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1 **CHARACTERIZATION AND IMPROVEMENT OF RHEOLOGICAL PROPERTIES OF SODIUM**
2 **CASEINATE GLYCATED WITH GALACTOSE, LACTOSE AND DEXTRAN**
3

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19 **Abstract**

20

21 The aim of this work was to investigate the effect of non-enzymatic glycosylation with
22 galactose, lactose, and 10 kDa dextran on the rheological properties of sodium caseinate. To
23 promote the formation of covalent complexes, the reaction was done in solid state (a_w=0.67), pH
24 7.0 (0.1 M sodium phosphate buffer), and temperature set at 50 and 60ºC. The progress of
25 Maillard reaction was indirectly traced by measuring the formation of the Amadori compound,
26 through furosine (2-furoylmethyl-lysine) analysis, and brown polymers, and the resulting
27 glycoconjugates were characterized by LC/ESI-MS and SEC. Results showed a higher reactivity
28 of galactose than lactose and dextran to form the glycoconjugates, due to its smaller molecular
29 weight. Glycation with galactose and lactose increased the viscosity of caseinate and also altered
30 its flow characteristics from Newtonian to shear-thinning. Oscillatory testing showed a higher
31 elastic modulus (G') in glycoconjugates when compared to non-glycated caseinate, especially
32 with galactose, where a gel like behavior was observed after long incubation times. Glycation with
33 dextran did not produce substantial improvements in the rheological properties of caseinate,
34 probably due to the limited extent of the reaction. Our results show that by controlling the rate and
35 extent of the Maillard reaction is a technologically feasible operation to improve the viscosity and
36 gelling properties of sodium caseinate-based ingredients.

37

38

39

40 **Keywords:** sodium caseinate; carbohydrates of different molecular weight; non-enzymatic
41 glycosylation; neoglycoproteins; rheological properties.

42

43

44 **1. Introduction**

45

46 Sodium caseinate (SC), a more soluble form of casein, is extensively used in food
47 industry as a functional ingredient in a wide variety of food products due to its simple production,
48 excellent nutritional value, and versatile functional properties, including heat stability, water-
49 holding capacity (WHC), fat binding, rheological properties (increase of viscosity and gel
50 formation) and foaming, emulsifying, and stabilizing capacities. Concretely, WHC and gelling
51 properties are used to improve rheological properties, texture, stability, and appearance of many
52 food products such as processed meats, surimi, cheese, yogurt and confectionary products.
53 However, SC is required in relatively high concentrations to produce viscous dispersions (Ennis &
54 Mulvihill, 2000), resulting in an expensive component in food formulations. Hence, improvements
55 in its rheological properties could produce an equivalent functionality with a smaller amount of
56 added protein, helping to reduce production costs. Initial attempts to improve the viscosity of SC
57 by reaction with carbohydrates were done by Colas, Gobin and Lorient (1988) and Courthaudon,
58 Colas and Lorient (1989). Nevertheless, the formation of these covalent glycoconjugates was
59 achieved following an alkylation reaction with a solution of cyanoborohydride, a highly toxic agent,
60 making the process unsuitable for food application.

61 Consumers demand for food ingredients with improved and desirable functional attributes
62 is increasing and the food industry is moving towards the search of procedures to obtain new
63 multifunctional ingredients. There is substantial evidence that controlled heating of proteins with
64 reducing sugars of different molecular weight to form glycoconjugates via the Maillard reaction
65 (so called neoglycoproteins), is one of the most efficient and safest methods to generate new
66 functional proteins with great potential as novel ingredients (Oliver, Melton & Stanley, 2006a).
67 Extensive glycation has been shown to decrease the solubility of proteins due to polymerization
68 and further cross-linking, produced at the most advanced stages of the reaction (Aoki, Hiidome,

69 Sugimoto, Ibrahim & Kato, 2001; Katayama, Shima & Saeki, 2002; Sato, Sawabe, Kishimura,
70 Hayashi & Saeki, 2000; Tanabe & Saeki, 2001), whereas the viscosity and gelling properties can
71 be improved by carefully controlling the extent of the Maillard reaction.

72 Under controlled conditions, globular proteins of different nature (e.g., whey and egg
73 white proteins, soy protein isolate) have been conjugated via the Maillard reaction with xylose,
74 ribose, glucose, fructose, lactose or galactomannan, to improve their gelling properties (Hill,
75 Mitchell & Armstrong, 1992; Armstrong, Hill, Schrooyen & Mitchell, 1994; Cabodevila, Hill,
76 Armstrong, De Sousa & Mitchell, 1994; Easa, Hill, Mitchell & Taylor, 1996; Handa & Kuroda,
77 1999; Rich & Foegeding, 2000; Lauber, Klostermeyer & Henle, 2001; Matsudomi, Nakano, Soma
78 & Ochi, 2002; Sun, Hayakawa & Izumori, 2004). However, despite the fact that caseins lack a
79 complex secondary and tertiary structure (Farrel, 1988) and could readily react with reducing
80 sugars via the Maillard reaction, to the best of our knowledge, hardly any research has been
81 conducted on this regard. Only recently, Oliver et al. (2006b) glycated SC with ribose, glucose,
82 fructose, lactose and inulin via the Maillard reaction with the aim of describing the effect of
83 reaction conditions, including relative humidity, and type and concentration of sugar, on viscosity
84 of SC. These authors reported interesting results, as SC glycoconjugates showed to possess an
85 increased viscosity compared to unmodified SC, as well as a non-Newtonian behavior.
86 Nevertheless, no further studies on the effect of glycation on the viscoelasticity of SC were
87 carried out. In this sense, the knowledge on viscoelastic properties is needed to completely
88 characterize the rheological behavior of SC glycoconjugates, which will largely determine the
89 quality of foods to which they are added (Herh, Colo, Roye & Hedman, 2000; Ramaswamy,
90 Basak, Abbatemarco & Sablani, 1995). Moreover, to the best of our knowledge, there is no
91 available information on the relationship between rheological properties of SC and structural
92 modifications in native protein induced by glycation with sugars of different molecular weight,
93 including polysaccharides.

94 The aim of this work was to investigate the influence of the sugar molecular weight on
95 the structural changes of SC following glycation via the Maillard reaction with galactose (Gal),
96 lactose (Lac) and 10 kDa dextran (DX), as well as to evaluate the effect of these changes on the
97 flow and viscoelastic properties of SC.
98
99 **2. Materials and methods**
100
101 ***2.1. Preparation of sodium caseinate – galactose /lactose / dextran conjugates***
102
103 Dextran with average molecular weight of 10 kDa, produced by *Leuconostoc*
104 *mesenteroides* strain no. 151-877 was dialyzed (Spectra/Por® 3 Biotech Dialysis Membrane
105 Tubing, 3.5 kDa molecular weight cut-off, Spectrum Europe, Breda, The Netherlands) versus
106 deionised water to remove low molecular weight oligosaccharides. Later, protein-reducing sugar
107 combinations were prepared at the following weight ratios: SC:Gal/Lac at 1:0.2 (600 mg SC:120
108 mg Gal/Lac per portion) and SC:DX at 1:2 (600 mg SC:1.2 g DX per portion). For this,
109 carbohydrates (Gal, Lac or DX) (Sigma-Aldrich, St. Louis, MO) and SC (Rovita FN 5, Proveedora
110 Hispano Holandesa, S.A., Barcelona, Spain) were dissolved in 0.1 M sodium phosphate buffer,
111 pH 7.0 (Merck, Darmstadt, Germany) and frozen at -20ºC for their subsequent lyophilization. The
112 protein-carbohydrate powders were kept at 50 and 60ºC for 96 and 24 hours, respectively, under
113 vacuum in a desiccator equilibrated at 67% of relative humidity (RH) (Oliver et al., 2006b),
114 achieved with a saturated solution of CuCl (Sigma-Aldrich, St. Louis, MO). At the equilibrium, the
115 a_w in the powders was then 0.67. In addition, control experiments were performed with SC stored
116 at 50 and 60ºC without reducing sugars during the same periods (control heated SC). Incubations
117 were performed in duplicate, and all analytical determinations were performed at least in
118 duplicate. After incubation all samples were stored at −20 ºC until used in the following analysis.

119 ***2.2. Assessment of the Maillard reaction evolution***

120

121 The initial steps of Maillard reaction (formation of the Amadori compounds) were
122 indirectly assessed through the determination by ion-pair RP-HPLC of the corresponding 2-
123 furoylmethyl amino acid (2-FM-AA) after the acid hydrolysis of the dry-heated products. Briefly,
124 8.7 mL of 8 N HCl was added to 54.6 mg of SC in hydrolysis tubes and heated at 110°C for 23 h
125 under inert conditions (helium), followed by the filtration through Whatman no. 40 filter paper. The
126 filtered hydrolysate (500 μL) was applied to a previously activated Sep-Pak C18 cartridge
127 (Millipore Corp., Billerica, MA, USA). Compounds retained in the cartridge were eluted with 3 mL
128 of 3 N HCl, and 50 μL were used for injection (Moreno, López-Fandiño & Olano, 2002). Analysis
129 was carried out via an ion-pair RP-HPLC method using a C_8 (Alltech furosine-dedicated; Alltech,
130 Nicolasville, KY) column (250 x 4.6 mm i.d.) and a variable wavelength detector at 280 nm (LDC
131 Analytical, SM 4000, Salem, NH). Operating conditions were as follows: column temperature, 35
132 °C; flow rate, 1.2 mL/min; solvent A, 0.4% HPLC grade acetic acid in double-distilled water;
133 solvent B, 0.3% KCl in solvent A (Resmini, Pellegrino & Batelli, 1990). Calibration was performed
134 by using known concentrations (0.52 to 5.2 mg/L) of a commercial pure standard of furosine
135 (Neosystem Laboratories, Strasbourg, France). Data were expressed as mg per 100 mg of
136 protein.

137 Browning of SC:Gal/Lac/DX conjugates (30 mg/mL in double-distilled water) was
138 measured at room temperature by absorbance at 420 nm in a Beckman DU 70
139 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA), as an index of the brown
140 polymers formed in more advanced stages of non-enzymatic browning (Ting & Rouseff, 1986).
141 Some insoluble conjugates were solubilized with sodium dodecyl sulfate (SDS) at 2.5% + heating
142 at 70 ºC for 10 min, before absorbance measure.

143

144 ***2.3. Structural characterization of glycoconjugates***

145

146 Before LC/ESI-MS separations, dry-heated samples were dissolved in 1 mL of 0.1 M
147 Tris/HCl pH 7.0 buffer (Sigma-Aldrich, St. Louis, MO) containing 8 M urea (Riedel-de Haën,
148 Seelze, Germany) and 20 mM dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO) to give a final
149 protein concentration of 10 mg/mL. After standing at 37ºC for 1 hour, samples were diluted (1/3)
150 with solvent A and filtrated with PVDF membranes (0.45 μm, Symta, Madrid, Spain). LC/ESI-MS
151 experiments were carried out on a Finnigan Surveyor pump with quaternary gradient system
152 coupled in-line to a Finnigan Surveyor PDA photodiode array detector and to a Finnigan LCQ
153 Deca ion trap mass spectrometer using an ESI interface. Sample injections (7 μL) were carried
154 out by a Finnigan Surveyor autosampler. All instruments were from Thermo Fisher Scientific (San
155 José, CA, USA). RP-HPLC separations were carried out with a BioBasic-4 (100 mm x 2.1 mm, 5
156 μm) column (Thermo Fisher Scientific) at 25 ºC using an adaptation of the method by Neveu,
157 Mollé, Moreno, Martin and Léonil (2002). Separation was achieved at a flow rate of 200 μL/min
158 and using 0.25% (v/v) of formic acid (analytical grade, Merck, Darmstadt, Germany) in double-
159 distilled water (Milli-Q water, Millipore, Bedfor, USA) as solvent A and 0.25% (v/v) of formic acid
160 in acetonitrile (LC–MS Chromasolv® grade, Riedel-de Haën, Seelz, Germany) as solvent B. The
161 elution program was applied as follows: at the start 20% B; after 2 min the percentage of B was
162 linearly increased to 40% in 3 min; 40-50% B linear from 5 to 12 min; 50-80% B linear from 12 to
163 15min; 80% B isocratic from 15 to 20 min; ramped to original composition in 1 min; and then
164 equilibrated for 15 min. The PDA detector operated in the wavelength range 190–600 nm in order
165 to gather UV-spectral data. The detection wavelength was set at 280 nm. The mass spectrometer
166 spray voltage was set at 4.5 kV, heated capillary temperature at 220 °C, nitrogen (99.5% purity)
167 was used as sheath (0.6 L min^{-1}) and auxiliary (6 L min^{-1}) gas, and helium (99.9990% purity) as
168 the collision gas. Mass spectra were recorded in the negative ion mode. The LC–MS system,

169 data acquisition and processing were managed by Xcalibur software (1.2 version, Thermo Fisher
170 Scientific). Spectra of the protein peaks detected were deconvoluted using the BIOMASS
171 deconvolution tool from BioWorks 3.1 software (Thermo Fisher Scientific).
172 With the aim to study the aggregation state, size exclusion chromatography (SEC) of
173 glycated SC was carried out under nondenaturing conditions (0.05 M sodium phosphate buffer,
174 pH 7.3, containing 0.15 M NaCl) using a 24 mL Superdex 75 column HR 10/30 (GE Healthcare
175 Bio-Sciences AB, Uppsala, Sweden) on an FPLC system. 100 μL of a 1 mg/mL sample was
176 applied to the column at room temperature. Elution was achieved in isocratic mode at 0.8 mL/min
177 for 30 min and detection of eluting proteins was performed at 214 nm. The standard proteins
178 used for calibration were human serum albumin (67 kDa), ovalbumin (43 kDa), α-
179 chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa) (GE Healthcare Bio-Sciences AB,
180 Uppsala, Sweden). The void volume was determined with Blue Dextran 2000. Additionally, native
181 SC (10 mg) was subjected to denaturing conditions with 1 mL of 0.1 M Tris/HCl pH 7.0 buffer
182 (Sigma-Aldrich, St. Louis, MO) containing 8 M urea (Riedel-de Haën, Seelze, Germany) and 20
183 mM dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO) and heated at 37 ºC for 1 hour prior to SEC
184 analysis performed under the conditions described above. Considering that: i) SEC separates on
185 the basis of the hydrodynamic volume and not of the molecular weight, and ii) all the standard
186 proteins used for calibration had a globular structure, the term Mr (relative molecular mass)
187 instead of absolute molecular mass will be used in the Results and Discussion section when SEC
188 analyses are described.
189
190 ***2.4. Rheological measurements***
191
192 Control heated and glycated SC dispersions were prepared in distilled water at 30 mg/mL
193 protein concentration for the determination of flow properties and at 150 mg/mL for viscoelastic

194 properties evaluation. Samples were left at room temperature (20ºC ± 2ºC, ~ 16 h), with
195 intermittent stirring, to allow complete hydration. Controls formed by SC and carbohydrate
196 separately heated and mixed at the moment of analysis (control heated SC+carbohydrate), were
197 also tested to study the effect of free carbohydrate on rheological properties of protein. The
198 rheological measurements were performed using a controlled stress rheometer (model AR2000,
199 TA Instruments, New Castle, DE) equipped with Peltier plate. The flow curves were obtained by
200 monitoring shear stress (τ) as a response to 0 to 120 s^{-1} shear strain rate ($\dot{\gamma}$) ramp, with data
201 recorded every 0.3 s during 1 min. All tests were performed at 25 ºC using the plate-plate
202 geometry (plate, 40 mm diameter; 0.3 mm gap) and a constant volume of 200 µL. The flow
203 curves were described using the power law model, appropriate for shear-thinning and shear-
204 thickening fluids.

205 $$\tau = K \cdot \dot{\gamma}^{n} \qquad (1)$$

206 where K is the consistency coefficient ($Pa \cdot s^n$) and the exponent n is the flow behavior index and
207 reflects the closeness to Newtonian flow. For the special case of a Newtonian fluid (n = 1), the
208 consistency coefficient K is the Newtonian viscosity of the fluid, whilst in power law fluids (shear-
209 thinning or pseudoplastic (0<n<1) and shear-thickening fluids (n>1)) apparent viscosity (η_a; Pa·s)
210 will change when the shear strain rate is increased. Apparent viscosity values between different
211 samples were compared at a shear strain rate of 60 s^{-1}.

212 For Oscillatory testing, a constant volume of 350 µL of sample was transferred to the
213 rheometer equipped with a cone and plate geometry (plate, 40 mm diameter; 0.03 mm gap) and a
214 Peltier plate set at 10 ºC. After 3 min of temperature stabilization, the sample was subjected to a
215 frequency sweep from 0.1 to 100 Hz (0.1% strain, within the linear region). Viscoelastic properties
216 were determined by monitoring the storage (G') and loss (G'') moduli as a response to the
217 applied dynamic deformation.

218

219 **3. Results and discussion**

220

221 ***3.1. Assessment of the Maillard reaction evolution during the formation of***
222 ***glycoconjugates***

223

224 It is well known that furosine (2-furoylmethyl-lysine), formed upon acid hydrolysis of
225 protein-bound Amadori product of lysine, can be a useful indicator to measure the glycation
226 extent of milk proteins (Moreno et al., 2002; Krause, Combs & Beauchemin, 2003; Fenaille,
227 Morgan, Parisod, Tabet & Guy, 2003; Jimenez-Castaño, López-Fandiño, Olano & Villamiel,
228 2005a and 2005b). Figures 1A and 1C show the evolution of furosine derived from the Amadori
229 products of the SC:Gal/Lac/DX conjugates upon storage at 50 and 60ºC for 96 and 24 hours,
230 respectively. The higher formation of furosine observed in galactosyl conjugates indicated that
231 Gal had a higher initial rate of utilization of ε-amino groups of lysine residues than lactose and
232 dextran at both temperatures. Thus, the maximum level of furosine in glycated SC:Gal system
233 was ~ 3-fold higher than in SC:Lac system and ~ 12-fold higher than in SC:DX system at 50 and
234 60ºC. Moreover, a noticeable decrease of furosine was also found in SC:Gal glycoconjugates
235 after 48 hours of incubation at 50ºC, indicating that the degradation of the Amadori compound
236 prevailed over its formation.

237 As it was expected, the sugar reactivity towards SC was galactose > lactose > dextran.
238 The order of reactivity according to which monosaccharides are more reactive than disaccharides
239 and these more reactive than polysaccharides is well stated and has been previously supported
240 in many studies (Lewis & Lea, 1950; Spark, 1969; Nacka, Chobert, Burova, Léonil, & Haertlé,
241 1998; Chevalier, Chobert, Mollé & Haertlé, 2001; Oliver et al., 2006c). Thus, it was observed that
242 the smaller the carbonic chain of the sugar is, the more acyclic forms exist and the more reactive
243 is the sugar with the amino groups of proteins. Likewise, it has been also described that DX

244 reacts poorly with proteins because of steric hindrance caused by the high branching degree of
245 their molecules, which difficult the reaction of the single reducing group of each dextran molecule
246 unavailable to react with free amino groups in the protein (Aminlari, Ramezani & Jadidi, 2005).
247 In good agreement with these results, the highest color development at the end of the
248 storage period was observed in galactosyl derivatives (dark brown) followed by SC:Lac (light
249 brown) and SC:DX (slight yellow) conjugates. As shown in figures 1B and 1D, for SC glycated
250 with Gal at 50 and 60ºC, the formation of brown compounds progressively increased over the
251 storage period, suggesting an extensive progress of the Maillard reaction toward the most
252 advanced stages. Lactosylated SC also showed an increase of color development over the
253 incubation time particularly evident at 60ºC, although much less marked than that of the
254 galactosylated SC. The lowest values of browning were observed in SC glycated with DX at both
255 temperatures, confirming a limited extent of the Maillard reaction. Such browning indices can not
256 be attributed to the sugar caramelisation because sugars heated alone failed to generate
257 compounds absorbing at 420 nm (data not shown). Furthermore, caramelisation mainly occurs at
258 higher temperature and alkaline pH in aqueous solution (Laroque, Inisan, Berger, Vouland &
259 Dufossé, 2008) and not under the relatively mild conditions assayed in this study (50-60ºC at a_w
260 0.67 and pH 7.0).
261
262 ***3.2. Structural characterization of glycoconjugates***
263
264 The fractions observed in the RP-HPLC profile of native SC were identified, in order of
265 increasing retention time, as κ-casein mono-phosphorylated genetic variant A (fraction 1), κ-
266 casein mono-phosphorylated genetic variant B and α_{S2}-casein non-phosphorylated genetic
267 variant A (fraction 2), α_{S1}-casein octa-phosphorylated genetic variant B (fraction 3), and β-
268 caseins penta-phosphorylated genetic variants A2 and A1 (fraction 4), and whose molecular

269 masses were 19,038 (fraction 1), 19,008 and 25,223 (fraction 2), 23,619 (fraction 3), and 23,987
270 and 24,030 Da (fraction 4), respectively, as previously reported by Léonil, Mollé, Gaucheron,
271 Arpino, Guénot, and Maubois (1995) and van der Ven (2002) (figure 2A).

272 Regarding glycated SC, table 1 summarizes the number of molecules of Gal and Lac
273 linked covalently to the main fractions of caseins determined experimentally by RP-HPLC/ESI-MS
274 analyses. Deconvoluted spectra of SC : Gal and SC : Lac conjugates showed a heterogeneous
275 mixture of various glycoform species differing in the number of carbohydrate residues linked to
276 the peptidic chain (table 1). Similarly, Oliver, Kher, McNaughton & Augustin (2009) recently
277 observed a similar behavior after glycation of SC with glucose in aqueous solutions. However, no
278 evidence of glycation of SC with DX was found after deconvolution of ESI spectra, which could be
279 attributed to both the low ionization efficiency of polysaccharides in ESI due to the low proton
280 affinity of carbohydrates (Hsu et al., 2007) and the slow progress of Maillard reaction using DX as
281 reducing sugar (figure 1). Jimenez-Castaño et al. (2007) needed 4 and 10 days at 0.44 a_w, 60ºC
282 and 0.65 a_w, 50ºC, respectively, to block a maximum of 1.73 lysine molecules per β-lactoglobulin
283 molecule. For reasons of simplicity, only the most abundant genetic variant of each casein was
284 included in table 1, i.e. κ-casein genetic variant A and β-casein genetic variant A2, as no
285 differences were found in the glycation patterns of the genetic variants corresponding to the same
286 casein. Furthermore, the corresponding deconvoluted spectra of glycated α_{S2}-casein were not
287 satisfactory due to the low ionization exhibited by fraction 2 (figure 2B). In general terms, a higher
288 number of Gal and Lac molecules were bound to the more abundant casein components (α_{s1}-
289 and β-casein) as compared to the κ-casein. α_{s1}-, α_{s2}-, β- and κ-caseins are present in SC in the
290 approximate proportions 4:1:4:1, respectively. Recent data concerning casein reactivity were in
291 good agreement with our results (Scaloni, Perillo, Franco, Fedele, Froio et al., 2002). Thus, a
292 maximum of 4, 3 and 1 Lac molecules linked to α_{s1}-, β- and κ-caseins, respectively, could be
293 detected after incubation of SC for 4 days at 50 ºC and for 1 day at 60 ºC (Table 1). As regards

294 SC : Gal conjugates, a maximum of 5, 4 and 3 Gal residues bound to α_{s1}-, β- and κ-caseins,
295 respectively, were detected after incubation of SC for 2 days at 50 ºC, and 6, 5 and 3 Gal
296 residues linked to α_{s1}-, β- and κ-caseins, respectively, after storage of SC for 4 hours at 60 ºC.
297 This data confirmed the higher reactivity of Gal in comparison to Lac. In addition, taking into
298 account the fast progress of the Maillard reaction between SC and Gal observed from the second
299 day of incubation at 50 ºC and from the eighth hour of storage at 60 ºC, as it can be deduced
300 from the high values of absorbance at 420 nm (Figures 1B and 1D), the deconvoluted spectra
301 corresponding to the two longest storage times (3 and 4 days at 50 ºC and 8 and 24 hours at 60
302 ºC) were very complex and confusing probably due to the elevated number of products formed
303 during the advanced stages of the Maillard reaction. Therefore, it is plausible that a higher
304 number of Gal molecules were actually attached to SC following long storage times as α_{s1}-, β-
305 and κ-casein contain 14, 11, and 9 lysine residues prone to participate in the Maillard reaction
306 (Whitney, 1988; Aminlari et al., 2005).
307 In an attempt to shed more light on the structural changes promoted by the reaction
308 between Gal, Lac or DX and SC, the polymerization degree of SC was investigated by means of
309 SEC under non-denaturing conditions (table 2). Native SC eluted as two partly resolved peaks,
310 eluting the first one very near the void volume, in agreement with several previous studies (Lynch,
311 Mulvihill, Law, Leaver & Horne, 1997; Lucey, Srinivasan, Singh & Munro, 2000). When SC was
312 treated with urea and DTT, the peak 1 substantially decreased with regard to peak 2
313 (chromatogram not shown), suggesting that the former was constituted by covalent and non-
314 covalent casein aggregates, concretely by κ-casein polymers and some α_{s1}- and β-casein
315 complexes (Lucey et al., 2000). Moreover, peak 2 presented a wide size distribution from Mr ~ 80
316 kDa at the start of peak, to Mr of 25-30 kDa, close to the theoretical molecular weight of casein
317 monomers, at the trailing end of the distribution, according to Lucey et al. (2000). The control
318 protein heated in absence of sugars showed a slight increase of the high Mw (HM_w) fraction as

319 compared to native SC, indicating the formation of some minor aggregated forms during heating,
320 probably due to hydrophobic interactions. For SC:Gal conjugates at both temperatures and
321 SC:Lac conjugates at 60ºC, a notable increase of HM_w fraction with regard to low M_w (LM_w)
322 fraction was observed with increasing incubation time, especially in galactosyl derivatives at 50ºC
323 (figure 3). This indicates that glycation of SC promoted its polymerization mainly through covalent
324 cross-linking, produced at the most advanced stages of the Maillard reaction. SEC profiles of
325 lactosyl derivatives at 50ºC and SC:DX conjugates at 50 and 60ºC were very similar to those of
326 unglycated protein, confirming the limited extent of the Maillard reaction to the more advanced
327 stages under these conditions, in agreement with the results derived from the furosine and
328 browning evaluation (figure 1). The greater browning development and accelerated
329 polymerization of SC:Gal might be due to the rapid degradation of the corresponding Amadori
330 compound, tagatosyl-lysine, into highly reactive Maillard reaction products and brown polymers.
331 Similar trend was previously described by Kato, Matsuda, Kato and Nakamura (1988) for the
332 ovalbumin glycated with glucose and lactose at 50ºC. These authors reported that the limited
333 extent of the Maillard reaction in the lactosyl conjugates is attributed to the galactopyranoside
334 group on the C-4 hydroxyl group of glucose which acts to protect the Amadori adduct from further
335 degradation.
336
337 ***3.3. Effect of glycation on flow and viscoelastic properties of sodium caseinate***
338
339 Figure 4 shows the consistency coefficient, *K*, and the flow behavior index, n, of native,
340 control heated, control heated+carbohydrate and SC glycated with Gal, Lac and DX upon
341 hydration at 30 mg/mL protein concentration incubated at 0.67 a_w for 96 and 24 hours at 50 and
342 60ºC, respectively. Unlike SC:Gal/Lac systems, no consistent differences between flow properties
343 of SC:DX conjugate and the control heated SC+DX were observed, indicating that the alteration

344 of the flow properties of SC glycated with DX was mainly due to the intrinsic properties of the
345 polysaccharide. So, from now, to study the effect of the Maillard reaction on rheological properties
346 of SC, we will focus on the caseinate glycated only with Gal and Lac.

347 In the shear strain rate range applied (0 to 120 s^{-1}), the flow of native and control heated
348 SC was very close to Newtonian (n ~ 1), with correlation coefficients (R^2) above 0.993. However,
349 for SC glycoconjugates, an increase of the consistency coefficient *K* as well as an evolution of the
350 flow behavior to markedly pseudoplastic was observed with increasing incubation time, showing
351 better R^2 values (> 0.997) with the power law model than with the Newtonian model. This effect
352 was particularly notable in galactosyl derivatives upon storage at 50 and 60ºC, and in lactosyl
353 derivatives after storage at 60ºC. Thus, the flow curves of glycoconjugates SC:Gal (72 hours,
354 50ºC), SC:Gal (8 hours, 60ºC), and SC:Lac (24 hours, 60ºC) had a flow behavior index (n) of 0.73
355 (vs. 0.96 for control heated), 0.76 (vs. 0.95 for control heated) and 0.80 (vs. 0.94 for control
356 heated), and consistency factors (*K*) 4.9-fold, 3.1-fold and 2.5-fold higher than that of the control
357 heated SC, respectively. This suggests a high level of internal structure within SC
358 glycoconjugates.

359 In agreement with these results, the viscosity for control SC+Gal/Lac at time 0 did not
360 depend on the shear rate, remaining fairly constant over the range investigated and displaying,
361 therefore, a very close Newtonian flow. In contrast, for SC:Gal and SC:Lac glycoconjugates the
362 viscosity decreased with increasing shear rate over the range investigated (figure 5). This
363 behavior is characteristic of pseudoplastic fluids, which are well described by a power law model.
364 Moreover, this shear rate dependence of the viscosity was particularly evident in SC glycated with
365 Gal at 50ºC followed by SC glycated with Gal at 60ºC > Lac at 60ºC > Lac at 50ºC. These results
366 indicate that glycation with Gal produce more substantial alteration in flow behavior of SC, from
367 Newtonian to markedly pseudoplastic with increasing incubation time, than glycation with Lac.

368 Likewise, apparent viscosity of glycated SC increased with the incubation time, whereas
369 the apparent viscosity of control heated SC remained low during all storage. The largest increase
370 in apparent viscosity was observed in SC glycated with Gal from 48 hours of incubation at 50ºC,
371 followed by SC:Gal and SC:Lac from 2 and 4 hours at 60ºC, respectively (figure 6). Because of
372 the greater reactivity and faster progress of the Maillard reaction with Gal, SC:Gal conjugates
373 upon storage for 96 and 24 hours at 50 and 60ºC, respectively, gave rise to dispersions
374 containing highly insoluble brown polymers ("insoluble particles"), which interfered with measures
375 of rheometer, being impossible to determine their viscosity. These results are in agreement with
376 those of Oliver et al. (2006b) who glycated SC with glucose, ribose, fructose, lactose and fructo-
377 oligosaccharide (inulin). However, these authors did not investigate the viscoelasticity of such
378 glycoconjugates, which would be needed to completely describe their flow behavior, since they
379 have both properties of solids and liquids.

380 Viscoelastic properties of glycoconjugates were determined upon hydration (at 150
381 mg/mL protein concentration) by means of oscillatory tests, monitoring the storage (*G´*) and loss
382 (*G´´*) moduli as a response to the applied dynamic deformation. Figure 7 depicts the dependence
383 of the *G´* modulus on frequency and incubation time for the glycoconjugates. The *G´* of native and
384 control heated SC at 50 and 60ºC remained low and constant over the storage period ($G' < 0.1$).
385 In contrast, *G´* of glycoconjugates showed a general trend to increase with increasing incubation
386 time, i.e. with the progress of the Maillard reaction. At longer incubation times, some
387 glycoconjugates showed a gel-like behaviour. Frequency sweeps confirmed gelled samples when
388 the *G´* was > 1 and greater than the loss modulus (*G´´*), the *G´* and *G´´* were parallel over the
389 frequency range. This behavior seemed to be clear in galactosyl derivatives from 48 and 8 hours
390 of storage at 50 and 60ºC, respectively). Lactosyl derivatives also formed gels from 8 hours at
391 60ºC. For these conjugates with gel-like behavior *G´* did not show a strong dependence on
392 frequency, displaying a slight increase at higher frequency (>50 rad/s), what is suggestive of a

well-organised and cohesive structure (true gel). *G´* of SC:Gal conjugates was higher than that of SC:Lac conjugates. Moreover, increasing frequency had less effect on gels with galactose than with lactose. This suggested that the gels containing galactose formed more elastic networks than the gels containing lactose. In SC:Lac systems upon storage for 72 and 96 hours at 50ºC, the *G´* was also greater than the *G´´* fairly over the frequency range; however we did not consider them as gelled systems since *G´* was $<$ 1. In these cases, *G´* and *G´´* increased over the frequency range studied, but at higher frequency, *G´* modulus slightly dropped, most probably due to rheometer inertial effects. These results confirm a significantly higher level of internal structure in SC glycated with Gal than with Lac, in agreement with the results of flow properties determination.

At shorter storage times, the viscoelastic behavior of glycoconjugates was characterized by a *G´´* greater than *G´* over the frequency range. Likewise, the *G´* remained low and fairly constant, ~ 0 (1 $>$ *G´* $>$ 0), over the frequency range (figure 7). These glycoconjugates displayed, therefore, a viscous-like flow characteristic of Newtonian or slightly pseudoplastic fluids.

A number of studies carried out with proteins from different origin, mainly globular proteins, support that protein aggregation and cross-linking associated with the advanced stages of the Maillard reaction have an important impact on the rheological properties of these proteins (Hill et al., 1992; Armstrong et al., 1994; Cabodevila et al., 1994; Oliver et al., 2006b). According to these authors, the alteration of flow and viscoelastic properties of SC glycated, particularly with galactose, observed in this work could be probably due to a caseinate polymerization through covalent cross-links induced by the advanced Maillard reaction.

4. Conclusions

417 Rheological changes associated with SC glycated via the Maillard reaction were found to
418 be influenced by the sugar molecular weight and reaction temperature. Due to the limited extent
419 of the Maillard reaction observed in SC when was glycated with DX at 50 and 60ºC, the effect of
420 DX on rheological properties of SC could be mainly attributed to the intrinsic properties of the
421 polysaccharide. In contrast, glycation with Gal and Lac at both temperatures altered the SC flow
422 characteristics with increasing storage time, so that SC glycoconjugates showed a higher
423 viscosity and more pseudoplastic flow behavior than native and control heated SC. Likewise,
424 these Maillard complexes were characterised by intense viscoelastic character. For glycated SC,
425 oscillatory testing showed an increasing G' modulus with incubation time, so that samples
426 exhibited a gradation in flow behavior from highly liquid of unmodified SC to viscous-like of
427 glycoconjugates for shorter incubation times and to gelled network of glycoconjugates for longer
428 incubation times. Such results suggest a great level of internal structure within SC
429 glycoconjugates. The most remarkable effects of glycation on protein flow and viscoelastic
430 properties were observed in galactosyl conjugates incubated at 50ºC, followed by SC:Gal 60ºC >
431 SC:Lac 60ºC > SC:Lac 50ºC, in agreement with the results derived from the assessment of the
432 Maillard reaction evolution and structural characterization of glycoconjugates. These showed a
433 higher reactivity of Gal during the initial stages of the Maillard reaction, as well as a greater
434 browning development and an accelerated polymerization of SC glycated with Gal as compared
435 to Lac and DX, suggesting that such improvement in rheological properties of glycated SC could
436 be attributed to protein aggregation through covalent cross-linking, produced at the most
437 advanced stages of the Maillard reaction.

438 Taken together, results of this study show that by controlling the rate and extent of the
439 Maillard reaction, it is possible to increase the viscosity and gelling properties of SC in a
440 technologically feasible and 'food-safe' way without the addition of toxic reagents. The
441 improvement of rheological characteristics of SC could produce an equivalent functionality with a

442 smaller amount of added protein, helping to reduce production costs, as well as to promote its
443 use as food ingredient with added value to develop customer-favored products with a competitive
444 edge in the marketplace.

445

446 **Acknowledgements**

447

448 This work was supported by projects Consolider Ingenio 2010 FUN-C-FOOD CSD2007-00063
449 (Ministerio de Educación y Ciencia), ALIBIRD S-0505/AGR/000153 (Comunidad de Madrid) and
450 Hatch project TEN00332 (University of Tennessee Institute of Agriculture). M. Corzo-Martínez
451 thanks the CSIC for an I3P PhD-grant. We also gratefully acknowledge Plácido Galindo-Iranzo
452 and Dr. Rosa Lebrón for their help in acquiring the LC/ESI-MS spectral data.

453

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581

582 **FIGURE CAPTIONS**

583

584 **Figure 1.** Evolution of the content of furosine derived from the Amadori products **(A** and
585 **C)** and absorbance at 420 nm **(B** and **D)** during storage at 0.67 a_w upon 96 hours at 50ºC (**A** and
586 **B**) and 24 hours at 60ºC (**C** and **D**) of the Maillard conjugates: —◆— SC:Gal; —✕— SC:Lac; —■—
587 SC:DX. Data points represent the mean of duplicate determinations with error bars indicating the
588 standard deviations.

589

590 **Figure 2.** UV- (**A**) and total ion current- (TIC) (**B**) chromatograms of native SC following
591 LC/ESI-MS analysis.

592

593 **Figure 3.** SEC elution profile of SC glycated with Gal at 50ºC for 96 hours (a_w=0.67)
594 using a Superdex 75 column on a FPLC system under non-denaturing conditions.

595

596 **Figure 4.** Changes in consistency factor *K* (Pa s^n) (fluid consistency) and flow index (n)
597 of —▬— control heated, control heated + sugar (---◇--- SC+Gal; ---✕--- SC+Lac; ---□--- SC+DX) and
598 glycated SC (—◆— SC:Gal; —✕— SC:Lac; —■— SC:DX), at 30 mg/mL of SC, incubated at 0.67 a_w
599 for 96 hours at 50ºC (**A** and **C**) and for 24 hours at 60ºC (**B** and **D**). Data points represent the
600 mean of duplicate determinations, being the standard deviation in all cases < 0.01.

601

602 **Figure 5.** Shear rate dependence of the apparent viscosity of SC:Gal/Lac
603 glycoconjugates (at 30 mg/mL of SC) upon storage for 4 days at 50ºC and 1 day at 60ºC.

604

605 **Figure 6.** Effect of glycation via the Maillard reaction on apparent viscosity of SC
606 glycated with Gal and Lac (at 30 mg/mL of SC) at 50 (**A**) and 60ºC (**B**).

607

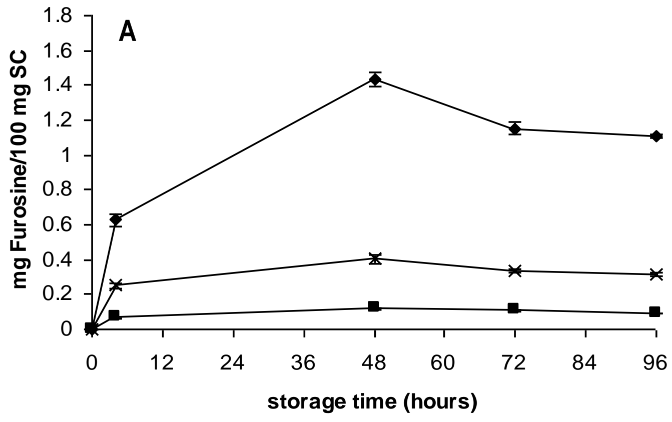
608 **Figure 7.** Dependence of the storage modulus (*G*') on frequency and incubation time for

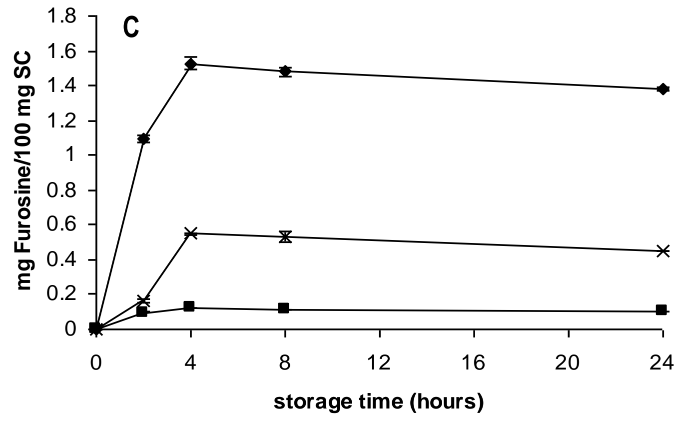
609 control heated SC and and SC:Gal/Lac conjugates (at 150 mg/mL of SC) incubated for 96 hours

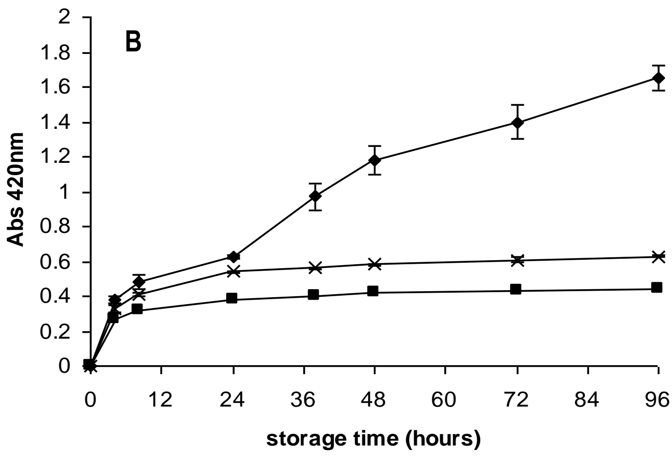
610 at 50ºC (**A**) and 24 hours at 60ºC (**B**).

Figure 1

Figure 1. ***Corzo-Martinez et al.***







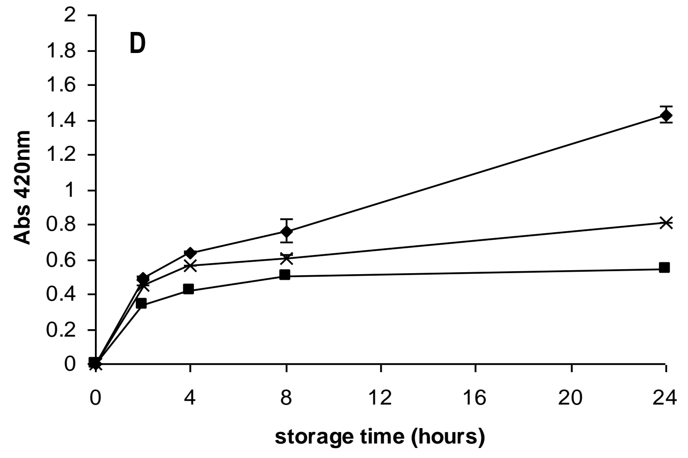


Figure 2

Figure 2. *Corzo-Martinez et al.*

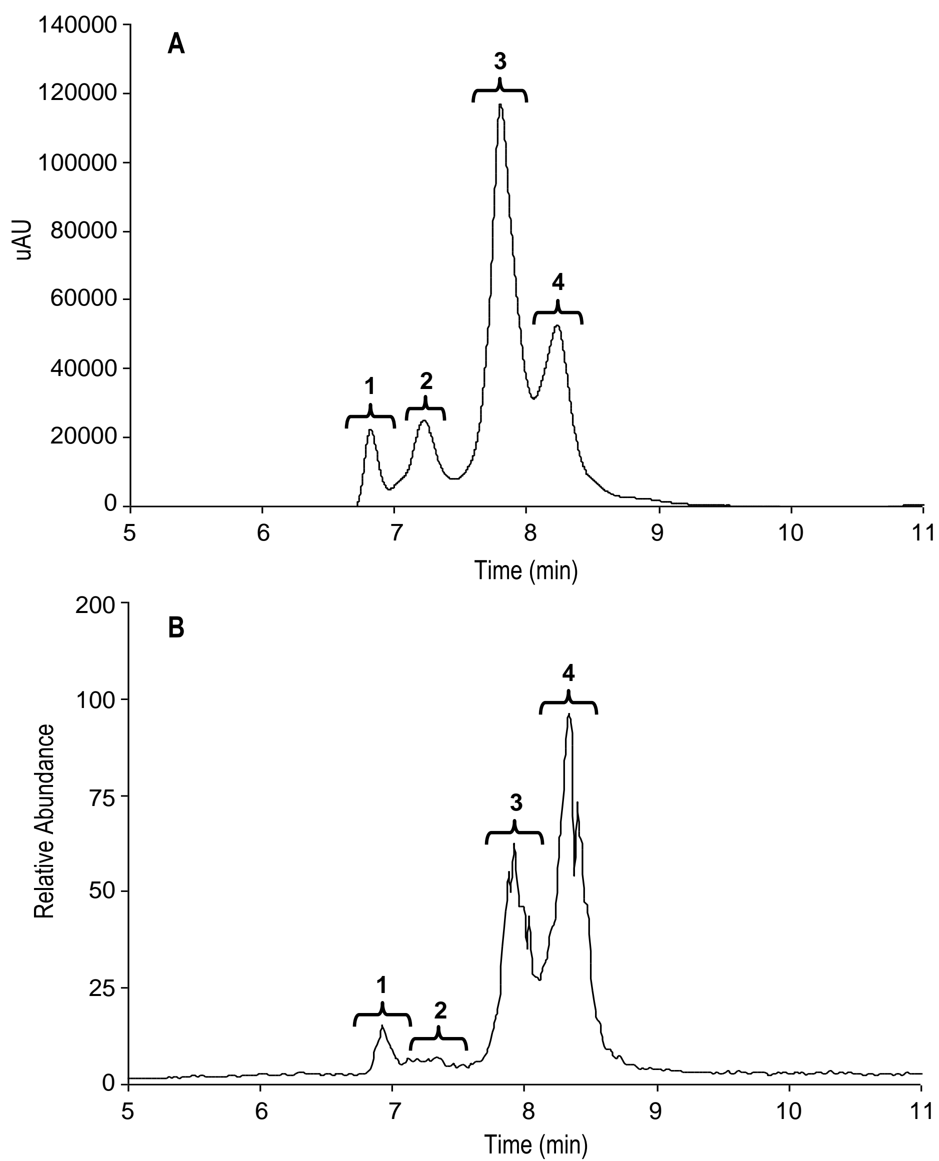


Figure 3

Figure 3. ***Corzo-Martinez et al.***

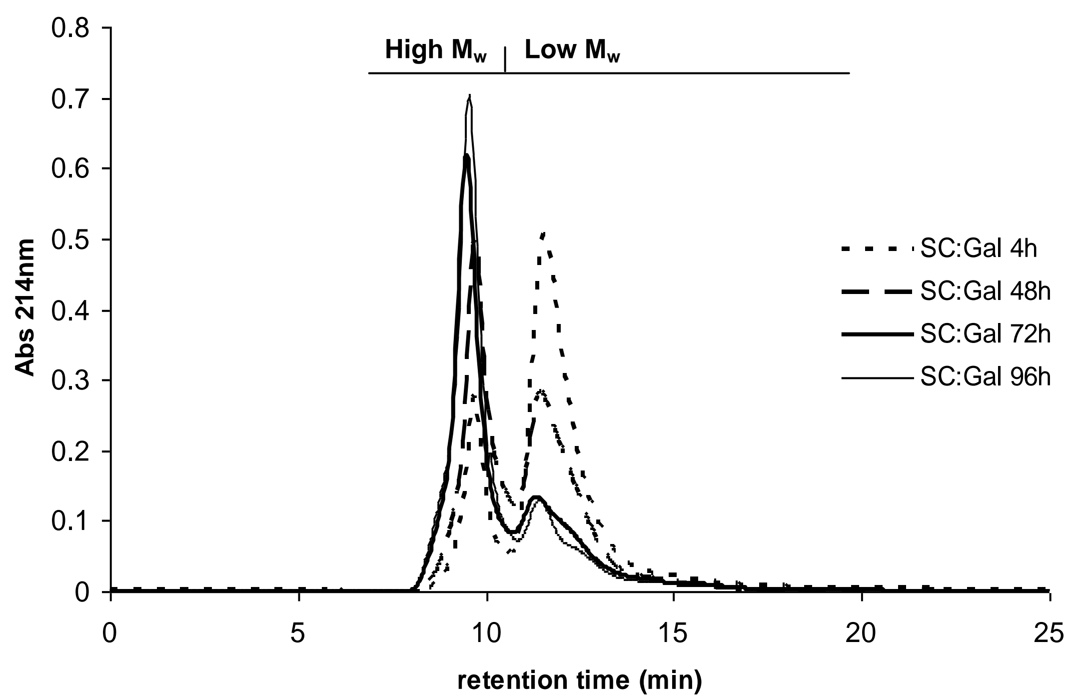


Figure 4

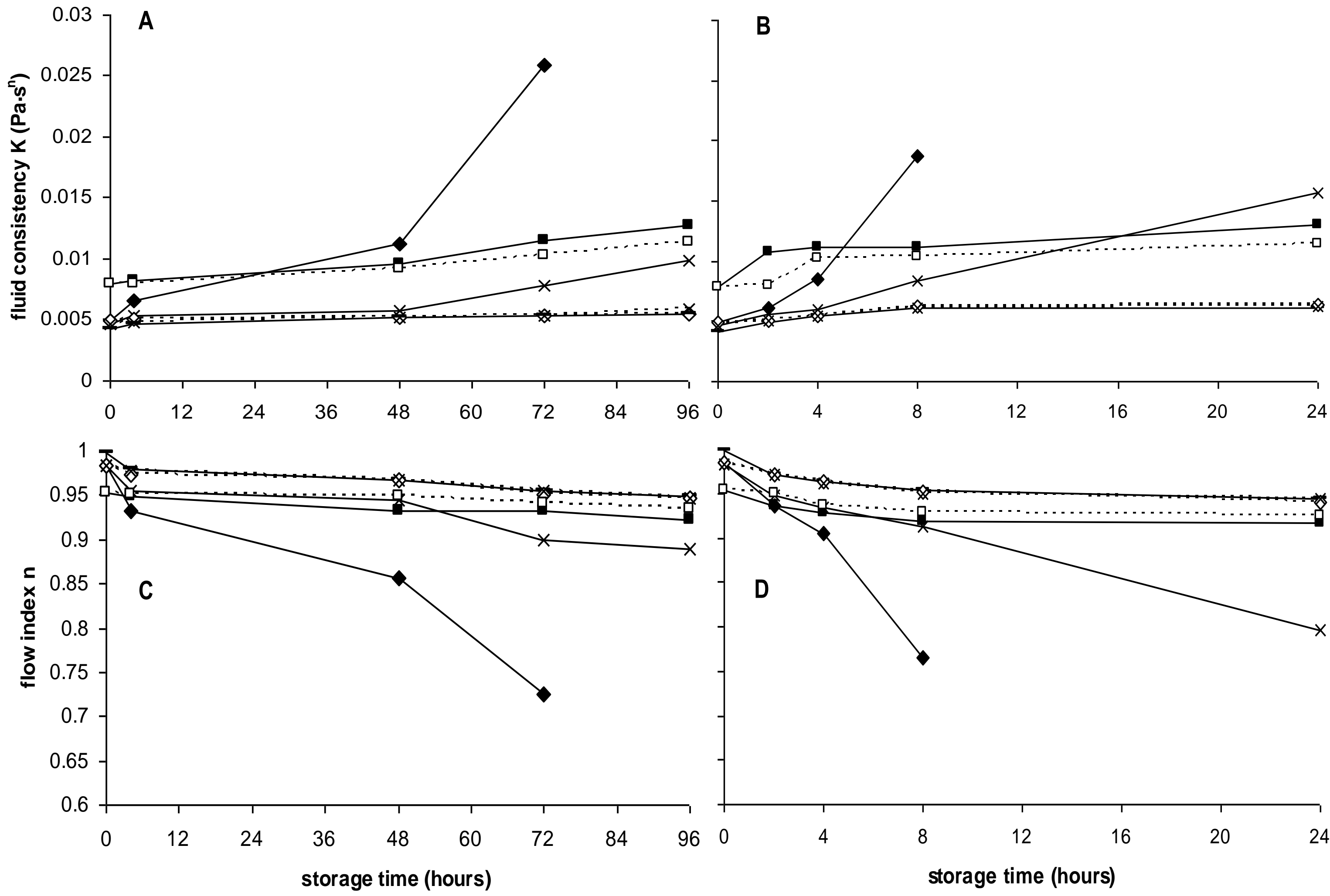


Figure 5

Figure 5. ***Corzo-Martinez et al.***

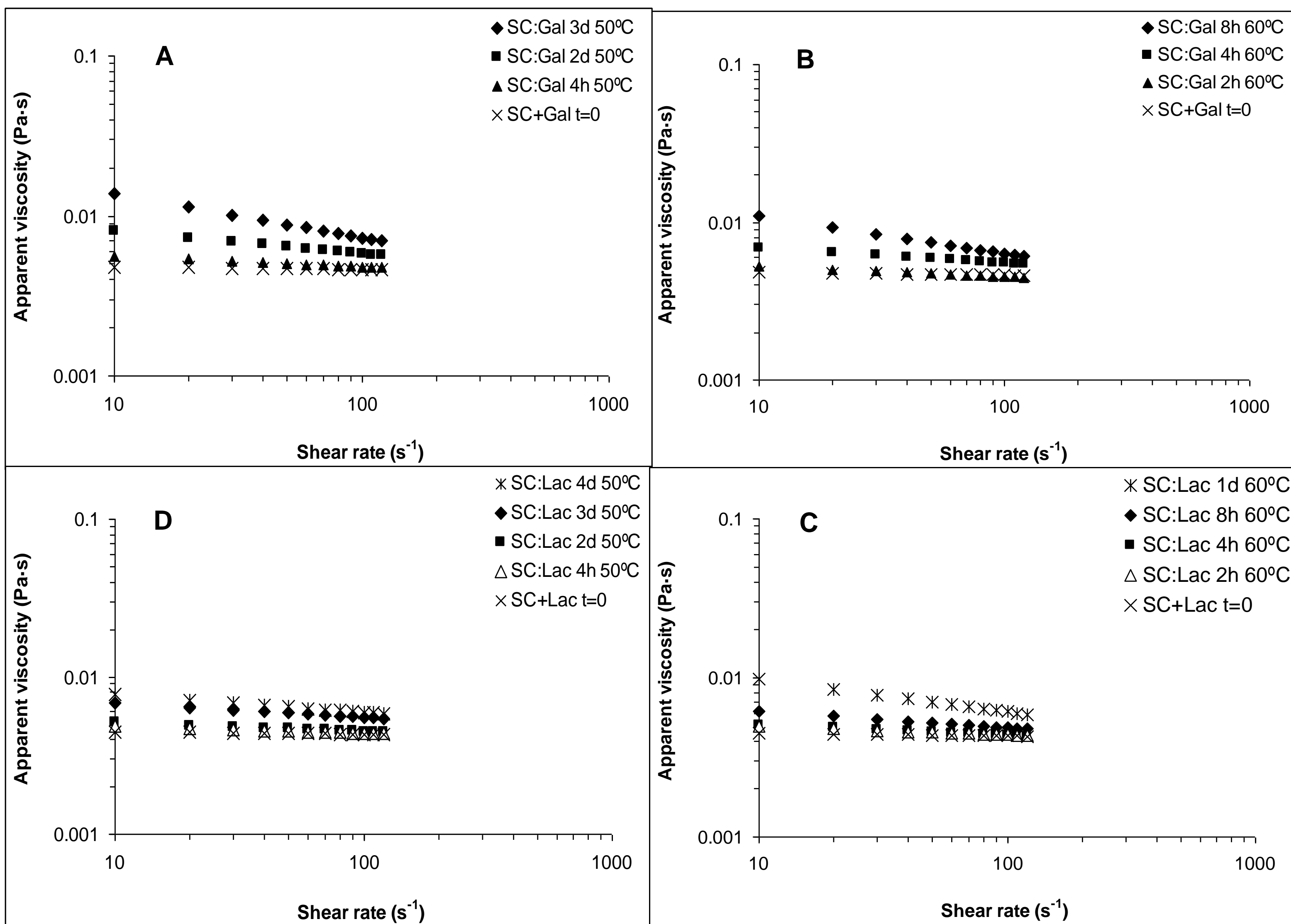


Figure 6

Figure 6. *Corzo-Martinez et al.*

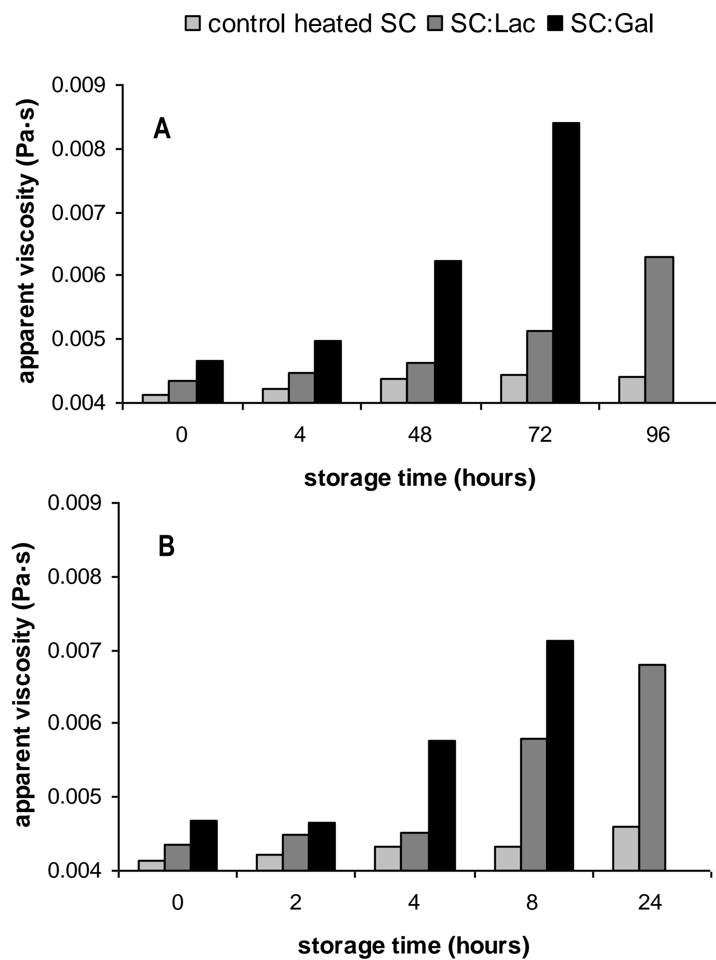
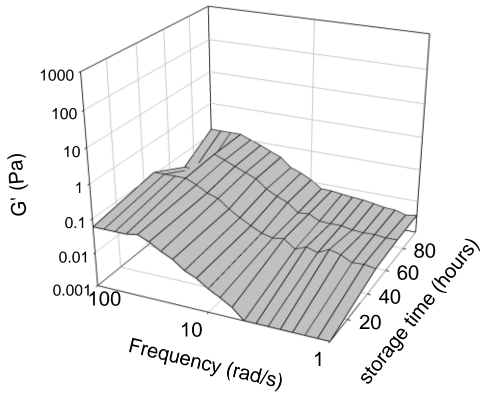


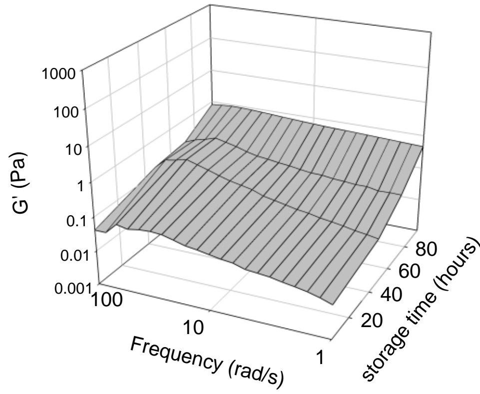
Figure 7

Figure 7. ***Corzo-Martinez et al.***

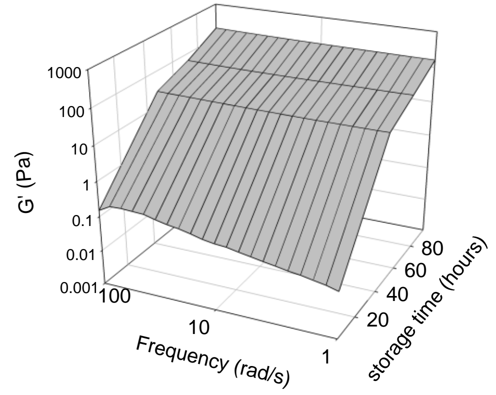
A



Control heated SC

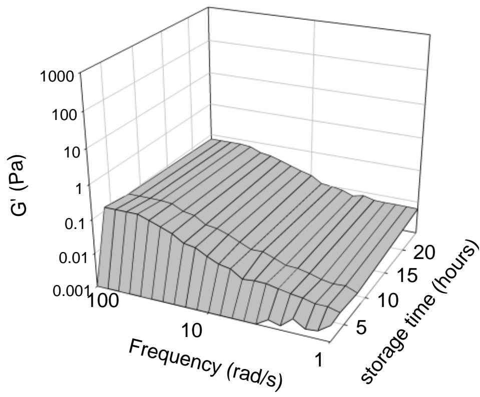


SC:Lac conjugates

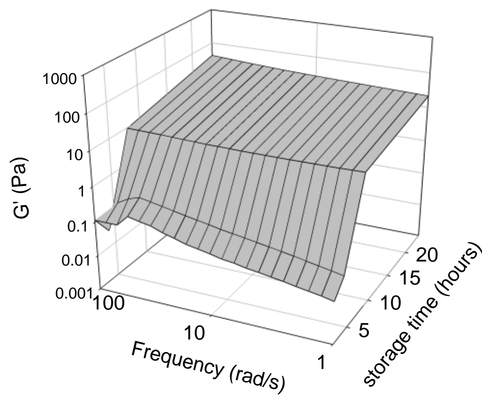


SC:Gal conjugates

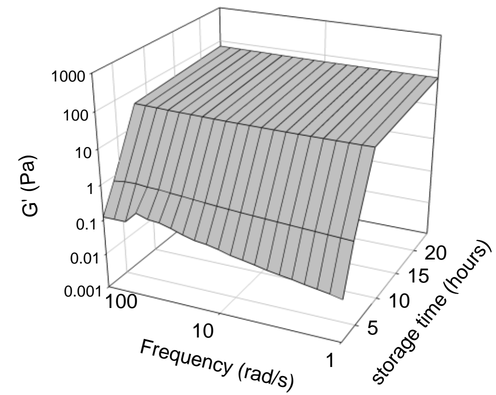
B



Control heated SC



SC:Lac conjugates



SC:Gal conjugates

Table 1

Table 1. Experimentally determined molecular masses of the different forms of κ-, α_{s1}- and β-casein and assigned number of galactose (Gal) and lactose (Lac) molecules bound to sodium caseinate (SC) derived from the LC-ESI mass spectrometry analyses.

Temperature	Sample	Storage time (hours)	Observed molecular masses of κ-casein A[a] (no. of linked sugar adducts)	Observed molecular masses of α_{s1}-casein B[b] (no. of linked sugar adducts)	Observed molecular masses of β-casein A2[c] (no. of linked sugar adducts)
50ºC	SC:Gal	4	19,038 (0 Gal) 19,200 (1 Gal) 19,362 (2 Gal)	23,619 (0 Gal) 23,781 (1 Gal) 23,943 (2 Gal) 24,105 (3 Gal) 24,267 (4 Gal)	23,987 (0 Gal) 24,149 (1 Gal) 24,311 (2 Gal)
		48	19,038 (0 Gal) 19,200 (1 Gal) 19,362 (2 Gal) 19,524 (3 Gal)	23,619 (0 Gal) 23,781 (1 Gal) 23,943 (2 Gal) 24,105 (3 Gal) 24,267 (4 Gal) 24,429 (5 Gal)	23,987 (0 Gal) 24,149 (1 Gal) 24,311 (2 Gal) 24,473 (3Gal) 24,635 (4 Gal)
	SC:Lac	4	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac)
		48	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac) 24,591 (3 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac)
		72	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac) 24,591 (3 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac)
		96	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac) 24,591 (3 Lac) 24,915 (4 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac) 24,959 (3 Lac)
60ºC	SC:Gal	2	19,038 (0 Gal) 19,200 (1 Gal) 19,362 (2 Gal)	23,619 (0 Gal) 23,781 (1 Gal) 23,943 (2 Gal) 24,105 (3 Gal) 24,267 (4 Gal) 24,429 (5 Gal)	23,987 (0 Gal) 24,149 (1 Gal) 24,311 (2 Gal) 24,473 (3Gal) 24,635 (4 Gal)
		4	19,038 (0 Gal) 19,200 (1 Gal) 19,362 (2 Gal) 19,524 (3 Gal)	23,619 (0 Gal) 23,781 (1 Gal) 23,943 (2 Gal) 24,105 (3 Gal) 24,267 (4 Gal) 24,429 (5 Gal) 24,591 (6 Gal)	23,987 (0 Gal) 24,149 (1 Gal) 24,311 (2 Gal) 24,473 (3Gal) 24,635 (4 Gal) 24,797 (5 Gal)

SC:Lac	2	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac)
	4	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac)
	8	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac) 24,591 (3 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac)
	24	19,038 (0 Lac) 19,362 (1 Lac)	23,619 (0 Lac) 23,943 (1 Lac) 24,267 (2 Lac) 24,591 (3 Lac) 24,915 (4 Lac)	23,987 (0 Lac) 24,311 (1 Lac) 24,635 (2 Lac) 24959 (3 Lac)

[a] Mono-phosphorylated.

[b] Octo-phosphorylated.

[c] Penta-phosphorylated.

Table 2

Table 2. High M_w and Low M_w fractions of native, control heated and glycated sodium caseinate (SC) with galactose (Gal), lactose (Lac) and dextran (DX) incubated at 50ºC for 96 hours and 60ºC for 24 hours, estimated after their analysis by size exclusion chromatography under nondenaturing conditions.

Temperature	Sample	Storage time (hours)	HM_w fraction (%)	LM_w fraction (%)	HM_w / LM_w ratio
50ºC	Native SC	0	23.99 ± 0.28	76.01± 0.28	0.31
	Heated SC	4	24.39 ± 0.41	75.61± 0.41	0.32
		48	30.71 ± 0.84	69.29 ± 0.84	0.44
		72	30.89 ± 0.33	69.11 ± 0.33	0.45
		96	32.17 ± 1.13	67.85 ± 1.13	0.47
	SC:Gal	4	25.02 ± 0.65	74.98 ± 0.65	0.33
		48	53.60 ± 0.85	46.40 ± 0.85	1.16
		72	76.49 ± 0.49	23.51 ± 0.49	3.25
		96	81.25 ± 0.71	18.75 ± 0.71	4.33
	SC:Lac	4	24.58 ± 1.56	75.42 ± 1.56	0.33
		48	33.35 ± 1.02	66.65 ± 1.02	0.50
		72	35.13 ± 0.36	64.87 ± 0.36	0.54
		96	37.22 ± 0.44	62.78 ± 0.44	0.59
	SC:DX	96	33.32 ± 0.23	66.68 ± 0.23	0.50
60ºC	Heated SC	2	23.82 ± 0.76	76.18 ± 0.76	0.31
		4	26.81 ± 0.88	73.19 ± 0.88	0.36
		8	27.05 ± 1.41	72.95 ± 1.41	0.37
		24	30.59 ± 0.94	69.41 ± 0.94	0.44
	SC:Gal	2	25.97 ± 0.76	74.03 ± 0.76	0.35
		4	33.13 ± 0.84	66.87 ± 0.84	0.50
		8	39.82 ± 0.54	60.18 ± 0.54	0.66
		24	78.40 ± 0.35	21.60 ± 0.35	3.63
	SC:Lac	2	24.44 ± 1.12	75.56 ± 1.12	0.32
		4	27.85 ± 0.89	72.15 ± 0.89	0.39
		8	36.52 ± 0.99	63.48 ± 0.99	0.57
		24	39.82 ± 1.70	60.18 ± 1.70	0.66
	SC:DX	24	33.90 ± 1.05	66.10 ± 1.05	0.51