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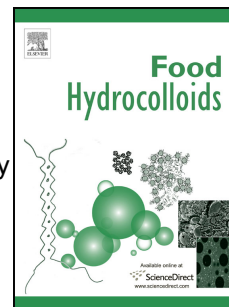
From Egg Yolk/κ-Carrageenan dispersions to gel systems: Linear Viscoelasticity and Texture Analysis

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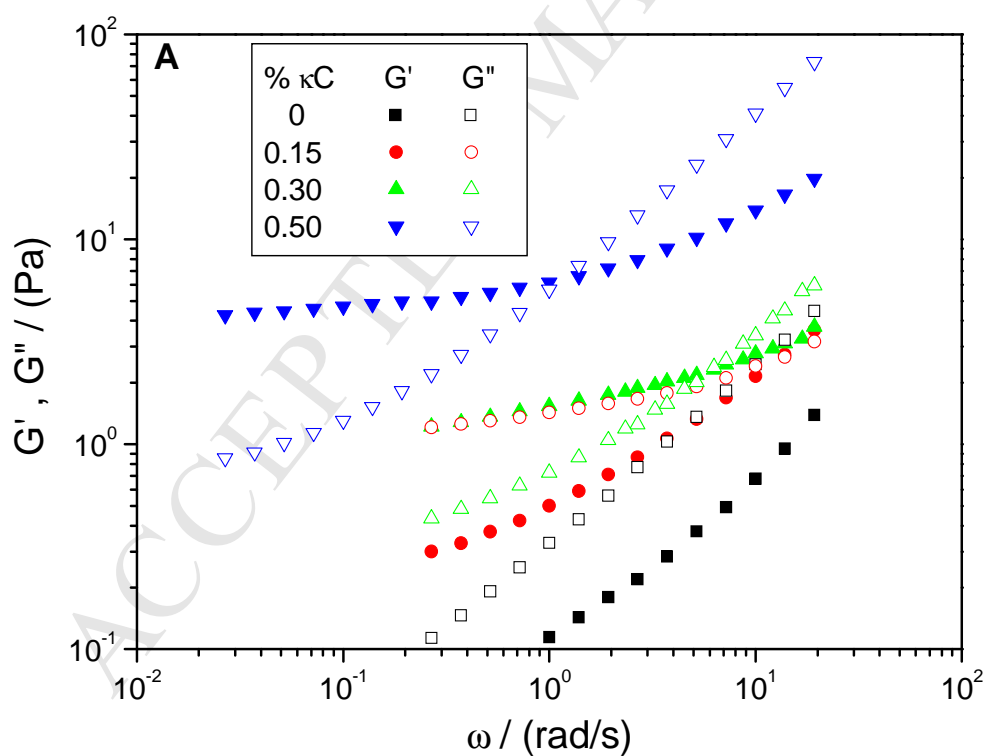
1 # From Egg Yolk/κ-Carrageenan dispersions to gel systems:

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9 ## Abstract

10 The effect of pH (3.5-6) and polysaccharide concentration (0-0.5 wt%) on the linear viscoelastic

11 behaviour of egg yolk/κ-Carrageenan (EY/κC) mixtures in aqueous solution was studied by using

12 small amplitude oscillatory shear (SAOS). Native egg yolk containing 45 wt% solids was used for

13 all the samples. Thermally set EY/κC gels were also studied by SAOS and texture analysis. A

14 variety of linear viscoelastic behaviours depending on κC concentration and pH were exhibited by

15 EY/κC dispersions that may be explained in terms of the contributions of electrostatic attractive

16 interactions and an exclusion volume effect between protein and polysaccharide macromolecules.

17 This last effect seems to be dominant as pH shifts towards the isoelectric point (IEP) of egg yolk

18 proteins, whereas, at pH far from the IEP a certain enhancement in the degree of compatibility, and

19 even some κC autohydrolysis, seems to take place. The results obtained either from rheological or

20 textural characterization of gels were consistent with that balance. In any case, the results obtained

21 suggest that the microstructure of gels is governed by the protein ability to form gels, where

22 hydrophobically driven interactions and subsequent cross linking among protein segments play a

23 dominant role.

24 **Keywords:** *Proteins, Polysaccharides, Gels, Viscoelasticity, Texture.*

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1. Introduction

34 Protein and polysaccharides are natural polymers that are widely used as functional ingredients in

35 food materials. The interactions of these biopolymers, which lead to extremely complicated and

36 interesting phenomena in various systems, impart an important effect on the macroscopic

37 properties of food products such as: flow, stability, texture and mouth feel (de Kruif & Tuinier,

38 2001). Tolstoguzov (2003) stressed out the importance of thermodynamic considerations in

39 protein-polysaccharide interactions to design food formulations with novel or improved properties.

40 Although for very dilute solutions the system is stable since the mixing entropy dominates and

41 proteins and polysaccharides are co-soluble, from a thermodynamic point of view, proteins and

42 polysaccharides may be compatible or incompatible in an aqueous solution. Thermodynamic

43 incompatibility occurs when the interaction between different biopolymers is energetically less

44 favourable than the average interaction between similar biopolymers. This incompatibility, where

45 biopolymers are mutually segregating one from the other, results in the formation of two separated

46 phases, each being rich in one of the biopolymers. If the interaction between both biopolymers is

47 favoured, a phenomenon known as coacervation or associative phase separation of both

48 biopolymers from a phase mainly containing solvent takes place. The main difference between

49 these two mechanisms is that thermodynamic incompatibility is predominantly entropically driven,

50 whereas complex coacervation, which has been attributed to an electrostatic attraction between

51 oppositely charged biopolymers is both entropically and enthalpically driven (Turgeon, Beaulieu,

52 Schmitt & Sánchez, 2003; de Kruif, Weinbreck & de Vries, 2004; Tolstoguzov, 2007).

53 The major non-covalent interactions between proteins and polysaccharides are: electrostatic

54 interactions, steric exclusion, hydrophobic interactions and hydrogen bonding. Thus, associative

55 separation is commonly governed by the electrostatic attraction between molecules with opposite

56 electrical charges, whereas the steric exclusion effect causes segregative separation. The relative

57 importance of these interactions in a particular system depends on the molecules involved, the

58 composition of the aqueous mixture and the environmental conditions. Knowledge of the origin and

59 nature of the interactions involved and the relative importance of these interactions in a particular

60 system can often be used by food scientist to engineer novel structures and physicochemical

61 properties in food systems. Furthermore, by modulating these parameters it is possible to control

62 the interactions between the biopolymers and therefore create microstructures that provide novel

63 textural and sensory properties to food systems, improve emulsion stability to environmental

64 stresses, or enable novel encapsulation-release characteristics (McClements, 2006).

65 In food technology there has been recent increasing interest in protein-polysaccharide complexes

66 and coacervates derived from novel industrial applications such as micro an nano-encapsulation

67 processes, the design of multi-layers structures, the formation and stabilization of food emulsions,

68 the formation of new food gels and the recovery of protein from industrial by-products (Turgeon,

69 Schmitt & Sánchez, 2007). Thus, the rheological and interfacial properties of protein-

70 polysaccharide complexes might be advantageously used for designing food products with various

71 textures, stability and delivery properties. In addition, a variety of applications of protein-

72 polysaccharide or protein-polyelectrolyte complexes have been described for non-food applications

73 such as microencapsulation for the pharmaceutical industry or its use in the synthesis of

74 biomaterials and the same conclusion might apply to formulate novel delivery systems (Schmitt,

75 2009).

76 The objective of this contribution was to analyse the effect of pH and protein-polysaccharide ratio

77 on the linear viscoelastic behaviour of egg yolk/κ-carrageenan mixtures in aqueous solution and

78 gels obtained after application of thermal treatment to these blends in order to obtain information

79 about the type of microstructures present in the system. Texture properties of gels were also

80 evaluated.

2 **Materials and methods**

82 *2.1 Materials*

83 Egg Yolk (EY) was obtained from fresh chicken eggs purchased in a local market according to

84 grade A and type L (63-73 g) commercial specifications, and all damaged or cracked eggs were

85 discarded. κ-Carrageenan (κC) (Satiagel^{TM} AMP45) was kindly provided by Degussa (France).

86 The composition for the counter ion of this κC was 1, 14 and 0.4 wt% of sodium, potassium and

87 calcium, respectively. Hydrochloric acid (analytical grade) was obtained from Merck (Germany).

88 *2.2 Methods*

89 Chicken eggs were hand broken and the white carefully separated from the yolk using the Harrison

90 and Cunningham (1986) preparation method. EY/ κC mixtures were made by dispersing different

91 amounts of κC (0-0.5 wt%) in native EY (50.43 $\pm$ 0.8 average solid content wt %), under

92 mechanical stirring (300 rpm, 1h) at room temperature. The natural pH of the system was modified

93 using 1M and 2M hydrochloric acid as acidulate and demineralised water was adding until 45 wt%

94 in solids EY content.

95 Small Amplitude Oscillatory Shear (SAOS) measurements of egg yolk dispersions containing 45

96 wt% in solids at different pH values an κC content, including either stress sweep or frequency

97 sweep, were performed by means of a controlled-stress rheometer Haake RS-300 (Thermo

98 Scientific, Germany), using 60 mm and 4^0 cone-plate geometry with 1 mm gap. A shear stress

99 sweep test was always done beforehand at 6.3 rad/s to establish the linear viscoelasticity range.

100 All the dynamic viscoelasticity frequency sweep measurements were carried out at 20ºC and at a

101 stress clearly lower than the critical value for linear viscoelasticity.

102 Measurements of the zeta potential of systems containing 45 wt % in EY and 0.3 wt % in κC at

103 different pH values were performed using a Zetasizer 2000 (Malvern Instruments, U.K.). Aliquots

104 of 0.5 g were diluted in deionised water up to 100 ml of total volume, keeping adjusted the pH

105 value of the dispersion with 0.1 N HCl. Prior to analysis, the samples were tempered at 20ºC and

106 stirred in order to keep the suspension homogeneous, avoiding segregation. The samples were

107 measured in triplicate and each measurement was an average of five determinations. During the

108 experiments, no flocculation was observed and each measurement was conducted at 20 °C. The zeta

109 potential was calculated from the electrophoretic mobility using the Henry equation and the

110 Smoluchowski approximation.

111 The gels were obtained by transferring the above samples without entrapping air into a 20 ml

112 plastic bottles (Ø= 20mm, 65mm height) and sealed prior to heat at 90ºC in a thermally controlled

113 water bath during 30 min. These gels were stored overnight at 5ºC in order to allow the maturation

114 of mixed gels. Dynamic linear viscoelastic properties were conducted by frequency sweep tests on

115 thin slices (1.5±0.3mm) of gel samples at 20ºC, using serrated plate-plate geometry with 1 mm gap

116 within the linear response regime. The pH values were also checked after processing and no

117 modification was detected.

118 Texture properties of the final gels were evaluated using a TA-XT2i (Stable Micro Systems, UK)

119 texturometer. In order to avoid any correction for non-ideal diameter/height ratios, the gel samples

120 were cut into 20mm diameter and 26 mm height specimens (Kuhn, 2000).and lubricated with low

121 viscosity silicon oil. Uniaxial compression tests were performed using a 75mm diameter flat platen

122 (15% strain, 600 s and 0.1 mm/s of crosshead speed). Before performing any measurements, gels

123 were allowed to equilibrate at 20ºC for approximately 3 h in a temperature-controlled room.

124 Textural results for each gel were performed at least three times.

125 ## 3. Results and discussion

126 *3.1. Linear viscoelastic properties of EY/κC dispersions*

127 Figure 1 displays the influence of both linear viscoelastic moduli (G' and G'') on frequency for EY

128 dispersions as a function of κC concentration at pH 6 (Fig. 1A) and pH 3.5 (Fig. 1B). As may be

129 observed in both graphs, in absence of polysaccharide, the EY dispersion showed a predominantly

130 viscous fluid-like behaviour, with both moduli showing higher values for the lower pH, due to the

131 electrostatic interactions among charged protein surfaces. The addition of κC to EY leaded to a

132 remarkable evolution in both viscoelastic funtions that depends on pH.

133 Near the mean isoelectric point (IEP) of yolk proteins (pH 6), when 0.15 wt% κC was added, a

134 crossover between G'' and G' took place at high frequency. Although a further increase in κC up to

135 0.3 wt% yielded similar values of both moduli at high frequency, the behaviour is rather different.

136 Thus, at low frequency G'' was always higher than G' for the 0.15 wt% κC/EY, whereas, the blends

137 containing 0.30 and 0.50 wt% κC were predominantly elastic, giving rise to a crossover point and a

138 shift to a predominantly viscous response at higher frequencies. This crossover took place at lower

139 frequency for the highest κC content and may be related to the increase in repulsive interactions

140 due to increasing negative charges. Thus, the zeta potential value at pH 6 increase from almost 0,

141 corresponding to the IEP in absence of κC, to -26.9 mV for 0.3 wt% κC (see Table 1). In any case,

142 no evolution to gel-like behaviour was found near the IEP. This behaviour is similar to that one

143 shown by amorphous uncrosslinked polymers of high molecular weight as described by Ferry

144 (1980). In accordance, the first above mentioned crossover point corresponds to the so-called

145 terminal transition of the generalized mechanical spectrum, whereas the second one corresponds

146 to the beginning of the transition region, with G'' dominating over G'. For uncrosslinked polymers of

147 high molecular weight, this region is preceded by a predominantly elastic entangled plateau region

148 that in this case was observed at the low frequency regime.

149 At pH 3.5 protein surfaces exhibit a positive net charge, as may be observed in Table 1. The

150 addition of κC leaded to an increase in G' and G'', as well as to a decrease in their respective

151 frequency slopes and loss tangent and a reduction in the zeta potential value. However since the

152 amount of protein is much higher than the polysaccharide content the electrostatic repulsions are

153 still noticeable. The EY dispersion containing 0.30% κC experienced a marked decrease in loss

154 tangent and frequency dependence, showing a weak gel-like behaviour, with G' higher than G'' for

155 the whole frequency range studied, which indicates that the presence of charges on protein

156 surfaces promoted the enhancement of network-like structures. A further increase in κC up to

157 0.50% resulted in a weakening of the dispersion leading to linear viscoelastic functions similar to

158 that one shown by the system containing 0.15% κC. In fact, occurrence of maximum consistency

159 for mixed biopolymers gels has been described in the literature of food hydrocolloids (Turquois,

160 Rochas & Taravel, 1992). Imeson (2009) has recently reviewed the interactions between κC and

161 other biopolymers, reporting a positive synergism with LBG or konjac glucomannan which yields

162   maximum gel strength at intermediate κC content for 1% total polysaccharide. A synergism

163   between κC and κ-casein protein was also reported, being attributed to interaction of an

164   electrostatic nature with positively charged amino acids at the surface of κ-casein micelles (Hood

165   and Allen, 1977; Langendorff, Cuvelier, Michon, Launay, Parker & de Kruif, 2000; Spagnuolo,

166   Dagleish, Goff & Morris, 2005, Tijssen, Canabady-Rochelle & Mellema, 2007).

167   Figure 2 shows the evolution of tan δ at 1 rad/s as a function of κC content for pH 3.5. Previous

168   results on EY dispersions at acidic pH (3 and 3.5) showed that the behaviour in absence of

169   polysaccharide is quite different from that obtained for 0.3 κC/EY blends, which corresponds to a

170   viscoelastic fluid showing the terminal transition crossover point at high frequency (Guerrero,

171   Carmona, Martínez & Partal, 2004; Aguilar, Cordobés & Guerrero 2007). The results obtained at

172   pH 3.5 suggest that protein-polysaccharide interactions of electrostatic nature may lead to the

173   formation of gel-like network structures, displaying maximum strength at 0.3 wt% κC (minimum

174   loss tangent), at which protein to polysaccharide optimum ratio seems to be found (Schmitt et al.

175   2009). A possible explanation for this increase in the loss tangent above 0.3% is a phenomenon

176   known as carrageenan autohydrolysis, which occurs at low pH values as carrageenan in the acid

177   form cleaves at the 3,6-anhydrogalactose linkage in the molecule (Hoffmann, Russell & Gidley,

178   1996). Although this phenomenon is favoured at high temperature, autohydrolysis would also take

179   place to some extent at temperatures above the cold-gelling point as long as κC content is high

180   enough (i.e. 0.5 % κC). The contribution of the decrease in electrostatic repulsions, although

181   moderate, may also be taken into account, being a consequence of the addition of negatively

182   charged κC.

183   *3.2. Linear viscoelastic properties of EY/κC gels*

184   The mechanical spectra of thermally processed EY/κC systems at pH 6 are shown in Figure 3. As

185   may be observed, these systems exhibit a gel behaviour with G' being much higher than G''. The

186   addition of κC leaded to a clear increase in both moduli, but did not produce any modification on

187   the shape of the mechanical spectra, in spite of the variety of viscoelastic behaviours exhibited by

188   the unheated EY/κC mixtures shown in Fig 1. These results indicate that the viscoelastic behaviour

189 of EY/κC gels were dominated by the ability of protein macromolecules to form a three dimensional

190 network, whereas the polysaccharide may contribute to reinforce the consistency of the gel without

191 any further modification on microstructure, which might be related to an exclusion volume effect of

192 incompatible biopolymers in a mixed solution according to the description of the so-called

193 molecular symbiosis reported by Tolstoguzov (2007).

194 As a consequence of the similar mechanical spectra shown in Fig. 3, a time-concentration

195 superposition method may be applied by normalizing both viscoelastic functions with the storage

196 modulus at 1 rad/s to obtain a master curve. As may be seen in Figure 4A all the mechanical

197 spectra obtained at different κc concentration and pH collapse into the master curve.

198 The values for the normalization parameter (G' at 1 rad/s), which are shown in Figure 4B,

199 underwent an increase with the addition of κC that became more apparent and steeply as pH was

200 closer to the IEP. In fact, at acidic pH, far from the IEP, this increase is dampened and tends to

201 vanish above 0.15 wt% κC. As a consequence, the higher electrostatic interactions obtained as κC

202 was raised seem to inhibit the enhancement of the gel network. This behaviour may be explained

203 in terms of mutual exclusion of each biopolymer, which seems to be more evident at a low level of

204 electrostatic interactions (pH 6 and 5), at which the evolution of G'_1 would correspond to an

205 increase in effective concentration of protein. This effect seems to be hindered by a certain degree

206 of compatibility between positively charged proteins (as pH departs from the IEP) and

207 polysaccharide anions or, alternatively, it may be a consequence of the above mentioned

208 autohydrolysis effect found for carrageenan macromolecules.

209 *3.3 Textural properties of EY/κC gels*

210 Figure 5 shows the results of some textural parameters obtained for EY/κC gels under uniaxial

211 compression as a function of κC concentration and pH. Springiness only exhibits a moderate

212 decrease with κC concentration for all the pH values tested. In contrast, the evolution of firmness is

213 more evident, being similar to that one found for viscoelastic properties (i.e. G'_1 in Fig. 3). Thus, a

214 marked increase in firmness takes place with increasing κC content near the IEP, but this effect

215 attenuated at pH 4.5 and even reversed at the lowest pH. These results of firmness are consistent

216 with the exclusion volume effect taking place near the IEP and with the reduction produced as a

217 consequence of the increase in electrostatic interactions.

218 ## 4. Concluding remarks

219 EY/κC dispersions exhibited a variety of linear viscoelastic behaviours depending on κC

220 concentration and pH, where three regions of the mechanical spectrum may be generally

221 observed: The terminal region obtained at low κC concentration for all the pH values studied; The

222 plateau region, observed at low frequency at 0.3 % κC and above; The beginning of the terminal

223 region at high κC content and frequency, which was particularly pronounced near the IEP.

224 The second or plateau region was enhanced as pH departs from the IEP even leading to a gel-like

225 behaviour. This change in the microstructure of the system at a controlled κC content and low pH,

226 when protein surfaces exhibited a marked positive net charge, might be due to a certain degree of

227 compatibility between both polymers.

228 The results obtained either from rheological or textural characterization of gels may be explained in

229 terms of an exclusion volume effect between protein and polysaccharide macromolecules that

230 seems to be more important in absence of net charge at protein surfaces. At pH far from the IEP a

231 certain enhancement in the degree of compatibility also seems to occur, although an

232 autohydrolysis effect typical of carrageenan macromolecules at low pH may also takes place. In

233 any case, as may be inferred from the unique master mechanical spectrum obtained by time-

234 concentration superposition, the microstructure of thermally treated EY/κC systems seems to be

235 governed by the protein ability to form gels, where hydrophobically driven interactions and

236 subsequent cross linking among protein segments play a dominant role.

237 ## Acknowledgments

240 ## References

241 Aguilar, J. M., Cordobés, F. & Guerrero, A. (2007). Influence of high pressure processing on the

242   linear viscoelastic properties of egg yolk dispersions. *Rheol. Acta, 46, 731*

243   de Kruif, C. G. & Tuinier, R. (2001). Polysaccharide protein interaccions. *Food Hydrocolloids,15,*

244   *555*

245   de Kruif, C. G. Weinbreck, F. & de Vries, R. (2004). Complex coacervation of proteins and anionic

246   polysaccharides. *Curr. Opinion Colloid Interface Sci., 9, 340*

247   Ferry, J. D. (1980). *Viscoelasctic properties of polymers, 3rd edn.* New York: Jonh Wiley & Sons.

248   Guerrero, A., Carmona, J. A., Martínez, I. & Partal, P. (2004). Effect of pH and added electrolyte

249   on the thermal-induced transitions of egg yolk. *Rheol. Acta, 43, 539*

250   Harrison, L. J. & Cunningham, F. E. (1986). Influence of salt on properties of liquid yolk and

251   functionality in mayonnaise. *Poultry Sci., 65, 915*

252   Hoffmann, R. A., Russell, A. R. & Gidley, M. J. (1996). Molecular weight distribution of

253   carrageenans. In G. O. Philips, P. J. Williams and D. J. Wedlock (eds.) Gums and Stabilisers for

254   the Food Industry 8 (pp. 137-148). IRL Press at the Oxford University Press, Oxford.

255   Hood, L. F. & Allen, J. E. (1977). Ultrastructure of carrageenanmilk sols and gels. *J. Food Sci., 42,*

256   *1062.*

257   Imeson, A. P. (2009). Carrageenan and furcellaran. In G. O. Philips, & P. A. Williams (Eds.),

258   *Handbook of hydrocolloids, 2nd edn.* (pp. 164-185). Boca Raton, FL: Woodhead Publishing Limited,

259   CRC Press.

260   H.A. Kuhn, H. A. (2000). Mechanical testing and evaluation. In: *Uniaxial compression testing, ASM*

261   *Handbook, vol. 8.*

262   Langendorff, V., Cuvelier, G., Michon, C., Launay, B., Parker, A. & de Kruijf, C. G. (2000). Effects

263   of carrageenan type on the behaviour of carrageenan/milk mixtures. *Food Hydrocollids, 14, 273.*

264   McClements, D. J. (2006). Non-covalent interactions between proteins and polysaccharides.

265   *Biotechnology Advances, 24, 621*

266   Schmitt, C. (2009). Protein-polysaccharide complexes and coacervates. In G. O. Philips, & P. A.

267   Williams (Eds.), *Handbook of hydrocolloids* (pp. 421-476). Boca Raton, FL: Woodhead Publishing

268   Limited, CRC Press.

269   Spagnoulo, P. A., Dagleish, D. G., Goff, H. D. & Morris, E. R. (2005). Kappa-carraggeenan

interactions in systems containing micelles and polysaccharide stabilizers. *Food Hydrocolloids, 19, 371.*

Tijssen, R. L. M., Canabady-Rochelle, L. S. & Mellema, M. (2007). Gelation upon long storage of milk drinks with carrageenan. *J. Dairy Sci., 90, 2604*

Tolstoguzov, V. B. (2003). Some thermodynamic considerations in food formulation. *Food Hydrocolloids, 17, 1*

Tolstoguzov, V. B. (2007). Ingredient interactions in complex foods: aggregation and phase separation. In D. Julian McClements (Ed.), *Understanding and controlling the microstructure of complex foods* (pp. 185-206). Boca Raton, FL: Woodhead Publishing Limited, CRC Press.

Turgeon, S. L., Beaulieu, M., Schmitt, C. & Sánchez, C. (2003). Protein-polysaccharide interactions: phase-ordering kinetics, thermodynamic and structural aspects. *Curr. Opinion Colloid Interface Sci., 8, 401*

Turgeon, S. L., Schmitt, C. & Sánchez, C. (2007). Protein-polysaccharide complexes and coacervates. *Curr. Opinion Colloid Interface Sci., 12, 166*

Turquois, T., Rochas C. & Taravel, F. R. (1992). Rheological studies of synergistic κappa carrageenan-carob galactomannan gels. *Carbohydrate Polymers 17:263-268.*

FIGURE CAPTIONS

Fig.1. Evolution of linear viscoelastic moduli of EY/ κC dispersions as a function of frequency and κC concentration: A) at pH 6 and B) at pH 3.5.

Fig.2. Evolution of loss tangent values at 1 rad/s for EY/ κC systems at pH 3.5, as a function of κC content.

Fig.3. Evolution of the mechanical spectra of EY/ κC heat set gels at pH 6, as function of κC content.

Fig.4. Normalized linear viscoelastic functions for EY/ κC gels: A) Master mechanical spectrum; B) Normalization parameter as a function of κC concentration and pH.

296   Fig. 5. Evolution of textural parameters (firmness and springiness) of EY/ κC gels as a function of κC

297   concentration at different pH values

Table 1. Z-potential values for EY/kC dispersions as a function of pH and kC concentration

pH	κC concentration	Z-Potential
(-)	(wt %)	(mV)
3.5	0	35.8 ± 0.5
3.5	0.3	35.0 ± 0.8
3.5	0.5	34.2 ± 0.8
4.5	0.3	- 0.2 ± 0.7
6.0	0.5	- 26.9 ± 1.4