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Evaluation of the effect of *Gleditsia amorphoides* gum on the properties of rennet-induced milk protein gels

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ABSTRACT

The study and characterisation of food gels obtained from phase-separated systems has gained interest since a wide variety of gel structures and textures can be developed. In this study, the phase and rheological behaviour of milk protein/espina corona gum (MP/ECG) mixtures were evaluated. These mixtures presented a segregative phase separation and a rheological behaviour proportional to the ECG concentration. Microstructural analysis, textural parameters and water-holding capacity of gels obtained from MP/ECG mixed systems using rennet as gelling agent were determined. At high ECG concentrations (≥0.05%, w/v), the gel microstructure changed from a coarse strand to a bicontinous microstructure. Such microstructural changes affected the textural parameters, firmness and break point, and the water-holding capacity of the gels. The results obtained in this work could be explained by the interplay between the segregative interaction of the biopolymers and the rennet-induced gelation rate.

1. Introduction

Protein-polysaccharide mixtures in aqueous solutions display different behaviours, which can be either associative or segregative (Goh, Sarkar, & Singh, 2014; Sadeghi, Kadkhodaee, Emadzadeh, & Phillips, 2018). The mixture components could interact with each other in such a way as to impart a desirable characteristic to food stuffs (Acero-Lopez, Alexander, & Corredig, 2010; Bulut-Solak & O'Mahony, 2015). The biopolymer mixtures that exhibit segregative behaviour (phase separation) are interesting for the creation of new gelled food matrices, as a result of the intertwining gelation and phase separation processes (Bulut-Solak & O'Mahony, 2015; Corredig, Sharafbafi, & Kristo, 2011; Renard, van de Velde, & Visschers, 2006; Rohart & Michon, 2016).

Galactomannans, which consist of a linear mannose backbone with galactose units linked as side chains, can be considered as one of the most widely used polysaccharides in the food industry. Guar gum (GG) and locust bean gum (LBG) are two of the most studied galactomannans since they offer a wide variety of applications (Barak & Mudgil, 2014; Sharma et al., 2018). Espina corona gum (ECG) is a galactomannan extracted from seeds of a leguminous tree, *Gleditsia amorphoides*, that grows in Argentina, Brazil and Paraguay. The chemical composition of ECG was described by Cerezo (1965) and, in recent decades, it has been gained relevance since its chemical composition is similar to that of GG and LBG (López, Galante, Alvarez, Risso, & Boeris, 2017b). It is a non-gelling biopolymer that could be used as a food stabiliser and thickener (Spotti, Santiago, Rubiolo, & Carrara, 2012). The study of new polysaccharides and protein-polysaccharide mixtures is important to replace food additives already in use or to develop new food formulations (Busch, Delgado, Santagapita, Wagner, & Buera, 2018). In this regard, Perduca et al. (2013) characterised ECG solutions and López et al. (2017b), studied sodium caseinate/ECG mixed systems.

Milk proteins (MP) are the primary building blocks for gelled dairy foods such as cheese or yoghurt. MP gel formation could take place by acid induction, enzymatic action or a combination of both mechanisms (Lucey, 2002). The destabilisation of casein micelles in milk is the basis of milk gel formation. Rennet addition to MP suspension induces casein micelle aggregation by loss of steric and electrostatic stabilisation since the rennet hydrolyses κ-casein (Corredig et al., 2011; Perreault, Morin, Pouliot, & Britten, 2017). Some authors have reported that the addition of polysaccharides to MP suspensions

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affects the final characteristics of the gels obtained from the MP/polysaccharide mixture (Bulut-Solak & O'Mahony, 2015; Galante, Boeris, Álvarez, & Risso, 2018). Therefore, the study and characterisation of the effect of polysaccharide addition on MP suspensions is important for the development of new gelled systems (Corredig et al., 2011; Nepovinnykh, Kliukina, Ptichkina, & Bostan, 2019).

There are previous reports on protein/ECG mixture gels. Spotti et al. (2012) studied mixed whey protein/ECG heat-induced gels whereas López, Galante, Alvarez, Risso, and Boeris (2017a) focused on acid-induced sodium caseinate/ECG gels. Nevertheless, there are no previous reports for MP/ECG mixed gels obtained by rennet addition. Therefore, the aim of this work was to study the effects of the presence of ECG on MP rennet-induced gel properties to explore future applications of this galactomannan in gelled food products.

2. Materials and methods

2.1. Materials

Milk protein (MP) suspension was prepared from commercial skim milk powder (Nestlé SA, Vevey, Switzerland) reconstituted to 20% (w/v) in 5 mM $CaCl_2$ (Merck, Germany) and held for 24 h at 4 °C before use. The concentration of MP was determined by the Kuaye (1994) method. Commercial liquid rennet was donated by C.O.T.A.R. S.A. (Santa Fe, Argentina). ECG was donated by Idea Supply Argentina S.A. (Chaco, Argentina). Solutions of 0.5% (w/v) ECG were prepared by dissolving the powder in distilled water.

2.2. Preparation of mixed MP/ECG systems

MP/ECG mixed systems were prepared by carefully mixing ECG and MP stock solutions (MP at 3.5%, w/v, and ECG concentrations ranged from 0.025 to 0.3%, w/v) in a media of 10 mM Tris–HCl buffer, pH 6.4.

2.3. Phase behaviour

MP/ECG mixed systems were prepared according to section 2.2 in a 10 mL graduated cylinder. Then, the MP/ECG systems were mixed using a vortex mixer. The phase behaviour (one-phase or two-phase) was verified after 1 and 24 h incubation at 35 °C.

2.4. Rheological measurement

The rheological measurements of MP and MP/ECG systems were performed at 35 °C using a viscometer Brookfield LVDV-II + CP (Wisconsin, USA) in steady shear with a cone-plate geometry (diameter 48 mm, angle 0.8°). During each measurement, the shear rate varied in a range of 7.5–750 s^{-1} and the shear stress and the apparent viscosity (η) were registered.

2.5. Rennet-induced milk protein gel preparation

Rennet-induced MP gels were prepared by adding rennet (2.7 RU) to the MP/ECG mixtures at a ratio of 5 μL mL^{-1}. Before rennet addition, reconstituted MP (7%, w/v) and ECG (0.5%, w/v) suspensions were mixed as described in Section 2.2 to obtain final concentrations of 3.5% (w/v) and 0–0.2% (w/v), respectively. After rennet addition, the mixtures were incubated for 1 h at a controlled temperature of 35 °C.

2.6. Microstructural analysis

Confocal scanning laser microscopy (CSLM) was performed to determine the ECG effect on the milk gel microstructure. The milk protein gels were obtained according to the protocol detailed in Section 2.5 with some modifications. Rhodamine B (red) was added to the MP/ECG mixtures before rennet addition. CSLM images were obtained according to the protocol published by Galante et al. (2018), for the MP/guar gum gels. Pore size determination was carried out by image analysis (Galante et al., 2018).

2.7. Textural analysis

Mechanical texture determination was performed according to the protocol previously reported (Galante et al., 2018). Briefly, MP/ECG gels were prepared as described in Section 2.5 in a suitable container (diameter, 35 mm; height, 30 mm) and a penetration test was performed using the textural analyser Mecmesin Multitest 2.5-d (Mecmesin, Barcelona, Spain) equipped with a dynamometer of a 25 N load cell. Two parameters, breaking force (N) and firmness (N mm^{-1}), were obtained from the force–displacement curves when the cylindrical plunger (diameter, 20 mm; height, 20 mm) penetrated the MP/ECG gel mesh during a total displacement of 10 mm.

2.8. Water-holding capacity determination

MP gels, with or without ECG, were formed in centrifuge tubes according to the protocol described in section 2.5. The water-holding capacity (WHC) was indirectly determined by the measurement of the serum expelled by the MP gels after 10 min of centrifugation at 200×*g* according to:

$$\text{WHC}\ (\%) = [(\text{MP gel weight} - \text{serum expelled weight})/(\text{MP gel weight})] \quad (1)$$

2.9. Statistical analysis

All determinations were performed at least in triplicate and the results were expressed as the media value with the associated standard deviation. ANOVA was used to analyse significant differences where $p < 0.05$.

3. Results and discussion

3.1. Characterisation of the MP/ECG mixtures

Fig. 1 shows the phase behaviour of the MP/ECG mixed systems after 1 and 24 h of incubation at 35 °C. After 1 h of incubation, the systems exhibit a macroscopic segregative behaviour (two-phase systems) when the ECG concentration in the mixtures was ≥0.15% (w/v). Before this limit in ECG concentration was reached, the mixtures exhibited one-phase behaviour.

After 24 h of incubation at 35 °C, two completely separated phases could be observed from the ECG limit concentration of 0.05% (w/v). In addition, among the MP/ECG systems that presented two phases, it was possible to visualise that the MP distribution in the graduated cylinder changed when the ECG concentration increased. The volume of the MP phase decreased and the volume of the polysaccharide phase increased when the ECG concentration in the mixtures increased.

Fig. 2A shows the flow curves displayed by the MP and MP/ECG mixture systems. The MP suspension, as previously reported, presented Newtonian behaviour (Vélez-Ruiz & Barbosa-Cánovas, 1998). Perduca et al. (2013) reported that ECG solutions exhibit a pseudoplastic behaviour in the ECG concentration range of 0.25–1.5% (w/w). In addition, Bordino (2015) reported that the ECG aqueous dispersions present Newtonian behaviour at

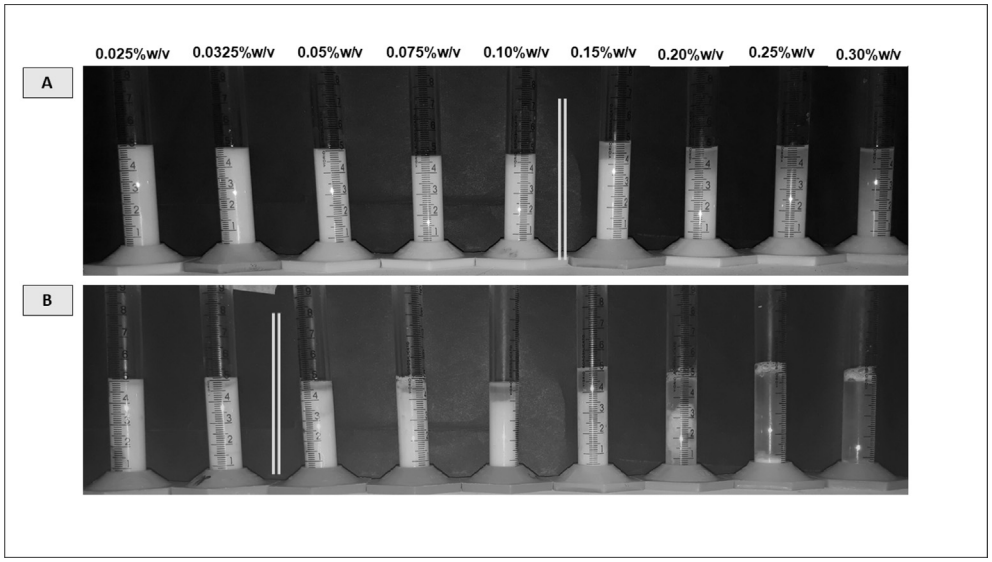

Fig. 1. Images of the milk protein/espina corona gum (MP/ECG) mixtures (3.5%, w/v, of MP and ECG concentrations ranged from 0.025 to 0.30%, w/v) prepared in buffer Tris–HCl 10 mM, pH 6.4, at 35 °C: (A) 1 h of incubation (one-phase systems: from 0.025 to 0.10%, w/v; two-phase systems: from 0.15 to 0.30%, w/v) and (B) 24 h of incubation (one-phase systems: from 0.025 to 0.0325%, w/v; two-phase systems: from 0.05 to 0.30%, w/v).

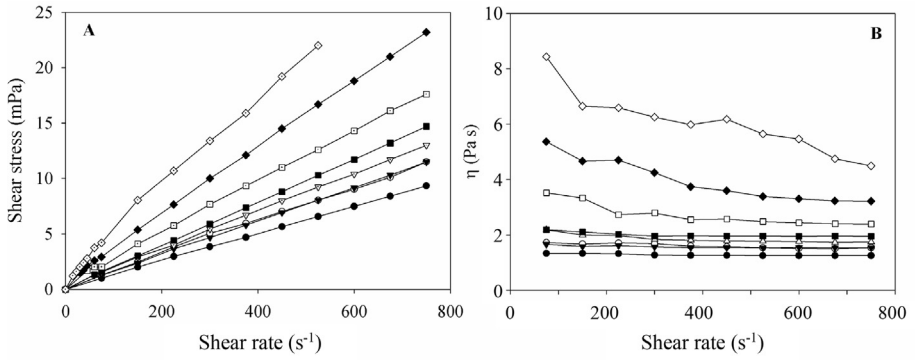

Fig. 2. Flow behavior (A) and apparent viscosity (η) versus shear rate (B) of the milk protein (MP) and MP/espina corona gum (ECG) mixtures at 35 °C: ●, 0.00% (w/v) ECG; ○, 0.025% (w/v) ECG; ▼, 0.0325% (w/v) ECG; △, 0.05% (w/v) ECG; ■, 0.075% (w/v) ECG; □, 0.10% (w/v) ECG; ♦, 0,15% (w/v) ECG; ◊, 0.20% (w/v) ECG.

concentrations ≤ 0.1% (w/v) while, at higher concentrations of ECG, a pseudoplastic behaviour was observed. According to Fig. 2A, the MP/ECG mixtures showed a linear correlation between shear stress and shear rate at low ECG concentrations (≤0.075%, w/v), which corresponds to Newtonian behaviour. Non-linear graphs were obtained for MP/ECG mixtures at higher ECG concentration (from 0.075%, w/v). Therefore, instead of exhibiting Newtonian behaviour, these systems displayed a pseudoplastic behaviour. In the latter case, the apparent viscosity (η) of MP/ECG mixtures decreased when the shear rate increased (Fig. 2B). This was since molecules in a resting state were randomly oriented, but when the shear rate increased they were oriented towards the flow, decreasing their flow resistance. Considering the previously reported flow behaviour of the MP and ECG suspensions, it is possible to note that the ECG concentration in the MP/ECG mixtures was responsible for the flow behaviour observed for such mixtures.

In addition, Fig. 2B shows that the η value of the MP/ECG mixtures increased when the ECG concentration in the samples increased. This result restates the possible use of the ECG as a thickening agent in dairy products (Spotti et al., 2012; Spotti & Perduca, 2019).

The rheological behaviour of the MP/ECG mixtures is very similar to those reported for MP/GG mixtures (Bourriot, Garnier, & Doublier, 1999a) and for MP/LBG mixtures (Schorsch, Jones, & Norton, 1999). This resemblance is probably related to the structural similarities among these galactomannans.

3.2. Characterisation of the rennet-induced MP/ECG gels

3.2.1. Gel microstructure

Fig. 3A–H shows the CSLM images from the MP/ECG gels stained with rhodamine B. The protein matrix appears as bright areas while the pores appear as black areas. Fig. 3I–K shows tridimensional image stacks representation of the MP gels and MP/ECG mixture gels at 0.05 and 0.2% (w/v) ECG concentration, respectively. From the CSLM images, it can be noted that the increase in the ECG concentration affects the gel microstructure, since the protein mesh seems to be less interconnected with bigger pore size. This was verified by measuring of the pore size of the gels by digital image analysis (Galante et al., 2018). Fig. 4 shows the pore size results for the MP and MP/ECG gels. This analysis agreed with the image observations. When the ECG concentration increased over 0.05% (w/v)

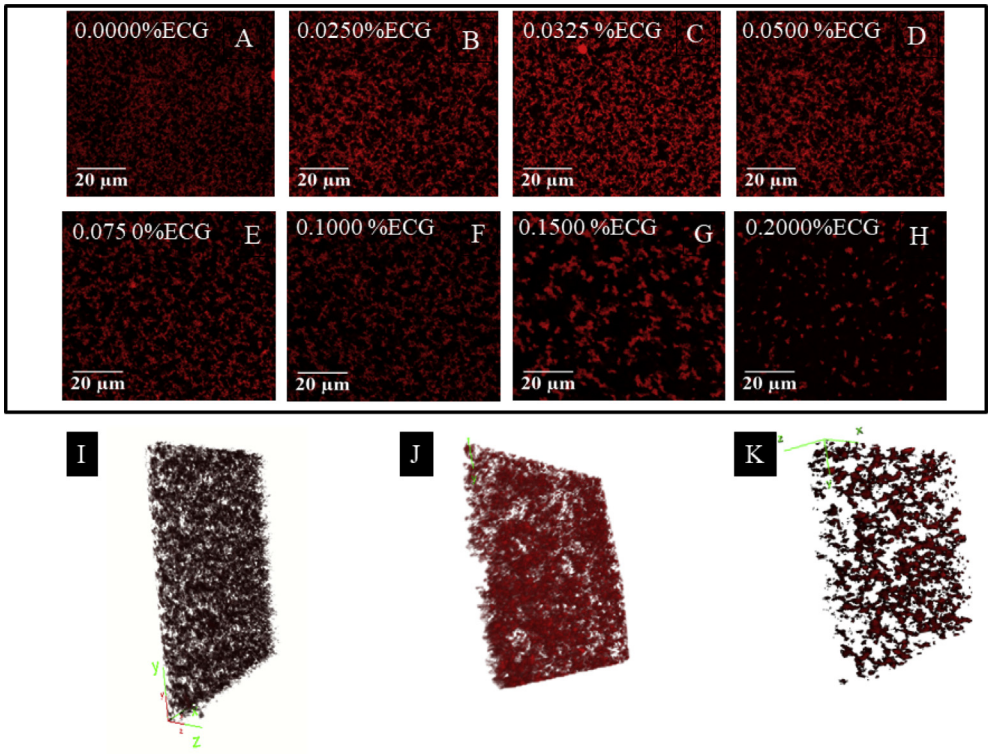

Fig. 3. CSLM images of (A) the milk protein (MP) gels and of (B–H) MP/espina corona gum (ECG) gels [0.025, 0.05, 0.0325, 0.075, 0.10, 0.15, and 0.20 (%, w/v) ECG concentration, respectively], with tridimensional representation of the image stacks of the MP gel (I) and MP/ECG mixture gels (J and K; 0.05 and 0.20%, w/v, ECG concentration, respectively).

in MP/ECG gels, bigger pore sizes were obtained. At an ECG concentration of 0.2% (w/v) the pore size could not be determined because the gel mesh was not interconnected enough.

The discontinuity of the protein network observed when the ECG concentration increased could be due to the microphase separation. After 1 h of incubation of a MP/ECG mixed system at 35 °C, the time at which rennet-induced gelation occurred, the macroscopic phase separation occurred at >0.15% (w/v) ECG concentration (Fig. 1). However, it is probable that a microphase separation had already taken place at a 0.05% (w/v) ECG concentration. After 24 h incubation at 35 °C, this separation becomes macroscopically evident at the latter concentration. In addition, the microphase separation was also reported by López et al. (2017b), for sodium caseinate/ECG mixtures.

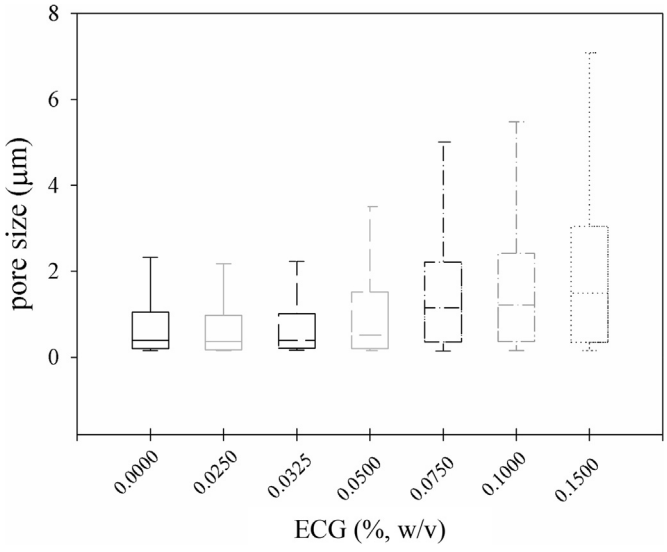

Fig. 4. Pore size distribution for milk protein (MP) and MP/espina corona gum (ECG) gels.

According to de Jong and van de Velde (2007), the microphase separation of protein/polysaccharide mixtures forces the protein and the galactomannan into local areas. Several authors have reported that this phase-separation behaviour is due to the fact that a depletion-flocculation mechanism of casein micelles occurs (Bourriot, Garnier, & Doublier, 1999b; Corredig et al., 2011; de Jong & van de Velde, 2007; Tuinier, Rieger, & de Kruif, 2003). The incompatibility between the biopolymers induces the formation of a depleted layer around the casein micelles and the local increase in MP concentration causes the casein micelles to attract each other and thus flocculate. The formation of these casein micelle clusters, like a pre-flocculated state, was already reported by Lopez, Corredig, and Alexander (2009).

From the microstructural analysis, it is possible to conclude that, at ECG concentration ≥0.05% (w/v) microphase separation process hinders the MP rearrangement that leads to an interconnected gel mesh formation. According to the classification for WPI/polysaccharide mixed gels reported by van den Berg, van Vliet, van der Linden, van Boekel, and van de Velde (2007), the MP/ECG gel microstructure changes from a coarse-stranded gel (a protein network distributed through the aqueous phase) to a bicontinuous gel (where both the serum phase and MP phase are continuous). Such microstructural changes occur at an ECG concentration of 0.05% (w/v) (Fig. 3). Finally, for MP/ECG gels with 0.2% (w/v) ECG concentration the discontinuity of the gel mesh gains relevance; therefore, a pore size determination could not be obtained from the CLSM gel image.

At ECG concentrations between 0.05 and 0.15% (w/v), phase separation kinetics was slower than the rennet-induced gelation

process due to the increase in background viscosity (Fig. 2B). Therefore, the formation of the gel network mesh occurs but with some modifications. After rennet addition, MP has restricted mobility due to its arrested state in the ECG-depleted zones as well as its aggregation state due to rennet effects. This reduced MP mobility might limit the possibility of rearrangements occurring and might lead to the formation of gel meshes with larger pore size (Figs. 3 and 4).

At 0.2% (w/v) ECG concentration, phase separation occurred at a faster rate than rennet gelation; consequently, no continuous gel mesh was formed.

3.2.2. Textural analysis

To evaluate MP/ECG gel characteristics, a penetration test was performed. Gels were penetrated with a cylindrical plunger and the force necessary to disrupt the gel mesh while the plunger was getting into the sample was measured. Firmness and break force parameters were obtained from the force–distance curves. The firmness values were obtained from the slope of the graphics and the break force was defined as the maximum force measured before the gels broke down. The textural parameters are presented in Table 1.

At low ECG concentration (≤0.05%, w/v), MP/ECG gel firmness slightly increased, and the MP/ECG gel breaking point increased as the ECG concentration increased. At higher ECG concentration, when the bicontinuous microstructure was obtained, the gel firmness decreased. These results could indicate that the addition of high ECG concentrations leads to weak gels.

Finally, when the ECG concentration added to MP was 0.2% (w/v), no break point was observed, since the gel obtained was too weak and presented a similar behaviour to a viscous liquid. In addition, in the CSLM images for this MP/ECG systems, (Fig. 3K), a poor interconnected protein mesh in a nonprotein phase (black areas) could be noted.

These effects are in agreement with the microstructural results previously discussed, since they are a consequence of MP/ECG mixture behaviour during the gelation process.

3.2.3. Water-holding capacity

Fig. 5 shows the WHC of MP gels and MP/ECG gels. It is notable that, for ECG concentrations <0.15% (w/v), the WHC of the MP/ECG gels did not change significantly ($p > 0.05$). Although a microstructural change above a concentration 0.05% (w/v) ECG could be noted (from coarse-stranded to bicontinuous microstructure), the pore sizes are small enough to retain the hydration water. Nevertheless, when the ECG concentrations were equal or higher than 0.15% (w/v), the WHC of the gels decreased significantly ($p < 0.05$), according to a significant increase of pore size (Fig. 4). The WHC decreased as a consequence of the protein mesh incapacity to retain water in the interstitial phase of the bicontinuous gel microstructure.

These results are consistent with the microstructural and textural analysis results discussed in previous sections.

Table 1
Textural parameters of MP/ECG rennet-induced gels.[a]

ECG (%, w/v)	Firmness (N mm^{-1})	Breaking point (N)
0.00	0.09 ± 0.01^{a}	0.16 ± 0.02^{a}
0.025	0.11 ± 0.01^{abc}	0.18 ± 0.04^{ab}
0.0325	0.10 ± 0.01^{abc}	0.17 ± 0.03^{a}
0.05	0.13 ± 0.01^{b}	0.18 ± 0.02^{ab}
0.075	0.10 ± 0.01a^{bc}	0.16 ± 0.03^{a}
0.10	0.09 ± 0.02^{c}	0.16 ± 0.01^{a}
0.15	0.04 ± 0.01^{d}	0.24 ± 0.05^{b}
0.20	0.01 ± 0.0003^{e}	–

[a] Abbreviations are: ECG, espina corona gum; MP, milk protein. The same superscript letters in the same column indicate that there was no significant difference among the samples ($p > 0.05$).

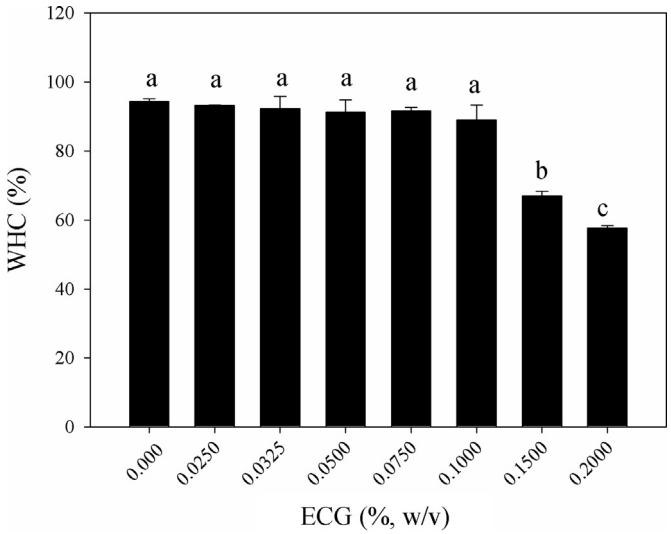

Fig. 5. Water-holding capacity (WHC) as a function of espina corona gum (ECG) concentration for rennet-induced mixed milk protein/ECG gels. Different letters indicate that there was significant difference among the samples ($p < 0.05$).

4. Conclusions

The MP/ECG mixtures present a segregative phase separation behaviour. Although this behaviour was not always macroscopically perceived during the experimental time assay, when rheological and microscopy determinations were performed, changes in the flow behaviour and in the microstructural and textural profiles were detected in MP/ECG mixtures and MP/ECG gels, respectively.

The addition of rennet to MP/ECG mixtures arrested the segregation process before the systems reached the equilibrium and the macroscopic phase separation took place. The gel characteristics obtained from these systems were markedly different and depended on the relationship between the kinetics of gelation and the segregation processes.

To correlate the microstructural and textural results, it is possible to note that coarse-stranded MP/ECG gels were obtained at low ECG concentration. When ECG increased, the microphase separation forced the MP and ECG into local areas and bicontinuous MP/ECG gels were obtained. This last kind of gel microstructure involves a bigger pore size and, the gel firmness and WHC decrease.

Therefore, as has been reported before, the study and characterisation of the gels obtained from these mixtures could be interesting to create new gelled food textures.

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