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# Characterization of the alginates from five madagascan brown algae

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ABSTRACT

In this paper, the influence of the conditions of bleaching on initial ground algae is discussed; it is demonstrated that it favours the yield of extraction of purified alginates but that it causes chain degradation and a decrease of the M/G ratio. These events are attributed to the sensitivity to hydrolysis of MM and MG osidic linkages. Nevertheless, from all the results it is shown that four of these original algae (except *Sargassum* sp. which has a M/G ratio around 1) from Madagascar are rich in guluronic units. Alginates obtained form strong gels in the presence of calcium (1 M $CaCl_2$) with ratios $G'/G''$ at 1 Hz larger than 6 (up to 8.6). In few cases, it is shown that bleaching favoured gel formation even if the molecular weight is decreased.



## 1. Introduction

Alginates are polysaccharides located in the cell wall and in the matrix of brown seaweeds (Rinaudo, 2007). They are produced also by some bacterial species (Fyfe & Govan, 1980; Linker & Jones, 1966; Russell & Gacesa, 1989; Sabra, Zeng, & Deckwer, 2001; Skjak-Break, Grasdalen, & Larsen, 1986). These polysaccharides are composed of two hexuronic acids: β-D-mannuronic acid (ManA or M) and α-L-guluronic acid (GulA or G), linked by 1–4 bonds. Alginates present two types of homopolymeric sequences: D-mannuronic acid blocks (MM) and L-guluronic acid blocks (GG), and additionally, heteropolymeric sequences of ManA and GulA (MG blocks) (Gacesa, 1988; Haug & Larsen, 1962; Haug, Larsen, & Smidsrod, 1967).

Alginates have widespread industrial uses especially due to their ability to form gels with calcium ions. The formation of these gels depends mainly of the presence of zones rich in GG blocks (Haug & Larsen, 1962). The physiological and the rheological properties of alginates as well as their applications as stabilizing, thickening, gelling or pharmaceutical additive, are strongly influenced by the uronic acids composition (ManA/GulA residues ratio), and by the distribution of the different blocks along the chains. The composition of alginates is directly dependent on the season and on the ecological conditions of algae growth (Rinaudo, 2008; Tonnesen & Karlsen, 2002). An interesting development is that alginates derived oligosaccharides were demonstrated as a potential source of biological active molecules (Tajima et al., 1999).

In native state, alginates exist as an insoluble salt form of mixed counterions found in seawater ($Na^+$, $Mg^{2+}$ and $Ca^{2+}$ especially) (Fourest & Volesky, 1997; Grant, Morris, Rees, Smith, & Thom, 1973). The extraction of alginates is based on the conversion from the insoluble to their soluble form using the $Na^+$ counterions in slightly basic conditions.

In this paper, extraction and characterization of alginates obtained from five brown algae collected from Madagascar coast are investigated at first. The determination of their rheological behaviour allows us to evaluate their quality as a function of the algae treatment adopted for alginate extraction. The current study is part of a program on polysaccharides from Madagascar seaweeds investigation.

## 2. Experimental

### 2.1. Materials

Five brown algae are studied: *Zonaria* sp. (EV) and *Chnoospora* sp. (IT) were collected from Fort-Dauphin, but *Sargassum* sp. (SG 4), *Spatoglossum* sp. (ST) from Toliary and *Sargassum* sp. (SG) from Fenerive-Est, respectively, in south-east, south-west and east coasts of Madagascar. The algae are washed with water, soaked quickly in ethanol to inactivate enzymes, dried overnight at 35 °C in an oven and ground.

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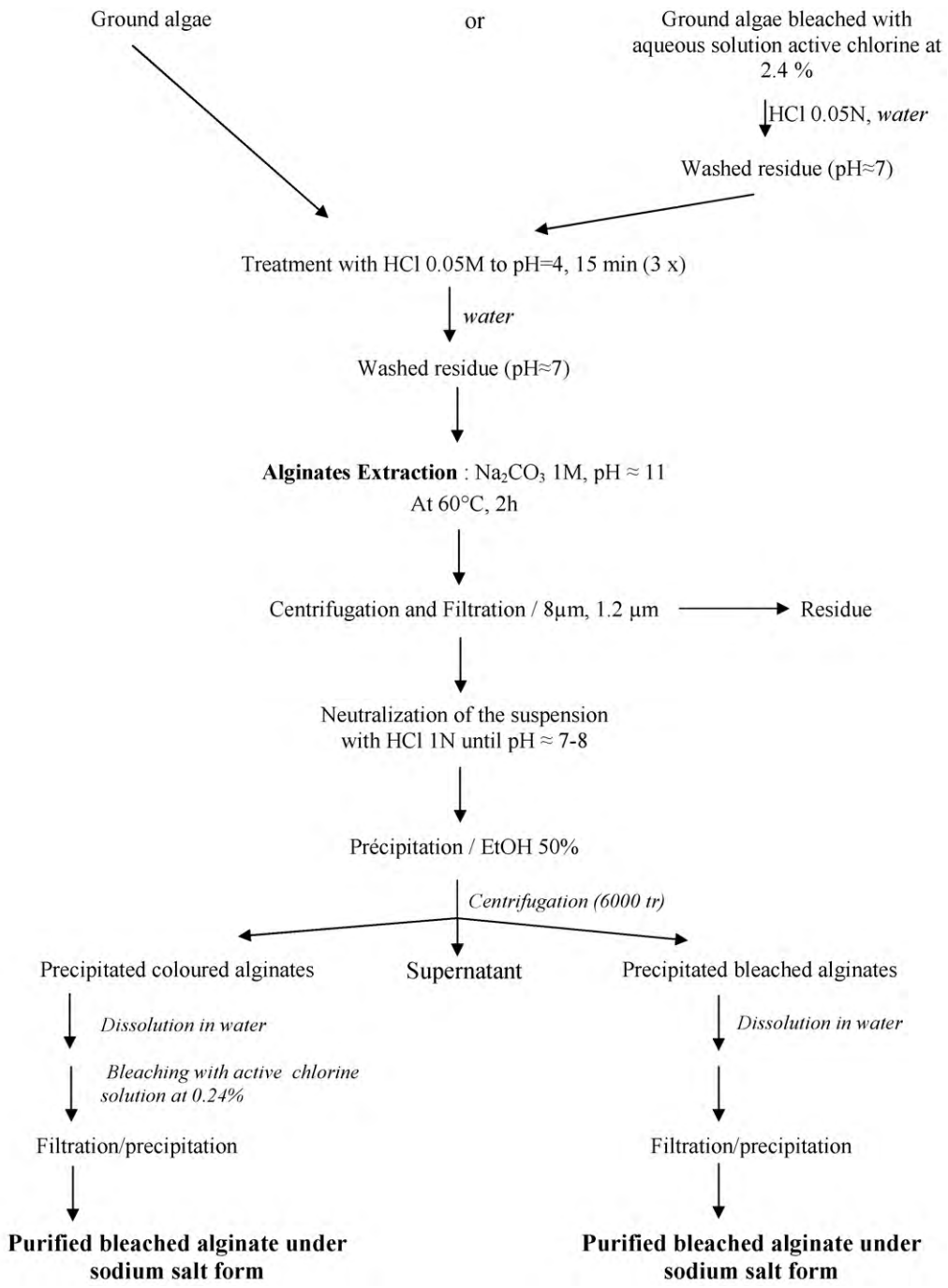


**Fig. 1.** Schema of treatment of algae to prepare sodium alginate.

### 2.2. Extraction of alginates

#### 2.2.1. Bleaching treatment of algae

The ground dark algae (2 g) are treated to eliminate the pigments by bleaching with 45 mL of aqueous solution of sodium hypochlorite with 2.4% active chlorine (obtained by dilution of commercial 9.6% active chlorine Javel Oxena, SA Pieri Chimie, France). The bleaching step will be examined in the following. The bleaching reaction with chlorine is stopped by neutralization with 0.05 N HCl. At end, residues are washed with water and centrifuged.

#### 2.2.2. Alginates extraction

Native or bleached algae residues are suspended in distilled water (200 mL) and treated as given in Fig. 1. An aqueous solution of 0.05 M HCl is added to reach pH 4 and the mixture is stirred during 15 min, at room temperature. The supernatant is eliminated by centrifugation. The moistened seaweeds are suspended in 300 mL of distilled water. By addition of an aqueous solution of 1 M $Na_2CO_3$, the pH of the suspension is adjusted at pH = 11. The algae are stirred at 60 °C for 2 h. After centrifugation, the supernatant is filtered successively on 8.0 µm, 3.0 µm and 1.2 µm porous membranes. The extracted solution is neutralized with 1 N HCl. The sodium alginate is precipitated with ethanol 50% in the presence of 0.5 M NaCl. At end, the sodium alginate is purified by a second precipitation with ethanol 50%. This precipitate is washed with ethanol/water (70/30, v/v), absolute ethanol and dried at room temperature.

#### 2.2.3. Bleaching of extracted alginates

Sodium alginate (100 mg) extracted from unbleached algae are suspended in water (4 mL) and treated with 20 mL of aqueous solution of sodium hypochlorite with 0.24% active chlorine (obtained by dilution of commercial 9.6% active chlorine Javel Oxena, SA Pieri Chimie, France). The suspension is stirred for 15 min. Water (10 mL) is added to the solution of bleached alginate which is neutralized with 0.05 N hydrochloric acid. Following, sodium alginate is precipitated in the presence of sodium chloride, with EtOH 50%, washed with EtOH 70%, EtOH 94%, and dried at ambient temperature.

### 2.3. Chemical analysis by $^1$H NMR

$^1$H NMR spectra of sodium alginates extracted from the five brown algae are successively recorded at 85 °C on 6 mg/mL solution in $D_2O$. $^1$H NMR experiments were performed using a Bruker Avance 400 spectrometer operating at 400.13 MHz. $^1$H NMR spectra were collected using 32K data points. Calibration was performed using the signal of the residual protons of the solvent as a secondary reference. Deuterium oxide was obtained from Euriso-Top, France.

### 2.4. Molecular weight distribution

Purified polymers under the sodium salt form are 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 injected was 0.5 g/L, with an injection volume of 100 μL using two columns in series (Shodex OH-pack 803 and 805). All samples are filtered on a 0.2 μm pore membrane (Sartorius AG; cellulose acetate filter) before injection, in order to retain large aggregates. The eluent used was 0.1 M $NaNO_3$, at 30 °C elution temperature and a flow rate of 0.5 mL/min; the dn/dc adopted is equal to 0.165 (Rinaudo, 2007). The molecular weight distribution, weight-average molecular weight ($M_w$), polydispersity index ($M_w/M_n$), and intrinsic viscosity ([$\eta$], mL/g) of the eluted polymers are obtained as characteristics of the biopolymers.

### 2.5. Gel characterization in the presence of calcium ions

Gel cylinders are prepared from an aqueous solution of alginates (20 g/L) by dialysis against 1 M $CaCl_2$, for 48 h (the bag used is made of a cellulosic membrane Spectra/Por; exclusion limit $M$ = 6–8000). Slices of gel (thickness around 2–3 mm) are cutted and rheological properties are measured at 25 °C using an AR2000 rheometer from TA Instruments (which allows to control the normal force). A two parallel plates geometry is used with a 2 cm diameter plate. For solution properties, an AR1000 rheometer from TA Instruments is used with cone and plate geometry (4 cm and 3°59 cone). Dynamic experiments are performed in the linear domain. The G′ and G″ moduli (Pa) as well as the complex viscosity $|\eta^*|$ (Pa s) are determined as a function of the frequency imposed (in Hz). The steady state viscosity $\eta$ (Pa s) is determined as a function of the shear rate ($\gamma$, $s^{-1}$) with the same geometry.

### 2.6. Swelling degree

Approximately, 200 mg of algae residues just after bleaching is recovered on a filter and weighted to get the wet weight; then, they are dried in an oven at 70 °C for 24 h to determine the dried weight of material. The degree of swelling ($\tau$) of the sample is given by $\tau$ equal to the mass (g) of water per g of dried bleached algae.

## 3. Results and discussion

### 3.1. Study of the bleaching step on native algae

Inspired from McHugh et al. studies on the bleaching of alginates (McHugh, Hernandez-Carmona, Arvizu-Higuera, & Rodriquez-Montesinos, 2001), the sodium hypochlorite is chosen as bleaching agent for Madagascan dark brown algae. The extraction after bleaching with NaOCl on algae gives white alginates with highest yields than after direct extraction as discussed later. The influence of the bleaching conditions is examined in the following on *Sargassum* sp. (SG) algae.

### 3.2. Influence of sodium hypochlorite concentration

Using different concentrations of sodium hypochlorite bleaching agent, namely 0.48%, 0.96%, 1.44%, 1.92% and 2.4% active chlorine (obtained by dilution of commercial 9.6% active chlorine Javel Oxena, SA Pieri Chimie, France), allows us to establish that the degree of bleaching of algae increases when hypochlorite concentration increases. An optimum at 2.4% active chlorine is observed where the samples are perfectly discoloured. This concentration is chosen to test the kinetic of bleaching and select a convenient time of reaction.

### 3.3. Kinetic of bleaching

For this study, the following experimental conditions were chosen: a 500 mg sample of ground algae SG is dispersed in 25 mL of aqueous solution of sodium hypochlorite with 2.4% active chlorine (obtained by dilution of commercial 9.6% active chlorine Javel Oxena, SA Pieri Chimie, France) at ambient temperature. After 10 min of treatment, cream algae are obtained. Bleaching reaction is continued up to 30 min to control the alginate extract yields and their characteristics. The influence of bleaching conditions on alginates characteristics are given in Table 1. According to the bleaching time, the alginate yield extracted increases up to 20 min of bleaching; in the same time, the accessibility of the bleached algae reflected by the swelling degree increases. The alginate samples obtained after different times of bleaching are firstly analyzed by $^1$H NMR spectroscopy (Fig. 2). The relative integrals of peaks H1 (G), H1 (M) + H5 (GM) and H5 (GG) signals at 5.1 ppm, 4.7 ppm and 4.5 ppm, respectively, contain information on the uronic acid composition of alginates (and gives the ratio M/G) (Grasdalen, 1983; Grasdalen, Larsen, & Smidsrod, 1979; Heyraud et al., 1996). In addition, it is observed that the ratio M/G is decreasing which indicates that mannuronic units are taken off the material, in direct rela-

**Table 1**
Characterization of samples obtained as a function of bleaching time on *Sargassum* sp. algae(SG).

| Time (min) | Swelling degree (g/g) | Alginate yield (%) | M/G from NMR | $M_w$ SEC | % Eluted SEC | [$\eta$] mL/g SEC |
|---|---|---|---|---|---|---|
| Native algae | 11.1 | 23.6 | 1.09 | 950,000[a] | 5[a] | 660 |
| 5 | 15.1 | 20.7 | 0.74 | 160,000 | 75 | 246 |
| 10 | 16.4 | 23.6 | 0.70 | 190,000 | 68 | 243 |
| 20 | 19.3 | 30.5 | 0.68 | 113,000 | 76 | 193 |
| 30 | 23.3 | 17.3 | 0.66 | 99,000 | 77 | 236 |

[a] Overestimated $M_w$ due to aggregation as indicated by the small fraction (5%) analyzed by SEC.

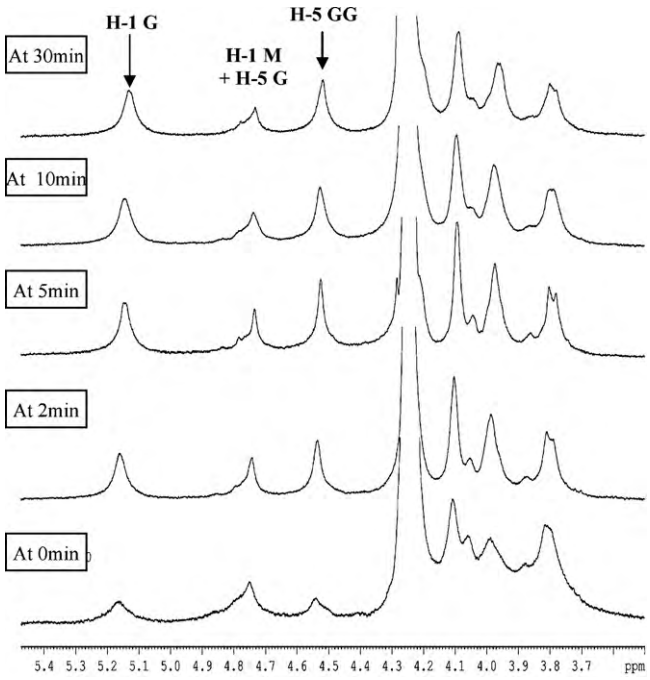


**Fig. 2.** Evolution of the alginate $^1$H NMR spectrum as a function of the bleaching time.

tion with the sensitivity of MG and MM blocks to hydrolysis. In the same time, from SEC experiments, it is found that around 75% of the material is eluted from the columns except for the extract from unbleached algae which is full of large aggregates. The molecular weight as well as the intrinsic viscosity decreases after bleaching which indicates a breakdown of the main chain. Taking into account these results, we have adopted the following conditions to bleach the different original algae: 2.4% active chlorine solution, ambient temperature, 20 min of contact.

### 3.4. Bleaching on extracted alginates

On one example (SG), the bleaching is performed on extracted alginates from unbleached algae following the method proposed in the literature (McHugh et al., 2001). The bleached alginate obtained in these conditions (named AbSG) is compared with the alginate extracted from the bleached algae (SG-b) in Table 2. In relation with the lower concentration in chlorine used, the AbSG sample has a higher molecular weight than SG-b but the yield recovered is larger for SG-b in relation with the larger accessibility of bleached algae.

## 4. Characterization of original alginates

The yields of sodium alginate extraction are given in Table 2. The yield of alginate obtained after bleaching of the algae is always higher than when it is recovered directly. It may be related with the swelling effect of the bleaching treatment as shown before.

The two routes represented in Fig. 1 are compared: (i) bleached alginate AbSG is recovered at a rate of 83.6% up to 93.7% from the SG extract (obtained from unbleached algae with a yield of 23.6%). Total of bleached alginate recovered is then 19.7% up to 22.0% on the basis of initial dried algae but (ii) one gets 30.5% from bleached algae (Table 2). The lower yield may be attributed to the lower accessibility of algae in the absence of bleaching treatment of algae and confirms, as mentioned previously, that higher alginate yield is obtained from bleached algae.

All the samples are characterized by NMR and SEC analysis; the data are given in Table 2. The $^1$H NMR spectra of sodium alginate from the five brown algae collected from Madagascar coast are characterized by low mannuronic acid content as shown by the low value of M/G ratio (especially after bleaching) except for SG algae (Table 2). Nevertheless, it is not a direct information on the M/G ratios of alginates *in situ* as it was shown that mannuronic units are more sensitive than guluronic unit to bleaching conditions.

It is shown that bleaching decreases the weight-average molecular weight and intrinsic viscosity of alginate (Table 2) as well as the viscosity of aqueous solutions. Firstly, the AbSG sample maintains a higher $M_w$ and a higher M/G ratio in relation with the lower amount of chlorine used for its bleaching. The decrease in viscosity upon bleaching on few samples is shown in Fig. 3. The decrease in viscosity is more important on SG4 samples for which the viscosity becomes Newtonian in the whole range of shear rates; this result is not explained in the present state of our study. From viscosity, the sample bleached after extraction (AbSG) looks less degraded than the extract from the bleached algae and, also, there is no significant change in M/G ratio. The differences observed among the

**Table 2**
Characteristics of alginates samples obtained by SEC and NMR.

| Extracted samples | Yields (%)[a] | $M_w$ | $[\eta]$ (mL/g) | M/G from $^1$H NMR | Viscosity at 1 s$^{-1}$[b] |
|---|---|---|---|---|---|
| *Zonaria* sp. (EV) | | | | | |
| EV[c] | 10.2 | 420,500 | 295 | – | – |
| EV-b[d] | 30.0 | 140,700 | 141 | 0.41 | – |
| *Chnoospora* sp. (IT) | | | | | |
| IT[c] | 9.2 | 228,700 | 590 | – | – |
| IT-b[d] | 50.8 | 149,300 | 162 | 0.51 | – |
| *Spatoglossum* sp. (ST) | | | | | |
| ST[c] | 9.7 | 203,800 | 342 | – | – |
| ST-b[d] | 17.4 | 95,000 | 135 | 0.75 | – |
| *Sargassum* sp. (SG) | | | | | |
| SG[c] | 23.6 | 385,000 | 1,910 | 1.09 | 44.7 |
| SG-b[d] | 30.5 | 113,000 | 192 | 0.68 | 3.00 |
| AbSG[e] | 22.0 | 225,000 | 850 | 1.00 | 5.50 |
| *Sargassum* sp. (SG4) | | | | | |
| SG4[c] | 26.6 | 190,000 | 565 | 0.76 | 25.20 |
| SG4-b[d] | 33.7 | 138,400 | 208 | 0.44 | 0.04 |

[a] Percentage weight on the basis of dried algae weight.
[b] On 20 g/L solution in water.
[c] From unbleached algae.
[d] From chlorine bleached algae.
[e] Chlorine bleached alginate extracted from unbleached algae.

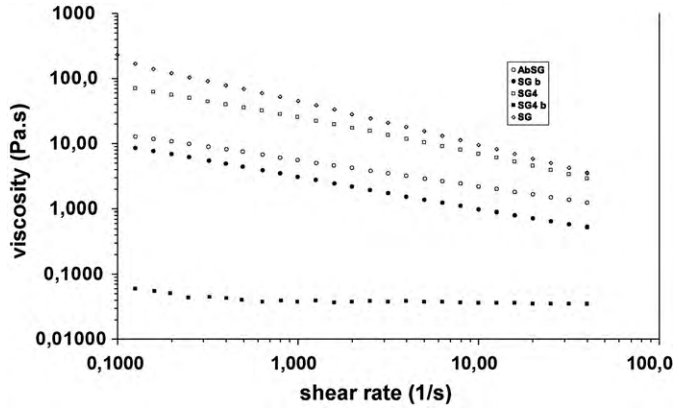


**Fig. 3.** Steady state viscosity as a function of shear rate for few samples dissolved in water at 20 g/L. Temperature is 25 °C.

different algae may be related to the microstructure of the alginates: the richer in MM and MG blocks is the more sensitive to degradative depolymerisation and decrease of M/G ratio. In addition, due to possible aggregation between alginate chains which remains partly in SEC experiments, it is very difficult to compare the characteristics of the alginates extracted from different algae (such as given in Table 2).

The conclusion of these characterizations is that at least four of the algae from Madagascar are rich in guluronic acid, which is the basis of gelation in the presence of calcium. Then, it is important to test for their gelling properties. Bleaching with chlorine usually increases the yield of alginate recovered, and increases the solubility in water of the extract (decreases of the turbidity and better elution from SEC) but decreases its molecular weight and the M/G ratio indicating a degradation of the MG and/or MM blocks.

## 5. Gels properties in the presence of calcium

The rheological characteristics of gels formed in the presence of calcium are determined first to examined the influence of bleaching on the gel performances and secondly to relate the physical properties to the microstructure of the polymers. Pictures of pieces of gel are shown in Fig. 4. These slices of calcium alginate gels were tested in rheometry.

In Figs. 5 and 6, the G′ and G″ as a function of frequency are given for few of the samples tested. For EV, the bleached alginates are much stronger in calcium form than the extract of unbleached algae which contains more coloured products (preventing probably association). EV looks particularly rich in polyphenols which may reach values up to 15% of the dried mass (they are condensed tannins with antioxidant activity) (Chkhikvishvili & Ramazanov, 2000; Parys et al., 2009). These hydrophobic species decrease the effective alginate content and may prevent dispersion of alginate chains in solution and consequently the interaction with calcium. The same behaviour is obtained with SG4 (data not shown). On the opposite, the samples of IT are very similar (unbleached sample being a little stronger). The data obtained for G′ and G″ moduli for the different samples are given in Table 3.

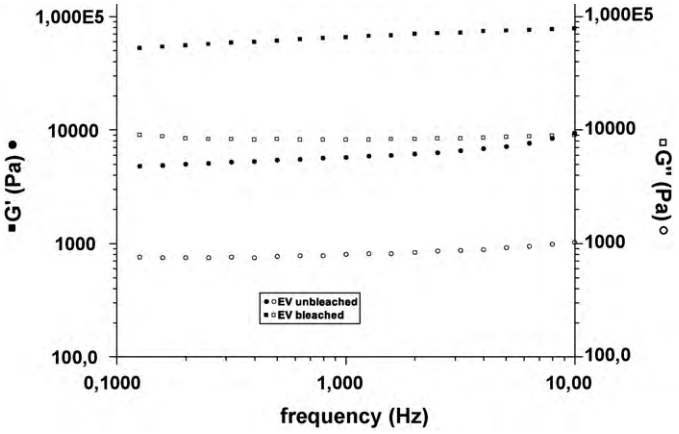


**Fig. 5.** Dynamic rheology of calcium gels from EV algae at 25 °C. Influence of bleaching.

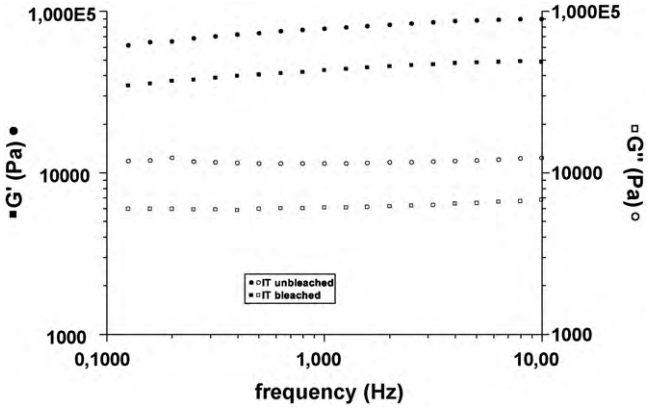


**Fig. 6.** Dynamic rheology of calcium gels from IT algae at 25 °C. Influence of bleaching.

**Table 3**
Comparison of G′ and G″ dynamic moduli at 1 Hz determined at 25 °C for the different alginates under calcium form (index b for bleached samples).

| Samples | G′ (Pa) at 1 Hz | G″ (Pa) at 1 Hz |
|---|---|---|
| EV | | |
| EV | 4,937 | 728 |
| EV-b | 56,370 | 7,366 |
| IT | | |
| IT | 65,295 | 9,565 |
| IT-b | 34,145 | 4,816 |
| SG4 | | |
| SG4 | 48,485 | 6,708 |
| SG4-b | 65,617 | 7,655 |
| ST | 30,570 | 4,731 |
| SG | 44,390 | 7,380 |

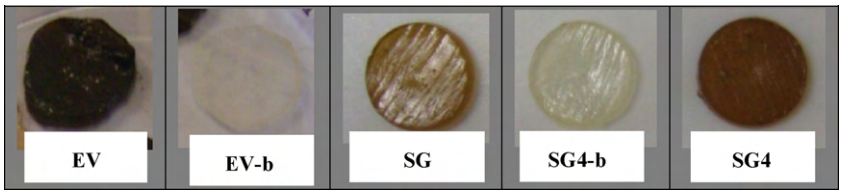


**Fig. 4.** Slices of calcium alginate of few studied gels.

From these data, it comes that EV, SG4 and IT give the stronger gels, usually after bleaching; these alginates have a molecular weight and a ratio M/G of the same order of magnitude and they are rich in guluronic acid units.

## 6. Conclusions

In this paper, five algae from Madagascar were studied to establish the quality of the alginate extracted after or before a step of bleaching. Firstly, the influence of conditions of bleaching was investigated: chlorine bleaching (with 2.4% of active chlorine) on grounded algae at ambient temperature was adopted with an optimum time of 20 min. Clearly, it is demonstrated that this treatment decreases the weight-average molecular weight and the M/G ratio. This indicates that the osidic linkages in MG and MM blocks are more sensitive to degradation than those of GG blocks. After bleaching, extraction of alginates is performed in alkaline conditions as usually recommended; it is shown that the yield in alginate extracted is favoured by chlorine bleaching possibly due to the swelling of the algae cell walls. It seems that bleaching on extracted impure alginates is less degrading than bleaching on algae (using a lower chlorine concentration) but, in these conditions, the yield of recovery of bleached alginate is lower. The performances of alginates obtained from the five algae tested were compared under calcium gel form. These gels were formed by dialysis against 1 M calcium chloride. The role of bleaching on elastic moduli G′ is discussed: on algae EV and SG4, bleaching is favourable but it is not a general conclusion valid for the other algae (as shown on IT).

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