesis of carbohydrates in seaweeds is similar than in plants, some of the polysaccharides finally synthesized are found only in seaweeds. These biopolymers are present principally in the cell wall, giving mechanical resistance to algae. Properties such as flexibility and softness in water to support currents are more important than rigidity. Therefore, the polysaccharides that prevail in seaweed are those that possess properties of gels and mucilage, being cellulose also present in a lower amount [1–6].

There are three principal groups of seaweeds classified by their colour: brown, red and green seaweeds. Each of them possesses the predominance of a typical polysaccharide [7], being alginate the most abundant in marine brown seaweed. Although there are different species of brown seaweed that contain alginates, these are no sufficiently abundant and suitably located for commercial production. The species of brown seaweed most commercially exploited are: *Laminaria hyperborea, Macrocystis pyrifera and Ascophyllum nodosum*. These species are mainly manufactured in countries such as USA, Japan, China, France, and Norway [8].

In natural environments, alginates exist in the cell wall as a mixture of calcium, potassium and sodium salts of alginic acid [9]. They constitute a family of linear copolymers of (1 → 4) β-D-mannuronic acid (M) and (1 → 4) α-L-guluronic acid (G) units. The chemical composition and sequence of M and G units depend on the biological source, growth, and stationary conditions. These polysaccharides have three types of diad sequences, i.e. MM, GG and MG blocks [10–12]. Sodium alginate is a water-soluble polymer, which gives highly viscous solutions. It can be used as a stabilizer of suspensions and as thickener in food industries among other applications [9]. Another characteristic property of the sodium alginate solutions is the ability of gel formation in the presence of polyvalent cations, such as Ca^{2+} [13]. Generally, the extraction and purification processes of alginates are based on the conversion from the insoluble form in the plant cell walls to the soluble one, normally the sodium salt, followed by successive dissolutions and precipitations to eliminate impurities [14,15]. Alginates extraction from brown seaweed has been studied during several decades in order to develop economic systems, obtaining high yields and a controlled molecular weight for different applications [16–18]. In some South American countries such as Argentina, there is no industry producing alginates despite the large amount of brown seaweed such as *M. pyrifera* along of its seacoast [19]. In this work we present a comparative analysis of three routes of sodium alginate purification in order to have a product with controlled molar mass and degree of

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purity. Samples of brown seaweeds were collected from Patagonian Argentine coast and used as raw material to carry out this study. Extracted alginate was characterized by SEC, ^{1}H NMR, rheological studies, ability to gel formation and swelling degree. On the other hand, the characteristic properties of extracted sodium alginates were studied and compared with those of a commercial sample.

2. Experimental

2.1. Materials and reagents

The raw material from which the alginate was extracted consisted of sheets and stems of brown seaweed (*M. pyrifera*) from Patagonian Argentine coast, which were dried, previously crushed and sifted (10–20 mesh). The following chemicals were also purchased and used in the extraction–purification processes: HCl 37 wt% (Cicarelli, Argentine); NaOH p.a. (Anedra, Argentine); Na_2CO_3 p.a. (Anedra, Argentine); $CaCl_2$ p.a. (Anedra, Argentine); ethanol 96% (Carries, Argentine); diatomaceous earth (Anedra, Argentine); eriochrome black-T p.a. (Fluka, Switzerland). A commercial sample of sodium alginate (N° 71238 Fluka, Switzerland) was employed as a reference. In addition, a solution of eriochrome black-T (5 g per 1 L of ethanol) was prepared, adding then 5 mL of the last one to 5 mL of a 0.5 M NH_4Cl/NH_3 buffer solution at pH 10, in order to obtain the indicator solution used for qualitative detection of calcium ions.

2.2. Extraction of alginates from seaweed

A crushed sample (10 g) of dry seaweed was moistened by addition of distilled water (400 mL) and a 0.1N HCl aqueous solution was added, under high stirring, in order to have a pH 4, following a similar extraction method reported by Arvizu Higuera et al. [16]. This mixture was stirred during 15 min at room temperature

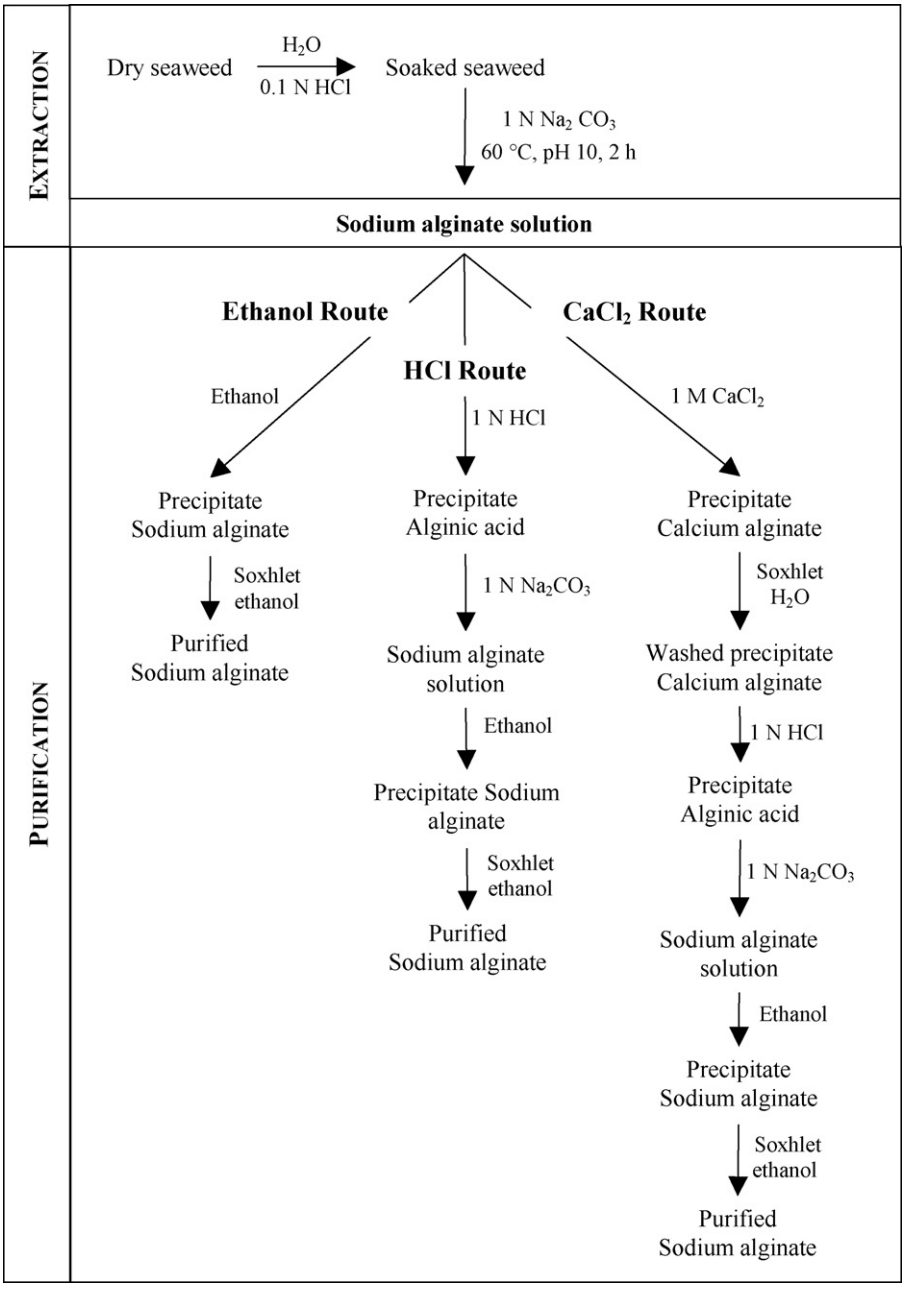

Fig. 1. Scheme corresponding to the three routes of alginate purification.

and then the supernatant was eliminated. This pre-treatment was repeated three times with every sample, using each time 40, 25 and 20 mL of HCl solution, respectively. Then, the moistened seaweed was placed in a beaker with 250 mL of a 1N Na_2CO_3 solution (pH 11.5) and mechanically stirred at 60 °C for 2 h. The extracted sodium alginate from the initial material was diluted to 800 mL with distilled water and 1 g of diatomaceous earth was added, stirring this mixture during 15 min. The insoluble material was separated by centrifugation, obtaining then sodium alginate in the supernatant.

2.3. Purification of sodium alginate extracts

Three different processes of purification were used with the solution of sodium alginate obtained in the above process, which have been named route of ethanol, HCl, and $CaCl_2$, respectively. A schematic representation of the steps developed in the processes is shown in Fig. 1.

2.3.1. Ethanol route (I)

Aqueous solution of sodium alginate obtained in the extraction step was directly precipitated, under stirring, by addition of ethanol until reaching a proportion 1:1 in volume, respectively. Thus, the insoluble polymer was separated and then exhaustively washed with ethanol by soxhlet for 100 h. Finally, the biopolymer was dried at room temperature under vacuum until constant mass.

2.3.2. HCl route (II)

In this route of purification, the solution of extracted sodium alginate and 250 mL of 1N HCl were mixed at room temperature under stirring for 1 h (pH 2). Next, the precipitate of alginic acid was separated by centrifugation adding later to it 100 mL of distilled water and 150 mL of 1N Na_2CO_3. Then, this mixture was left under stirring at room temperature for 1 h in order to obtain the soluble form sodium alginate. Later, the polymer was precipitated from this solution by slow addition of ethanol (1/1 volume ratio), employing the same procedure of washing and drying described in Section 2.3.1.

2.3.3. $CaCl_2$ route (III)

The aqueous solution of sodium alginate was precipitated by the addition of 1 M $CaCl_2$ (200 mL). The precipitate of calcium alginate was exhaustively washed with distilled water using a soxhlet for 64 h. Then, 150 mL of distilled water was added to the precipitate followed by the addition of 1N HCl until pH 2 in the supernatant, maintaining this mixture under agitation at room temperature for 3.5 h. The insoluble material obtained (alginic acid) was separated from the supernatant by centrifugation. Next, the alginic acid was washed with 1.5 L of 0.05N HCl under stirring at room temperature, without detecting the presence of Ca^{2+} in the supernatant when it was analysed by eriochrome black-T indicator. Later, the precipitate of alginic acid was treated as in Section 2.3.2.

2.4. Calcium determination assays

Qualitative analysis of the presence of calcium ion in the supernatant was performed in the $CaCl_2$ route. Here, an indicator solution of eriochome black-T (1 mL) was mixed with an equal volume of the sample supernatant, which changes its colour from blue to pink in the presence of calcium ions. Quantitative determination of the sodium and calcium content on the alginates samples were performed at the CNRS central microanalysis service at Solaize (France).

2.5. Molecular weight distribution

Purified alginates isolated under the sodium salt form were characterized by size exclusion chromatography (SEC) using a Waters Alliance GPCV2000 (USA) equipped with three detectors on line: a differential refractometer, a viscometric detector, and a multi angle laser light scattering (MALLS) detector from Wyatt (USA). The concentration of polymer injected was in the range of 0.5–5 g/L (depending on the range of molecular weights), with an injection volume of 108 μL using two columns in series (Shodex OH-pack 805 and 806). The dn/dc adopted for the analysis was 0.165. All the samples were filtrated on a 0.2 μm pore membrane ("Sartorius AG" cellulose acetate filter) before injection, in order to retain large aggregates. The eluent used was a 0.1 M $NaNO_3$ aqueous solution, at 30 °C as elution temperature and a flow rate of 0.5 mL/min; the molecular weight distribution, weight-average molecular weight (M_w), polydispersity index (M_w/M_n), and intrinsic viscosity of the eluted polymers were obtained as characteristics of the biopolymers.

2.6. Chemical structure

Chemical composition of extracted alginates was obtained by NMR. Polymer samples were dissolved in D_2O with a concentration 6 mg/mL. ^1H NMR experiments were performed using a Bruker AC-300 (Germany) spectrometer. The delay adopted was 20 s. Chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm). Deuterium oxide was obtained from SDS (Vitry, France). ^1H NMR spectroscopy is suitable for characterizing both the composition and the distribution sequence of the two-uronate residues in alginate samples [21]. We have assigned the signals at 5, 4.6, and 4.37 ppm, like those corresponding to H_1-G (pick 1), H_1-M + H_5-GM (pick 2), and H_5-GG (pick 3), respectively (Fig. 2). Taking into account that protons areas of H_1-G and H_5-G are equals, H_5-GM is attained from the difference between the picks 1 and 3. Then, making the difference between the pick 2 and H_5-GM, the H_1-M area is obtained. From these values, the M/G ratio is calculated as H_1-M/H_1-G and the fraction GG as H_5-GG/H_1-G.

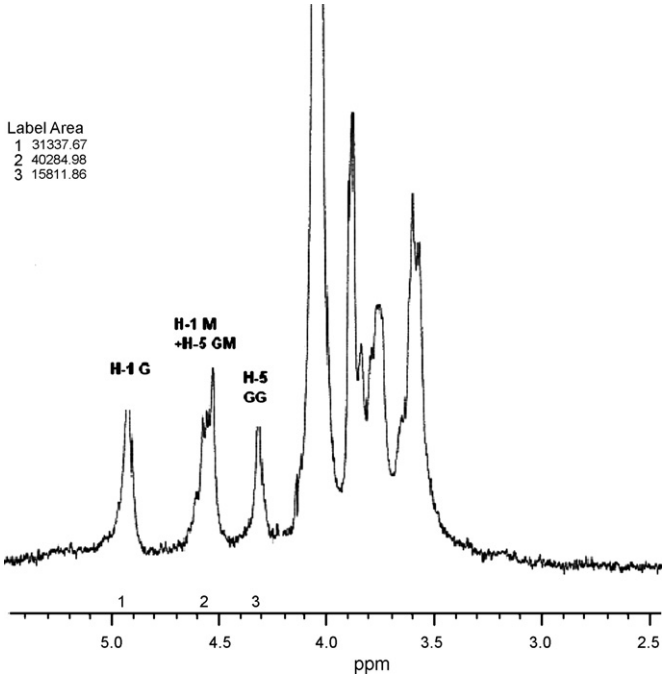

Fig. 2. ^1H NMR spectrum of sample I.1 in deuterium water and its characteristic signals.

2.7. Gelation ability of the polymeric backbone in the presence of calcium ions and the degree of swelling

Gelation ability of sodium alginate solutions in the presence of calcium ions was carried out by dialysis through a porous cellulosic membrane "Spectra/Por" (exclusion limit M = 12–14,000) [20]. Here, a sodium alginate aqueous solution at a concentration of 20 g/L was prepared and then introduced (20 mL) in the dialysis bag against a 1 M $CaCl_2$ solution during 48 h to assure equilibrium. A slice of gel (near 4 mm thick) was cut and placed between the plates of the rheometer and its rheological properties were measured. Moreover, the swelling degree (SD) of the gel was obtained and expressed as grams of regained water per gram of dry sodium alginate. Swelling in water was determined by difference of weight between the swollen gel and the dry mass of sodium alginate (based on the amount of polymer introduced in the bag). Later, the gel formed in excess of $CaCl_2$ was dried, and then swollen again in water until reaching equilibrium. The swelling liquid was removed, repeating the practice 5 times in order to eliminate the excess of $CaCl_2$ included, and then the re-swelling degree (RSD) was measured. Here, the gel was weighted and dried at 70 °C until achieve constant mass, and then RSD was expressed as grams of water per gram of dried polymer under calcium form.

2.8. Rheology of the sodium alginate solutions and the calcium alginate gels

The rheological behaviour of the solutions and gels was determined using an AR 1000 rheometer from "TA Instruments" (USA) at 25 °C. Parallel plate geometry with 2 cm diameter plate was used for gels, and a cone and plate geometry (4 cm diameter plate with 3.59° cone) for solutions. Dynamic experiments were performed in the linear viscoelastic region. Dynamic moduli (G' and G'') as well as complex viscosity $|\eta^*|$ were determined as a function of the angular frequency (ω).

3. Results and discussion

Brown seaweeds (*M. pyrifera*) from Patagonian Argentine coast were used in a study of extraction and purification of sodium alginate in order to attain the highest molar mass and purity degree. The products were obtained by three ways, named "Ethanol (I)", "HCl (II)", and "$CaCl_2$ (III)" route. Acid pre-treatment was used in order to eliminate polyvalent cations. After that, the soluble polymer diffuses out of the seaweed under basic conditions, and then it is purified by different ways. The final products were analysed and their chemical composition as well as physical properties were compared.

3.1. Purification process of sodium alginate

Purification process is based on successive solubilizations and precipitations of the polymer in aqueous medium. Different conditions are employed to eliminate the soluble impurities and all multivalent counter ions in order to obtain the polymer under sodium salt form. As can be seen in Fig. 1, there is a step corresponding to alginate extraction using a Na_2CO_3 solution at 60 °C, in which cellular matrix is hydrolysed and the alginate polymer is liberated to the solution followed by different purification ways. For most of the cases, the alginate yields were higher than other values published previously [14,19]. Fig. 1 and Table 1 show a logic effect of the number of steps and the extraction conditions on purity and extracted polymer yield. Moreover, the highest yield of purified sodium alginate was obtained by ethanol route, which presents the lower number of purification steps (Table 1). This behaviour reflects the already well-known low stability of the links of these polysaccharides principally in acid medium and in less extent in basic conditions. In addition, the extraction yield values given in Table 1 were calculated on the basis of corrected dry weight of the samples without the extra materials such as sand occluded in the initial algae.

Table 1
Performance of the extraction–purification routes of sodium alginate.

Sample	Purification		Ca^{2+} content (ppm)	Extraction yield (wt%)
	Route	Number of steps		
I.1	Ethanol	2	2300	33
I.2			–	34
I.3			–	32
II.1	HCl	4	–	25
II.2			2300	29
III.1	$CaCl_2$	6	1500	27
III.2			–	29

Calcium content for commercial sodium alginate was 1200 ppm.

3.2. M/G composition of the polymers and calcium content

The chemical composition of the extracted sodium alginate was determined using ^{1}H NMR spectroscopy. As can be seen in Fig. 2, the signal at 4.95 ppm corresponds to the H-1 of guluronic units, whereas the H-5 of guluronic units in GG block appears at 4.31 ppm, and the large signal between 4.45 and 4.7 ppm belongs to H-1 of mannuronic units and H-5 of guluronic units in GM moieties as assigned previously [21]. From the integration of these signals both the M/G ratio and the fraction of G units included in GG blocks were estimated. The results are given in Table 2, showing that M/G ratios of alginates obtained from *M. pyrifera* are higher than the value corresponding to the commercial sample, since the last one was probably extracted from a different algae species. Table 2 also shows that an average value of M/G ~ 1.15 is found for the polymers purified through different ways, from which a large fraction of G units (0.7) is involved in GG blocks. No significant dispersion in this ratio was obtained for the three routes. On the other hand, Table 1 indicates that the residual calcium content in the samples (1500–2000 ppm) was close to the value obtained for the commercial alginate (1200 ppm). Relatively lower values of calcium content were obtained when acid route was employed. An increase in the proton concentration lead to a higher polycations exchange, and then a product with a higher purity can be obtained. However, this variation gives also a decrease of the average molecular weight by hydrolysis of the ether linkages.

3.3. Molecular weight distribution

Molecular weight distribution of the samples was determined by SEC at 30 °C. Weight-average molecular weight (M_w) and the polydispersity index (M_w/M_n) are given in Table 2, where it is possible to observe that the best purification condition is the direct precipitation by ethanol. Ethanol route gives the highest weight-average molecular weight (M_w) with a relatively low polydispersity. Moreover, the molecular weight distribution of the extracted alginates was compared with the commercial sample (Fig. 3). Here, the maximum in the molecular weight distribution moves to higher molar mass when a lower number of steps in the purification route are used, which can be related to chains degradation. In addition, from SEC measurements it was possible to estimate the percentage of soluble components in each sample. Table 2 shows that the soluble percentage of every extracted alginate by ethanol and acid routes was higher than 60%, which is related to a successful purification of the polymer. Intrinsic viscosity data shown in Fig. 4 and Table 2, indicate that Mark Houwink exponent is, in the range of molecular

Table 2
Rheological behaviour and characterization by SEC and ^{1}H NMR of the different alginates.

Sample	GG fraction	M/G	$M_w \times 10^3$ [g/mol]	M_w/M_n	Soluble % (SEC)	[η] (mL/g) (SEC)	$\lvert\eta^*\rvert$[a] (0.1 Hz) [Pa s]
Commercial	0.48	0.43	279	1.9	70	660	1.07
I.1	0.69	1.17	297	1.8	72	575	0.60
I.2	–	–	350	1.6	61	1100	–
I.3	–	–	396	1.5	62	1185	–
II.1	0.76	1.29	227	1.6	69	530	0.39
II.2	0.59	1.30	213	1.6	70	480	0.19
III.1	0.69	0.97	94	3.0	57	170	0.02
III.2	0.77	1.12	56	1.9	58	150	0.01

[a]Sample in water solution (20 g/L; 25 °C), being (I), (II) and (III) the purification routes of ethanol, HCl and $CaCl_2$, respectively.

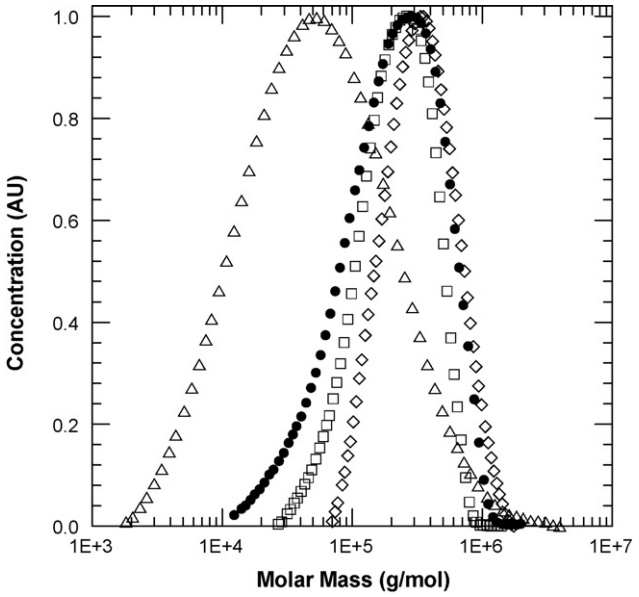

Fig. 3. Molecular weight distributions obtained by size exclusion chromatography. Symbols: (●) commercial; (◊) I.3; (□) II.1; (△) III.1.

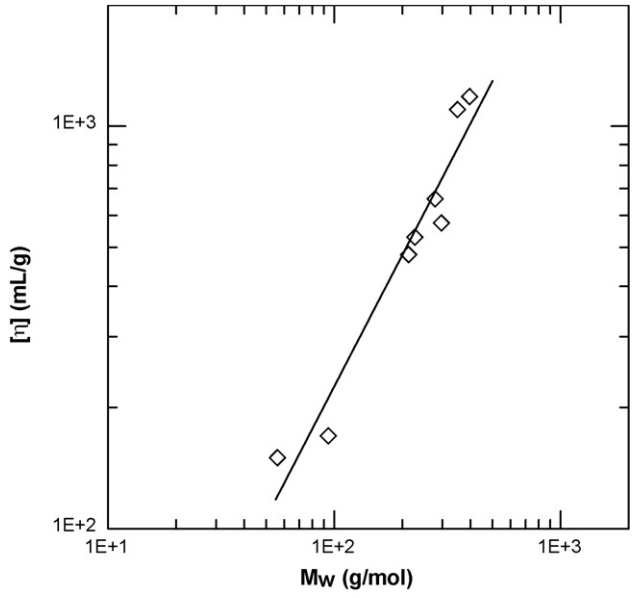

Fig. 4. Intrinsic viscosity [η] in 0.1 M $NaNO_3$ as a function of weight-average molar mass (M_w) for extracted sodium alginates; all the experimental data are obtained from SEC (Table 2).

weight analysed, $\alpha = 1.09$. This value is in good agreement with that determined in a previous work ($\alpha = 0.97$ in 0.1 M NaCl) [22]. Then, it is possible to confirm that alginates are semi-rigid molecules, which can be characterized by a persistence length depending slightly on their composition as discussed previously [23].

3.4. Rheology

Rheology of polymer solutions at 20 g/L in water was tested in steady state and dynamic experiments. All the solutions have a Newtonian plateau at low shear rate. As it was expected, loss modulus (G'') is always higher than storage modulus (G') in the range of frequency covered [24]. An example is given in Fig. 5, where the behaviour of the commercial and I.1 sample is compared, showing the former a higher performance as thickener. From these rheological data, it can also be concluded that the direct precipitation in the purification of sodium alginate through "ethanol route" gave the extracted polymer solution with the highest viscosity (Table 2). Given that the polymeric chain degradation is high in presence of HCl solution during the acid pre-treatment of the algae before to the extraction process, the acidity must also be carefully kept at pH 4. Moreover, the purification by the "HCl route" gives, in both samples (II.1 and II.2), relatively similar results with only few aggregates as it can be seen from soluble % (Table 2). Nevertheless, the use in this route of an acidic treatment produces hydrolysis of some linkages with a decrease in the molecular weight and a lower rheological performance for these samples.

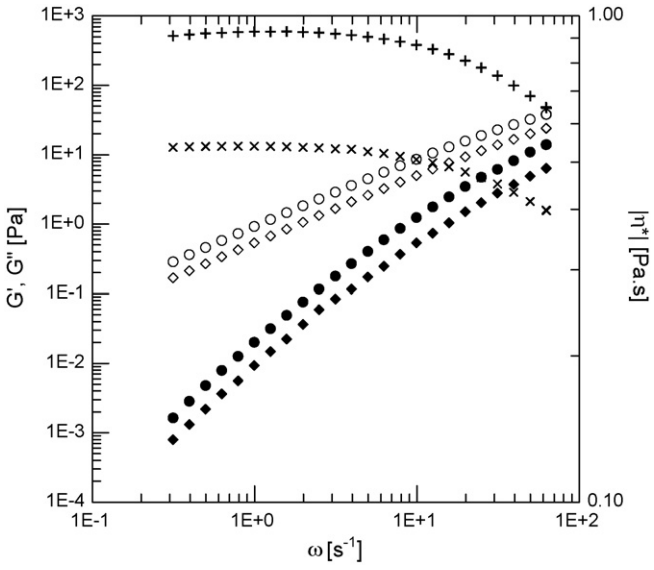

Fig. 5. Dynamic rheological behaviour of 20 g/L sodium alginate aqueous solutions performed at 25 °C. Elastic and viscous moduli, and complex viscosity: (●, ○, +) commercial; (♦, ◊, ×) I.1 sample.

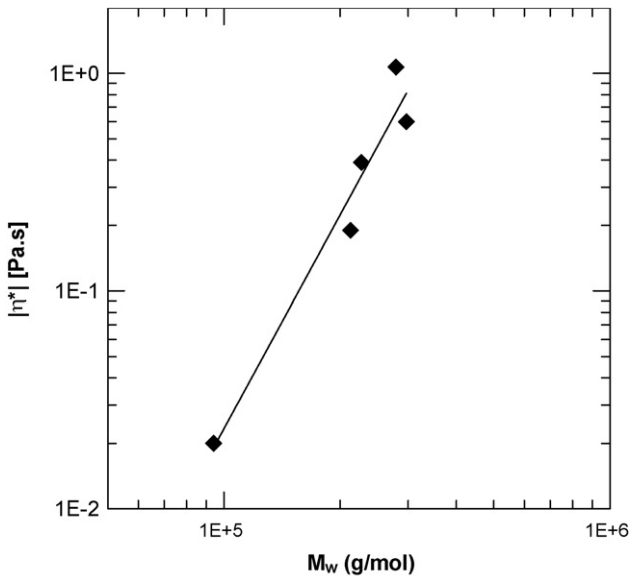

Fig. 6. Complex viscosity measured at 0.1 Hz for alginate solutions (20 g/L in water) as a function of weight-average molar mass (M_w) for extracted sodium alginate. Slope 3.26.

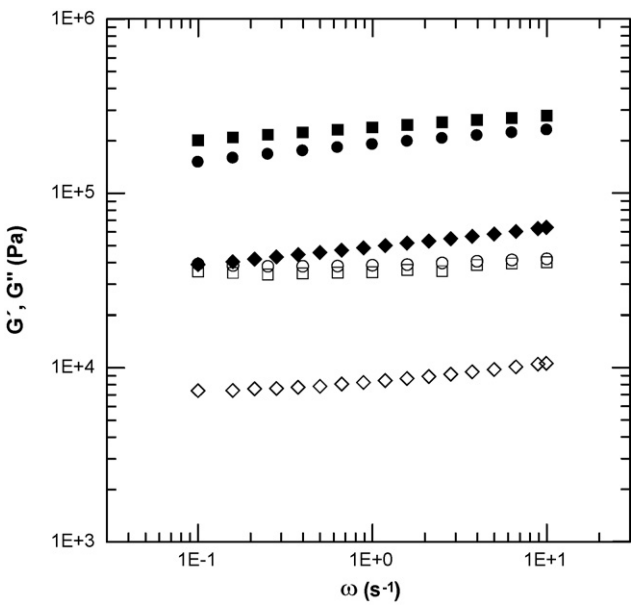

Fig. 7. Dynamic rheological behaviour of calcium alginate gels at 25 °C. Elastic and viscous moduli of (●, ○) commercial, (♦, ◊) I.1 and (■, □) I.3 samples.

On the other hand, the "$CaCl_2$ route" has the highest number of purification steps, including an acid treatment (Fig. 1), giving the worst results (III.1 and III.2 samples). Table 2 shows that samples III present the lowest molecular weight and viscosity with a lot of aggregates and a relatively high content of the residual calcium ions. The complex viscosity as a function of the angular frequency at 0.1 Hz for the different samples was obtained, and then their values compared (Table 2). There are not enough data to determine the critical value of the average molecular weight for effect of entanglement coupling on viscosity (M_c). Nevertheless, the values of weight-average molecular weight obtained seem to be over M_c, at the polymer concentration used (20 g/L in water), which screens the long range electrostatic repulsions. Viscosity values as a function of weight average molecular weight show a power trend (Fig. 6) with a slope of approximately 3.26 as it is expected at high molecular weights, due to the entanglement coupling [25].

3.5. Gelation ability of the polymers

Gelation ability of sodium alginate polymers was evaluated using a 20 g/L solution of polymer against a 1 M $CaCl_2$ solution for 48 h. Rheological properties of formed gels were obtained by dynamic measurements in parallel plate geometry. Fig. 7 shows the evolution of dynamic moduli (G' and G'') as a function of the frequency for formed gels of commercial, I.1 and I.3 sample. An elastic modulus (G') higher than the viscous one (G'') in all the range of frequency measured is obtained. From the relationship between the polymeric chain and gel properties (Tables 2 and 3), it is possible to observe that viscoelastic properties are dependent on both the chemical composition and the polymer molar mass. Those alginates with a lower M/G ratio displayed a higher G' value (Table 3) as result of a higher degree of calcium chelation, which is directly related to a higher content of guluronic groups. Although the moduli are lower when GG fraction is higher (Tables 2 and 3, Fig. 7), the total GG fraction (TGF) of the commercial sample (36%) is higher than sample I.1 (32%). This is in agreement with the "egg box" binding model of calcium by polyguluronate. The TGF % value was calculated from M/G ratio and GG-block fraction (Table 2), where TGF % = [100 × GG-block fraction]/[(M/G) + 1] was expressed as the percentage of G units presents in the GG block fraction referred to the total monomer (M + G) content.

Table 3
Viscoelastic properties at 25 °C and swelling degree of calcium gels obtained from aqueous solution of alginates with a Cp = 20 g/L.

Sample	G' (at 1 Hz) [Pa]	G'' (at 1 Hz) [Pa]	SD[a]	SD[b]
			[g H_2O/g dry polymer]	
Commercial	191,200	38,000	25	4.8
I.1	51,800	8,800	24	4.7
I.2	295,000	55,000	26	5.3
I.3	240,000	38,200	24	5.4
II.1	43,400	7,100	26	4.4
II.2	49,400	8,300	27	5.2
III.1	50,600	7,900	30	5.3
III.2	38,900	5,400	31	6.0

[a] Swelling degree.
[b] Re-swelling degree.

On the other hand, the control of acidity at pH 4 during acid pre-treatment allowed attaining the samples I.2 and I.3 with a very good reproducibility. These alginates (Table 2 and Fig. 3) displayed a narrower molecular weight distribution and a higher molar mass than the commercial sample. Therefore, the gelation ability of the extracted polymers was improved, showing the highest viscosity and improved viscoelastic properties (Tables 2 and 3). Extracted alginates with a higher molecular weight form stronger gels (Fig. 7) due to an increase in the number of cross-linking points (calcium chelation). On the other hand, the lower values of swelling and re-swelling degrees obtained with samples I.1 to I.3 also indicates a higher degree of cross-linking in those gels.

4. Conclusions

In this work a basic extraction of sodium alginate from brown seaweeds (*M. pyrifera*) was performed in order to examinate the influence of three purification methods on the yield and final properties of the polymer. Clearly the use of a step of precipitation in presence of calcium ions followed by the cations exchange in acid medium gives alginates with poor viscoelastic properties. Acid treatments degrade the polymer chain when it is used in the process. Ethanol route results the most successful process using the lowest number of steps. Direct polymer precipitation with ethanol (1/1 volume ratio) leads to the best yield. Sample I.1 with an weight

average molecular weight and M_w/M_n similar to the commercial sample shows lower viscoelastic properties, both in solution and in gel form. This behaviour can be explained considering the higher G unit content (TGF %) in the commercial alginate, which promotes a more successful calcium chelation. A pH higher than 3.5 in the acid pre-treatment enhanced the "ethanol route", avoiding the rupture of ether linkages. Then, extracted alginates I.2 and I.3 were obtained with a higher M_w and a narrower molecular weight distribution, presenting rheological properties comparable to or even better than the commercial sample.

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