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## Sulfate groups position determines the ionic selectivity and syneresis properties of carrageenan systems

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1 **Sulfate groups position determines the ionic selectivity and syneresis**  
2 **properties of carrageenan systems**

3

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9

10 **Abstract**

11 The salt sensitivity and selectivity feature of  $\alpha$ -carrageenan ( $\alpha$ -Car) were investigated and  
12 compared with  $\kappa$ -carrageenan ( $\kappa$ -Car) and iota-carrageenan ( $\iota$ -Car). These carrageenans are  
13 identified by one sulfate group on the 3,6-anhydro-D-galactose (DA) for  $\alpha$ -Car, D-galactose  
14 (G) for  $\kappa$ -Car and on both carrabiose moieties (G and DA) for  $\iota$ -Car. The viscosity and  
15 temperature, where order-disorder transition have been observed, were greater in presence of  
16  $\text{CaCl}_2$  for  $\alpha$ -Car and  $\iota$ -Car compared with KCl and NaCl. Conversely, the reactivity of  $\kappa$ -Car  
17 systems were greater in presence of KCl than  $\text{CaCl}_2$ . Unlike  $\kappa$ -Car systems, the gelation of  $\alpha$ -  
18 Car in presence of KCl was observed without syneresis. Thus, the position of sulfate group on  
19 the carrabiose determines the importance of counterion valency too. The  $\alpha$ -Car could be a  
20 good alternative to  $\kappa$ -Car to reduce the syneresis effects.

21 **Keywords:** Alpha-carrageenan; Sulfate group position; Anhydro-ring; Viscosity; Salt  
22 sensitivity; Syneresis

## 23 **1 Introduction**

24 Numerous applications of natural polymers are limited by the ability of the polymers to give a  
25 gel with exudation (syneresis) (Chen, Xu & Wang, 2007; Gonzalez, Loubes, Bertotto, Baeza  
26 & Tolaba, 2021; Laplante, Turgeon & Paquin, 2006). Some of the applications are relevant  
27 for human health management like cell cultures in hydrogels for disease diagnostics and tissue  
28 engineering (Divoux, Mao & Snabre, 2015; Hoffman, 2012; Jafari, Farahani, Sedighi, Rabiee  
29 & Savoji, 2022; Peppas, Bures, Leobandung & Ichikawa, 2000; Zhang et al., 2020). To  
30 understand the physicochemical causes of syneresis of natural polymers hydrogels, kappa-  
31 carrageenan polysaccharide is used as the material of this investigation, because this material  
32 exhibits severe syneresis in relatively short experimental time with a dependence to many  
33 physicochemical factors (Ako, 2015; Elmarhoum & Ako, 2021). In this study, the target is the  
34 sulfate,  $-\text{SO}_3^-$ , ionic group position in the monomer of this polysaccharide.

35 Carrageenan (Car) defines a family of sulfated galactans extracted from marine algae  
36 (Prechoux, Genicot, Rogniaux & Helbert, 2013; Souza, Hilliou, Bastos & Gonçalves, 2011;  
37 Usov, 2011). The disaccharide repetition moieties (i.e. carrabiose) of the polysaccharide is  
38 made with two galactose units identified as G and DA for respectively D-galactose and 3,6-  
39 anhydro-D-galactose, each of which can carry different number of sulfate functional group  
40 (Ueda, Saiki & Brady, 2001; Usov, 2011) (Fig.1). The number and position of the sulfate  
41 group on the carrabiose identifies the type of carrageenan (Campo, Kawano, da Silva Jr &  
42 Carvalho, 2009; Millane, Chandrasekaran & Arnott, 1988; Perez & Claudio, 2020; Souza,  
43 Hilliou, Bastos & Gonçalves, 2011; Viebke, Borgström & Piculell, 1995). Kappa-carrageenan  
44 ( $\kappa$ -Car) has one sulfate group at the position 4 of the G units and is coded as G4S-DA. Alpha-  
45 Carrageenan ( $\alpha$ -Car) has one sulfate group at the position 2 of the DA units and is coded as G-  
46 DA2S accordingly (Bourgoin, Zablackis & Poli, 2008). Iota-carrageenan ( $\iota$ -Car) has two

47 sulfates on the carrabiose units on the same carbon position as for  $\kappa$ - and  $\alpha$ -Car, and thus, is  
48 coded as G4S-DA2S. The number, the position and the distribution of ester sulfate along the  
49 carrageenans chain define the physicochemical properties of the polysaccharides (Millane,  
50 Chandrasekaran & Arnott, 1988; Souza, Hilliou, Bastos & Gonçalves, 2011). As an example,  
51 with the same concentration of positive charge counterions, the divalent counterions like  
52 calcium ( $\text{Ca}^{2+}$ ) influence strongly the flow behavior of  $\iota$ -Car solution than the monovalent  
53 counterions like potassium ( $\text{K}^+$ ) (Bui, Nguyen, Renou & Nicolai, 2019; Elfaruk, Wen, Chi &  
54 Li, 2021). Conversely,  $\text{K}^+$  ions change drastically the flow behavior of  $\kappa$ -Car solution  
55 compared with  $\iota$ -Car (Lai, Wong & Lii, 2000; Norton & Goodall, 1983b). The selectivity of  $\iota$ -  
56 Car and  $\kappa$ -Car to respectively divalent and monovalent counterions and the fact that trivalent  
57 counterion induced gelation in  $\lambda$ -Car, which carries three sulfate groups on the carrabiose,  
58 lead to claim that the number of sulfate on the carrabiose is what determines the importance  
59 of counterion valency (Running, Falshaw & Janaswamy, 2012).

60 The exact role of the sulfate position on the counterions binding and importance of the  
61 counterion valency in carrageenan systems are unclear (Klevens, 1950; Lindman, Karlstrom  
62 & Stigsson, 2010; Running, Falshaw & Janaswamy, 2012). The  $\kappa$ -Car hydrogels, for example,  
63 exhibit severe syneresis behavior in presence of  $\text{K}^+$  than  $\iota$ -Car and other types of salts which  
64 are commonly used in food and biological domains (Thrimawithana, Young, Dunstan &  
65 Alany, 2010). Therefore, in order to understand the influence of the sulfate position on the  
66 rheological behavior of the carrageenans, we took advantage of the recent discovery of the  
67 endo-4O- $\iota$ -carrageenan sulfatase secreted by the marine bacteria *Pseudoalteromonas atlantica*  
68 T6c. This enzyme removes specifically the sulfate ester group located at the position 4 of the  
69  $\iota$ -carrabiose moiety and allows completely converting the  $\iota$ -Car into  $\alpha$ -Car (Prechoux,  
70 Genicot, Rogniaux & Helbert, 2013). We have investigated the rheological behavior of  $\kappa$ -Car

71 and  $\alpha$ -Car solution in presence of different types of salts to emphasize the role that the  
72 positions of  $-\text{SO}_3^-$  may have in the flow properties of the polysaccharide.

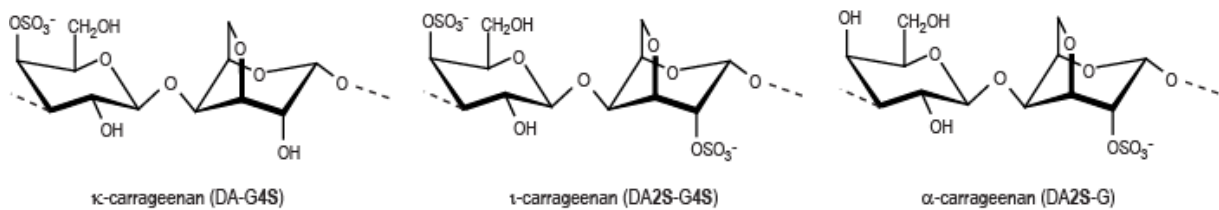
## 73 **2 Material and methods**

### 74 *2.1 Samples characteristics*

75 **Molecular weight.** The samples were solubilized in 0.1 M  $\text{NaNO}_3$  and filtered on 0.2  $\mu\text{m}$   
76 membrane. The samples were injected on two OHPak SB 806 M HQ Shodex columns  
77 mounted in series and eluted at 30°C in 0.1 M  $\text{NaNO}_3$ . Detection was monitored by a Optilab  
78 rEX Wyatt refractometer and a mini DAWN TREOS Wyatt light scattering working at three  
79 angles. The  $\text{dn}/\text{dC}$  was 0.142 mL/g. Polydispersity indices was calculated  $I_p = M_w/M_n$ . All  
80 measurements were done in triplicate.

81 **Enzymatic preparation of alpha-carrageenan ( $\alpha$ -Car).** The  $\alpha$ -carrageenan (Fig1) was  
82 prepared according to the method reported by (Prechoux, Genicot, Rogniaux & Helbert, 2013)  
83 using recombinant 4O-iota-carrageenan sulfatase. Briefly, the genePatl\_0889 (GenBank  
84 accession number ABG39415) encoding the sulfatase with a N-terminal poly-histidine tag  
85 was over expressed in BL21-DE3 *Escherichia coli* strain and purified on a Nickel affinity  
86 column. Iota-carrageenan provided by Danisco (Brabrand, Denmark) was incubated with the  
87 purified sulfatase in 50 mM Tris-HCl pH 7.5. Incubation with additional fresh enzymes was  
88 repeated until the complete conversion to  $\alpha$ -carrageenan was achieved. The yield of  
89 desulfation was measured by  $^1\text{H}$  NMR (Prechoux, Genicot, Rogniaux & Helbert, 2013). The  
90 polysaccharide was ultra-filtered on 10 Da membrane in water and lyophilized. The average  
91 molecular weight of the polysaccharide was  $M_w = 3.8 \times 10^5$  g/mol, with an average  
92 polydispersity index of  $M_w/M_n = 1.78$ .

93 **Kappa-carrageenan (k-Car)**. The  $\kappa$ -Car polysaccharide (Fig1) is a gift from Rhodia Food  
94 Switzerland, product name and reference: MEYPRO-GEL 01/2001 WG95-37 K-Car. The  
95 dialyzed product may contain a weak amount of glucose and was found in the pure potassium  
96  $\kappa$ -Car form of total potassium at no more than 4.2 % (w/w); the other salts were considered to  
97 be only trace amounts. The measurement of its molecular weight was done in triplicate. The  
98 average molecular weight of the polysaccharide was  $M_w = 3.3 \times 10^5$  g/mol, with an average  
99 polydispersity index of  $M_w/M_n = 2.02$ .



100

101 *Figure 1: Structure of the carrabiose moieties composing kappa-, alpha- and iota-*  
102 *carrageenan.*

## 103 2.2 Samples preparation

104 A 20 g/L solution was prepared by dissolving the  $\kappa$ -Car polysaccharides powder in  
105 demineralized water with 200 ppm sodium azide as the bacteriostatic agent under heat and  
106 stirring conditions. The resulting solution was dialyzed and filtered through 0.45  $\mu$ m pore  
107 size. A 9 g/L of solution was prepared by dilution of the 20 g/L solution and was stocked in  
108 fridge at 5  $^{\circ}$ C, with which, all the samples were prepared by dilution with demineralized  
109 water containing 200 ppm sodium azide with salt at the appropriate concentration. The  
110 dilution gives the desired final concentration of polysaccharide and salt concentration at the  
111 appropriate temperature. The  $\alpha$ -Car polysaccharide in the lyophilized form were dissolved in  
112 demineralized water with 200 ppm sodium azide under heat and stirring conditions to 9 g/l  
113 stock solution. The samples were prepared in the same way like the  $\kappa$ -Car using the  
114 appropriate salt.

115 *2.3 samples characterization*

116 **Viscosity measurements.** The viscosity measurements were performed in Couette geometry  
117 using a DHR3 Rheometer (TA Instrument). The Couette geometry consisted of a concentric  
118 cylinder geometry of an inner rotor cylinder (bob) and outer stator cylinder (cup) of  
119 respectively a radius  $R_1$  of 14 mm and  $R_2$  of 15 mm defining a horizontal gap ( $R_2 - R_1$ ) of 1  
120 mm and an average radius  $R$  of 14.5 mm as  $(R_1 + R_2)/2$ . The height of the bob was 42 mm and  
121 the vertical gap between the bob and cup was set at 2 mm. The minimum and maximum  
122 torque of the instrument were  $5 \mu\text{Nm}$  and  $5 \times 10^3 \text{ Nm}$  respectively. Both the torque ( $M$ ) and the  
123 angular velocity ( $\Omega$ ) of the bob, which were proportional with respectively the stress ( $\sigma$ ) and  
124 shear rate ( $\dot{\gamma}$ ), were measured by the rheometer, from which the viscosity ( $\eta$ ) was displayed  
125 as  $\sigma / \dot{\gamma}$ . The viscosity was determined as a function of the shear rate from  $0.3 \text{ s}^{-1}$  to  $30 \text{ s}^{-1}$  to  
126 define the Newtonian flow domain at a constant temperature in the range between  $5 \text{ }^\circ\text{C}$  and  $60$   
127  $^\circ\text{C}$ . The viscosity was also measured at a constant shear rate of  $15 \text{ s}^{-1}$  while cooling or heating  
128 at  $2 \text{ }^\circ\text{C min}^{-1}$  or a lower rate  $0.5 \text{ }^\circ\text{C.min}^{-1}$ .

129 **Viscoelastic modulus measurements.** The rheological measurements were performed using a  
130 DHR3 Rheometer (TA Instrument) with etching conic geometry ( $\text{\O}50 \text{ mm}$ ,  $2.0^\circ$ ,  
131 TADC2<sup>o</sup>InR500) and etching plate. The conic geometry has a truncation gap of  $110.0 \mu\text{m}$ . A  
132 portion of the prepared hot sample solution was loaded onto the Peltier of the rheometer and  
133 set at a temperature above the gelation temperature of the sample. After loading the sample,  
134 the surface was covered with a thin layer of mineral oil to avoid evaporation. Then, the  
135 temperature and the measurement program were run. The condition of each measurement will  
136 be specified in the text.

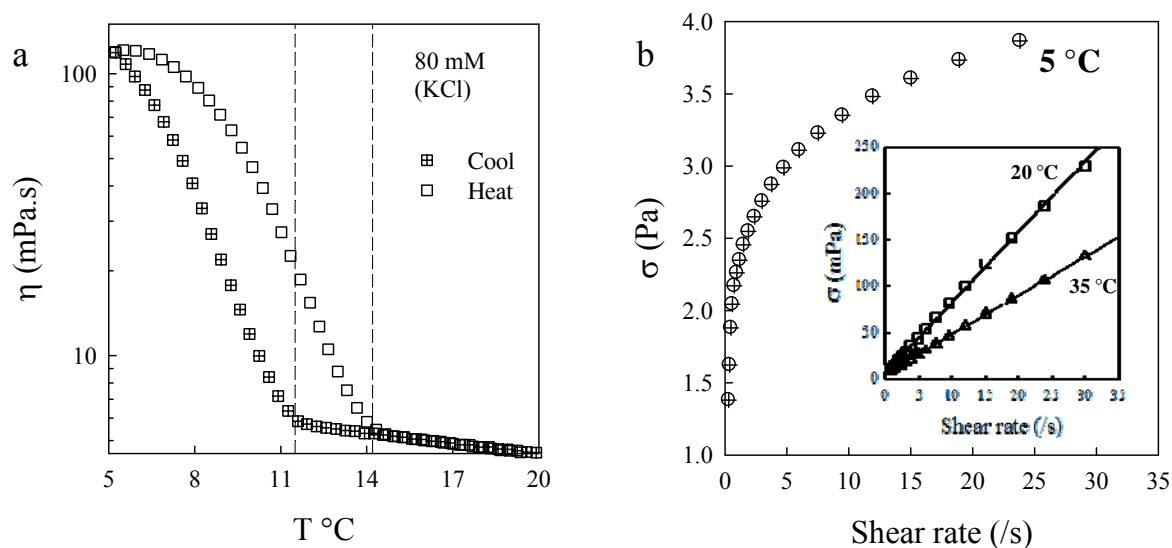
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138

### 139 3 Results and discussion

#### 140 3.1 Flow behavior of $\alpha$ -carrageenan solution

141 The viscosity of  $\alpha$ -Car in presence of 80 mM KCl is displayed in Fig. 2a for cooling and  
142 heating ramps. The viscosity increases when the temperature is decreased, and sharply from  $\approx$   
143 11.5  $^{\circ}$ C. The sharp increase of the viscosity was shown by Rochas and Rinaudo (1984) to be  
144 concomitant with the coil-helix transition. If the temperature is increased immediately from 5  
145  $^{\circ}$ C, the viscosity decreases sharply and the helix-coil transition was found at  $\approx$  14.3  $^{\circ}$ C above  
146 the coil-helix transition (Rochas & Rinaudo, 1984). The thermal hysteresis was reported as a  
147 result of inter-helical aggregation (Morris, Rees & Robinson, 1980; Piculell, Nilsson &  
148 Muhrbeck, 1992).



149  
150 *Figure 2: a) temperature dependence of  $\alpha$ -Car solution viscosity for 3 g/L, 80 mM KCl, b)*  
151 *shear rate and stress relation displayed for the solution to show the viscosity behavior, at 5*  
152  *$^{\circ}$ C in the helices domain the viscosity changes with the shear rate, in the coil domain the*  
153 *viscosity is constant (insert).*



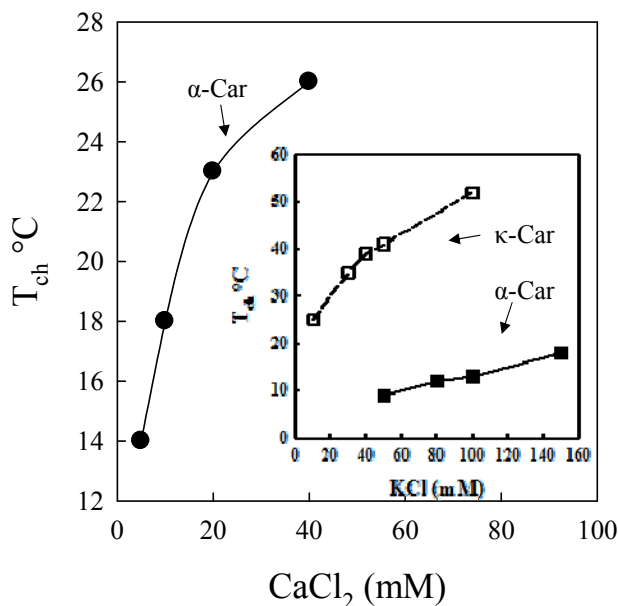
154 The thermal hysteresis defines three temperature domains which are identified in the Fig.2a  
155 as: (1) domain below the coil-helix transition temperature,  $< 11.5\text{ }^{\circ}\text{C}$ , (2) domain between the  
156 coil-helix and helix-coil transition temperature,  $[11.5\text{ }^{\circ}\text{C}, 14\text{ }^{\circ}\text{C}]$  and (3) domain above the  
157 helix-coil transition temperature,  $> 14\text{ }^{\circ}\text{C}$ . The conformational state of the carrageenan in  
158 temperature domain (2) depends on the temperature ramp (cooling or heating) but in the  
159 domain (1) or (3) the conformations of the polysaccharide chains were respectively either  
160 helices or coils. The shear rate dependence of the applied stress plots is shown in the Fig.2b.  
161 A non-linear relationship is observed, at  $5\text{ }^{\circ}\text{C}$ , in the domain below the coil-helix transition  
162 temperature and a linear relationship is observed at temperatures in the domain above the  
163 helix-coil transition temperature (insert of Fig.2b). We have observed that the flow behavior  
164 of  $\kappa$ -Car turns from Newtonian to non-Newtonian flow when the conformations of the  
165 polysaccharides turn from coils to helices (Elmarhoum & Ako, 2021). We think that similar  
166 conformational change could explain the temperature dependence of the flow behavior of  $\alpha$ -  
167 Car. The impact of salt on the flow behavior of carrageenan solutions depends mainly on the  
168 kinetics of the helices aggregation and the kinetics increases with decreasing temperature  
169 below the coil-helix transition temperature (Meunier, Nicolai, Durand & Parker, 1999). We  
170 didn't observe the gelation for  $\alpha$ -Car solutions in the interval of temperature explored in  
171 Fig.2a, when in the same KCl and polysaccharide concentration the  $\kappa$ -Car solutions gelled at  
172 temperature below  $50\text{ }^{\circ}\text{C}$  with syneresis (Ako, 2015, 2017). A lower helices aggregation  
173 kinetics is expected in relation with the weak coil-helix transition temperature in comparison  
174 with  $\kappa$ -Car. We didn't exclude the fact that the  $\alpha$ -Car solutions at temperature below  $12\text{ }^{\circ}\text{C}$   
175 behave like a suspension of aggregates (Garrec, Guthrie & Norton, 2013).

### 176 *3.2 Coil-helix and helix-coil transition temperature: effect of salt*

177 The transition from random coils to helices conformation, which is measured using optical  
178 rotation technique, have been observed to be concomitance with the steep rising of the

179 viscosity (Rochas & Rinaudo, 1984). Therefore, the onset of helix formation during cooling  
 180 ramp is characterized by the temperature at which the viscosity starts to increase and is  
 181 denoted as the coil-helix temperature ( $T_{ch}$ ). Salt concentration dependence of the  $T_{ch}$  is shown  
 182 in the Fig.3. The results for  $CaCl_2$  salt are displayed for  $\alpha$ -Car and the result for KCl salt is  
 183 displayed in the insert of the Fig.3 for  $\alpha$ -Car and  $\kappa$ -Car. The polysaccharides sensitivity to salt  
 184 as a polyelectrolyte is expected (Ako, 2015; Ciancia, Milas & Rinaudo, 1997), but the fact  
 185 that the coil-helix transition of  $\alpha$ -Car is more sensitive to  $CaCl_2$  than KCl was not obvious  
 186 regarding the literature on  $\kappa$ -Car (Elmarhoum & Ako, 2021). Moreover, the sensitivity of  $\kappa$ -  
 187 Car to KCl result in a greater coil-helix transition temperature than the sensitivity of  $\alpha$ -Car to  
 188 both KCl and  $CaCl_2$ . This finding emphasis the importance of the  $-SO_3^-$  position in the  
 189 monomer as a physicochemical factor, which determines the polysaccharide reaction to salt.

190



191

192 *Figure 3:  $CaCl_2$  dependence of the coil-helix transition temperature of  $\alpha$ -Car and in the insert*  
 193 *the KCl concentration dependence of the coil-helix transition of  $\alpha$ -Car and  $\kappa$ -Car.*

194 For  $\alpha$ -Car, if  $\text{CaCl}_2$  concentration is increased from 5 mM to 40 mM, the  $T_{\text{ch}}$  increases from  $\approx$   
195 14 °C to  $\approx$  26 °C. The  $T_{\text{ch}}$  increases from  $\approx$  10 °C to 20 °C, if the KCl concentration is  
196 increased from 45 mM to  $\approx$  150 mM and the coil-helix transition was not observed for 20 mM  
197 KCl concentration, which is two time greater than 5 mM  $\text{CaCl}_2$  in term of the concentration of  
198 positive charge counterions. Therefore,  $\alpha$ -Car is more sensitive to divalent than the  
199 monovalent counterions. Based on this result, the behavior of  $\alpha$ -Car with one sulfate anionic  
200 functional group on its monomer is rather similar to that of  $\iota$ -Car with two sulfates anionic  
201 functional groups than  $\kappa$ -Car (Morris, Rees & Robinson, 1980). The  $\kappa$ -Car systems with one  
202 sulfate anionic functional group on its monomer react strongly in presence of KCl salt than  
203 the  $\alpha$ -Car systems. The  $T_{\text{ch}}$  increases from  $\approx$  25 °C to  $\approx$  50 °C, if the KCl concentration is  
204 increased from 10 mM to 100 mM, with the hysteresis loop effect.

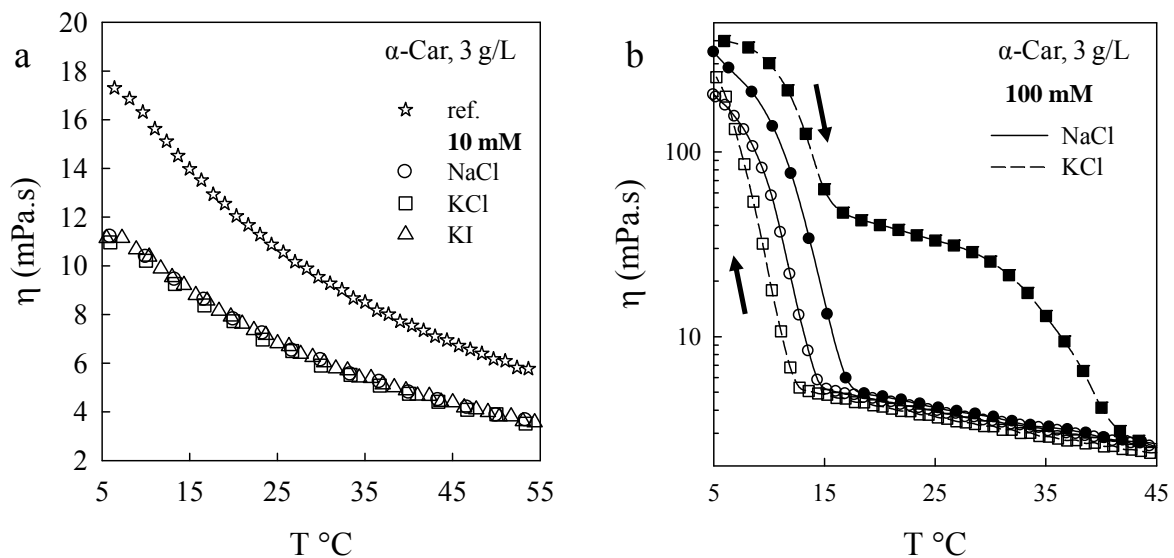
### 205 *3.3 Monovalent salt sensitivity of $\alpha$ -Car*

206 The fact that the conformational change of  $\alpha$ -Car happens at a greater temperature in presence  
207 of calcium than potassium counterions, characterizes its greater sensitivity for the divalent salt  
208 than the monovalent salt (Morris, Rees & Robinson, 1980). At any rate, in the conditions  
209 where the coils conformations prevail, the viscosity of the solution doesn't depend on the  
210 cooling or heating ramps and the solution flow is Newtonian. In this section we have tried to  
211 bring some more results on the sensitivity of the polysaccharide for monovalent salts like;  
212 NaCl, KCl and KI.

213 In the Fig.4a, are shown the temperature dependence of the viscosity for the solution of 3 g/L  
214 as the reference (ref.) to show the effect of 10 mM of the selected monovalent salt. The results  
215 show a decrease of the viscosity in presence of the salts with no particular effect due to the  
216 type of salt for the concentration of 10 mM. Moreover, the KCl salt concentration is not  
217 enough to induce the coil-helix transition in the temperature range of the measurements as it

218 was observed for  $\kappa$ -Car. Based on the fact that, the temperature dependence of  $\kappa$ -Car solutions  
 219 viscosity for 10 mM of NaCl, KI or KCl are different (Ciancia, Milas & Rinaudo, 1997), we  
 220 didn't expect that the temperature dependence of  $\alpha$ -Car solutions viscosity being similar. We  
 221 cannot explain the difference between the behavior of  $\alpha$ -Car and  $\kappa$ -Car, without considering  
 222 the sulfate group position. If the NaCl concentration is increased to 100 mM, the solutions  
 223 exhibited a coil-helix transition around 15 °C and a helix-coil transition around 17 °C. This  
 224 may appear to be a small hysteresis but when scan rate dependence is accounted for these  
 225 would be superimposable (Fig.4b). In presence of 100 mM of KCl, the coil-helix transition is  
 226 observed around 12 °C and two transitional temperatures were observed for the heating ramp;  
 227 the first one around 17 °C and the second one correspond to the helix-coil transition around  
 228 42 °C.

229



230

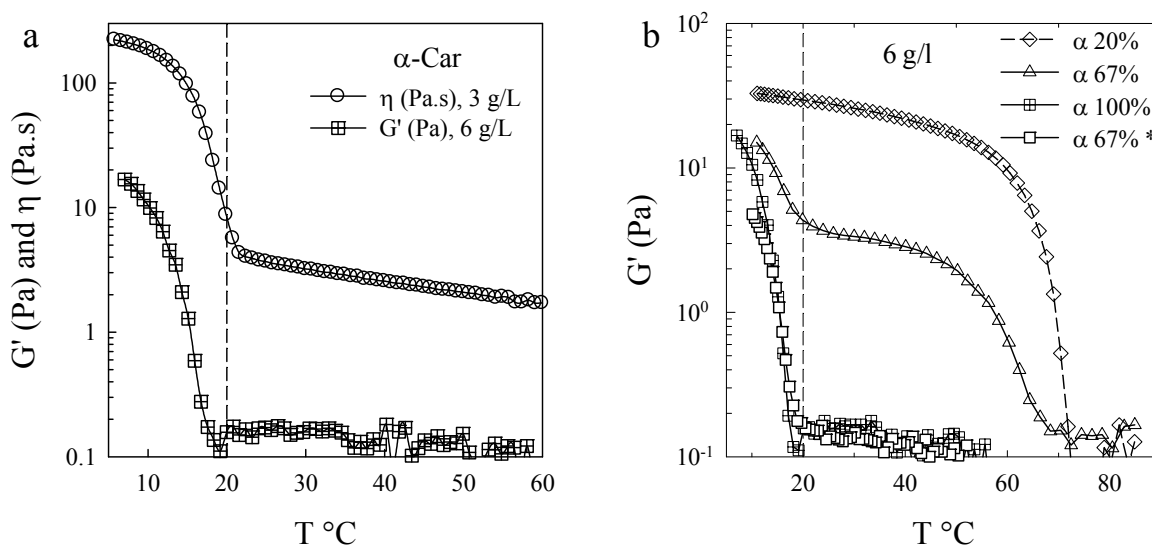
231 *Figure 4: temperature dependence of the viscosity of  $\alpha$ -Car solution, 3 g/L in presence of a) 0*  
 232 *mM (ref.) and 10 mM of NaCl, KCl or KI, the cooling and heating ramps give similar results*  
 233 *and b) 100 mM of NaCl or KCl, the cooling (open symbols) and heating (full symbols) results*  
 234 *are displayed.*

235 The thermal hysteresis of the solution of  $\alpha$ -Car with 100 mM KCl indicates an extensive  
236 aggregation of the helices which led the conserved solution to gel at 5 °C. The solution with  
237 100 mM NaCl didn't gel after several days at 5 °C. The shift of the coil-helix transition  
238 temperature  $T_{ch}$  from 12 °C to 15 °C, when KCl is replaced by NaCl, leads to say that the  
239 conformational change of  $\alpha$ -Car would be rather sensitive to NaCl than KCl. The role of  
240 monovalent salt to promote helix formation and then gelation for  $\kappa$ -Car systems was reported  
241 to follow the sequence RbCl > CsCl, KCl > NaCl > LiCl, HCl (Ciancia, Milas & Rinaudo,  
242 1997; Rochas & Rinaudo, 1984). The superiority of the potassium on sodium to induce coil-  
243 helix conformational change of  $\kappa$ -Car is inversed in the case of  $\alpha$ -Car. This inversion may  
244 indicate that the anhydro-ring promotes the accessibility to the sulfate group on the 3,6-  
245 anhydro-D-Galactose unit by small size counterions. However, extensive aggregation of the  
246 helices and gelation in presence of 100 mM KCl, indicate that the ion-pair formation of  $K^+$   
247 with the  $-SO_3^-$  is a time dependent process (Grasdalen & Smidsrod, 1981; Norton & Goodall,  
248 1983a). The reduction of the sulfate charge by counterions would be fast for  $\kappa$ -Car, and thus,  
249 promotes immediate coil-helix transition and aggregation of the helices to gelation.

#### 250 *3.4 Influence of the degree of carrageenan sulfation*

251 The gelation of different types of mono-composite (MC) of carrageenan polysaccharides  
252 made of  $\alpha$ -carrabiose and  $\iota$ -carrabiose on the same backbone are proposed in this section to  
253 show how the degree of conversion of  $\iota$ -Car to  $\alpha$ -Car can influence the coil-helix transition  
254 temperature. The gelation of a bi-composite (BC) solution, which is a blending of  $\alpha$ -Car and  $\iota$ -  
255 Car solution to the proportion of 67 wt.% and 33 wt.% respectively, is also shown. Given that  
256 the  $\alpha$ -Car is obtained by conversion of  $\iota$ -Car, the result of the bi-composite will indicate the  
257 influence that could have a few content of  $\iota$ -Car in the  $\alpha$ -Car solution on the coil-helix  
258 transition temperature (Piculell, Nilsson & Muhrbeck, 1992). The concentration of all the

259 solutions is 6 g/L and were prepared with 20 mM of CaCl<sub>2</sub> then cooled to give the results in  
 260 Fig.5. The temperature dependence of the G' of the solutions were measured during the  
 261 cooling ramp. The result of the BC solution is marked with a star in the legend of Fig5.b. The  
 262 gelation of carrageenan systems is a time dependent process which is connected to the helices  
 263 aggregation kinetic, thus the gelling point as the point where G' = G'' depends on the scan rate.  
 264 The onset of elastic behavior of aqueous iota-carrageenan has been observed by Piculell et al.,  
 265 (1992) to closely follow the onset of helix formation, and the modulus of elasticity increases  
 266 monotonically with an increasing helical content (Piculell, Nilsson & Muhrbeck, 1992).  
 267 Therefore, if G' > G'' is observed, the temperature at which the helices are formed, is  
 268 considered here as the gelation temperature. In this section, we have worked in the conditions  
 269 where for all the samples, G' > G'' was observed during the cooling ramps.



270  
 271 *Figure 5: Temperature dependence of the G' and the viscosity of a) α-Car in presence of 20*  
 272 *mM CaCl<sub>2</sub> and b) different composition of α-Car and ι-Car carrabiose in the polysaccharide*  
 273 *backbone. The composition α 67 %\* is a solution made with 67 wt% of α-Car and 33 wt% of*  
 274 *ι-Car. The solutions were prepared with 20 mM CaCl<sub>2</sub>.*

275 In Fig.5a, the gelation temperature is observed to be almost equal to the temperature at which  
276 the G' of the system increases sharply while cooling the solution. We can see that the domain  
277 of temperature where the viscosity increases coincides with that of G', which we said is  
278 concomitant with the formation of helices (Belton, Chilvers, Morris & Tanner, 1984; Ciancia,  
279 Milas & Rinaudo, 1997; Elmarhoun & Ako, 2021; Meunier, Nicolai, Durand & Parker, 1999;  
280 Norton & Goodall, 1983b; Rochas & Rinaudo, 1984). The  $\alpha$ -Car solution and the solution of  
281 67 wt.%  $\alpha$ -Car with 33 wt.%  $\iota$ -Car, indicated in the Fig.5b respectively as  $\alpha$  100 % and  $\alpha$  67  
282 %\*, show the same gelation temperature. Therefore, presumably a few content of  $\iota$ -Car in the  
283  $\alpha$ -Car solution would not affect the coil-helix transition temperature of  $\alpha$ -Car and its  
284 sensitivity to salt. The thermal behavior of  $\alpha$ -Car solution seems to be very stable regarding a  
285 small variation of  $\iota$ -Car concentration in wt.%. Considering the molecular weight of each  
286 composite of the blending solution, the 67 wt.% of  $\alpha$ -Car may correspond to 90 mol%, which  
287 means that one molecules of  $\iota$ -Car is surrounded by nine molecules of  $\alpha$ -Car and this could  
288 explain the similarity between the thermal properties of the 100 mol% and 90 mol%  $\alpha$ -Car  
289 solution. In the MC polysaccharide, the proportion of  $\alpha$ -carrabiose and  $\iota$ -carrabiose are in  
290 mol% and 67 % of  $\alpha$ -Car in the MC shows the gelation temperature of the part related to both  
291  $\alpha$ -Car and  $\iota$ -Car. This result, may indicate that the coil-helix transition would be rather an  
292 intramolecular mechanism. In the condition where the  $\alpha$ -carrabioses are randomly distributed  
293 along the hybrid carrageenan chain (Prechoux, Genicot, Rogniaux & Helbert, 2013), we  
294 should expect one gelation temperature. However, the probability to have a bloc of  $\alpha$ -  
295 carrabioses along the hybrid chain which behave like a free chain of  $\alpha$ -Car is increased by  
296 increasing the degree of  $\iota$ -Car conversion to  $\alpha$ -Car and this fact seems to correspond to the  
297 results in Fig.5b. Below 50 % of  $\alpha$ -Car in the MC, the gelation temperature of  $\alpha$ -Car was not  
298 clearly detectable. Supposed that the 20 mol% / 80 mol% of  $\alpha$ -/ $\iota$ -Car were distributed like a  
299 bloc copolymer, then the temperature dependence of G' would have shown distinctly the

300 gelation temperature of  $\alpha$ -Car and  $\iota$ -Car. Bui et al., (2019) have mixed 50 wt.% of  $\kappa$ -Car and  
301 50 wt.% of  $\iota$ -Car in 50 mM  $\text{CaCl}_2$  aqueous solution and they have observed two distinct  
302 gelation temperatures of the BC solution indicating independent coil-helix transition (Bui,  
303 Nguyen, Renou & Nicolai, 2019). However the authors observe that  $\kappa$ -Car network is more  
304 homogeneous in the mixed gels than in the corresponding individual gels and the mixed gels  
305 were stiffer than the individual gels. The independent coil-helix transition of the  
306 polysaccharide which occurs in both BC and MC systems may indicate that the reduction of  
307 the polymers net charge is preferentially intramolecular by which the helices and aggregates  
308 are formed.

309 The claim of intramolecular mechanism to explain the reduction of the polysaccharide charge  
310 or ion-pair formation could also refer to the finding of Rochas and Rinaudo (1984). The  
311 authors found that the ionic selectivity for potassium counterions work also even if the  $\kappa$ -Car  
312 is in the coil conformation. If so, then the coil-helix conformational change may take place in  
313 concentration well below the critical concentration, where the polysaccharide are supposed to  
314 be individual. Hence, the polysaccharide conformation would be single helix. However, the  
315 authors have rejected the single-helix formation presumably because they didn't observe a  
316 specific role of the potassium ion in the crosslinking mechanism of the polysaccharide. It is  
317 not clear the way the intramolecular charge neutralization mechanism conciliates the  
318 hypothesis of double helices and the aggregation of the helices in BC and MC model, except  
319 if the double helices are considered as aggregate of two single-helices.

320 We can note the absence of peak or maximum point for the  $G'$  plots in Fig.5, which several  
321 authors reported for  $\kappa$ -Car gel systems as characteristic of slipping effect due to sever  
322 syneresis (release of liquid phenomena by the gels). The gelation of  $\kappa$ -Car solutions with  $\text{K}^+$ ,  
323  $\text{Cs}^+$ ,  $\text{Ca}^{2+}$  gives gels with syneresis was reported by several authors (Ako, 2015; Lai, Wong &  
324 Lii, 2000; Piculell, Borgström, Chronakis, Quist & Viebke, 1997; Richardson & Goycoolea,



1994). The salt concentrations at which the syneresis was observed are relatively very weak compared with  $\iota$ -Car (Piculell, Nilsson & Muhrbeck, 1992; Thrimawithana, Young, Dunstan & Alany, 2010) and if the syneresis mechanism may exist for  $\alpha$ -Car, then it should be caused by salt concentrations larger than those used for  $\kappa$ -Car and presumably over the experimental time conditions of this study. The gels coarsening and contraction cause syneresis in general, but in condition which is determined by the polysaccharide net charge reduction or neutralization, extensive helices aggregation induced syneresis reflects the salt ability to neutralize or reduce the intra and/or intermolecular repulsion forces. Therefore, sulfate group position on D-galactose unit is more favorable to neutralization by salt than the sulfate group position on 3,6-anhydro-D-galactose unit.

### 3.5 Discussion

Alpha-carrageenan and kappa-carrageenan are characterized respectively, by a mono sulfate group on the 3,6-anhydro-D-galactose unit and a mono sulfate group on the D-galactose unit. Zablackis et al., have studied the carrageenan extracted from *Catenella nipa Zanardini*, which according to the work of Falshaw et al., (1996) may correspond to hybrid  $\alpha$ -/ $\iota$ -Car of variable proportion (Bourgoin, Zablackis & Poli, 2008; Falshaw, Furneaux, Wong, Liao, Bacic & Chandkrachang, 1996), and the content of  $\alpha$ -carrabiose in the native hybrid polysaccharide would not exceed 40 mol% (Prechoux, Genicot, Rogniaux & Helbert, 2013). The sodium form of this polysaccharide (in 100 mM NaCl) did not gel but exhibit a greater viscosity than  $\kappa$ -Car (Zablackis & Santos, 1986), while the sodium form of  $\iota$ -Car (in 100 mM NaCl) has been observed to form a gel (Piculell, Nilsson & Muhrbeck, 1992). In presence of salt the viscosity of the solutions of  $\alpha$ -Car and  $\kappa$ -Car depends on the degree of their helices formation and aggregation. However, the decrease of the gelling capacity when the proportion of  $\alpha$ -carrabiose increases in the hybrid polysaccharide indicates that the sulfate on the anhydro-D-galactose unit is less favorable for the inter-helical aggregation compared with the

350 sulfate on the D-galactose unit. Therefore, lower inter-helical aggregation activity and higher  
351 inter-helical aggregation activity, respectively, characterize the function of the sulfate on the  
352 anhydro-D-galactose unit and the function of the sulfate on the D-galactose unit, which  
353 explain, respectively, the weak gelling capacity of  $\alpha$ -Car and the strong gelling capacity of  $\kappa$ -  
354 Car.

355 The viscosity of the solution of 3 g/L of  $\alpha$ -Car and  $\kappa$ -Car at 20 °C were respectively 12.2  
356 mPa.s and 7.8 mPa.s. The viscosity of the free salt solution of  $\alpha$ -Car is observed to be greater  
357 than the viscosity of the free salt solution of  $\kappa$ -Car at a similar molecular weight,  
358 polysaccharide concentration and temperature conditions. Intrachain repulsive forces and  
359 water molecules binding capacity would be greater for  $\alpha$ -Car than  $\kappa$ -Car (Ako, Elmarhoum &  
360 Munialo, 2022), and accordingly, the sulfate on the anhydro-D-galactose unit promotes more  
361 expansion of the polysaccharide than the sulfate on the D-galactose unit.

362 It is appropriate to emphasize that the  $\alpha$ -Car system ionic selectivity for  $\text{CaCl}_2$  rather than  $\text{KCl}$   
363 was not expected, because the  $\kappa$ -Car system, which could derive from  $\iota$ -Car too, is known to  
364 be more sensitive to  $\text{KCl}$  than  $\text{CaCl}_2$ . Therefore, the sensitivity of carrageenan systems to  
365 counterions is not only a matter of number of sulfate groups on the carrabiose (Running,  
366 Falshaw & Janaswamy, 2012) but a result of many physicochemical factors. The size of  
367 hydrated counterions is quoted to play a role in the distance between the cation and the sulfate  
368 group (Piculell, Nilsson & Muhrbeck, 1992). The cation mobility can also affect the ion-pair  
369 formation stability or equilibrium constant, i.e., the small size cations could have a greater  
370 mobility, and thus, lead to a less stable or strong ion-pair formation (Ciancia, Milas &  
371 Rinaudo, 1997; Grasdalen & Smidsrod, 1981). Although  $\alpha$ -Car and  $\iota$ -Car system are rather  
372 sensitive to  $\text{CaCl}_2$ , the conformational transition temperature of  $\alpha$ -Car systems is found well  
373 below that of  $\iota$ -Car systems, which reflect the intramolecular characteristic of the charge  
374 reduction mechanism in carrageenan systems. The ability of salt to reduce the charge carried

375 by carrageenan polysaccharides could be pictured as an in-to-out process. The in-to-out  
376 neutralization process means that the system tries to neutralize first the charge carried by the  
377 carrabiose (intramolecular process), and if this fails like in the case of  $\alpha$ -Car with calcium, the  
378 system tries to neutralize the charge carried by the carrageenan chain (intrachain process). In  
379 the intrachain process or interaction between carrabiose of same chain, if a block of carrabiose  
380 behave the same, like a block of  $\alpha$ -Car in  $\alpha$ -/ $\iota$ -Car hybrid polysaccharide, then the  
381 conformation transition characterizing the block could be detectable. The neutralization  
382 process could continue or stop by interchain/intermolecular process (aggregation), in this  
383 process the two sulfates or carrabiose which bind the multi valence counterions do not belong  
384 to the same chain.

#### 385 **4. Conclusion**

386 The importance of counterions in the helical formation of carrageenan depends on many  
387 physicochemical factors. We show in this treatise that the influence of the counterion valency  
388 depends on the position and number of the sulfate group carried by the carrabiose. The sulfate  
389 on the 3,6-anhydro-D-galactose unit, which characterizes  $\alpha$ -carrageenan (G-DA2S), is rather  
390 sensitive to divalent counterions and the sulfate group on the D-galactose unit, which  
391 characterizes  $\kappa$ -carrageenan (G4S-DA), is rather sensitive to monovalent counterions. The  
392 two types of sulfate, which characterize  $\iota$ -carrageenan (G4S-DA2S), is more sensitive to  
393 divalent counterions than the one type of sulfate carried by  $\alpha$ -carrageenan (G-DA2S). The  
394 temperature dependence of the  $G'$  of  $\alpha$ -/ $\iota$ -carrageenan, as a carrageenan block copolymer  
395 system in  $\text{CaCl}_2$  solution, has shown distinctly the helical formation temperature of  $\alpha$ -  
396 carrageenan and  $\iota$ -carrageenan. Based on the results of this study, the counterion influence,  
397 which we qualify as in-to-out process, would be preferentially and gradually intramolecular,  
398 then intrachain and finally interchain process.

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