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1 **Effect of ethanol on the stability of sodium caseinate stabilised emulsions**

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10 **Abstract**

11 Proteins are commonly used as emulsifying agent in food applications, although they are highly
12 affected by their environment, with the presence of ethanol being just one parameter known to
13 influence their properties. An increasing level of ethanol is expected to result in a reduced
14 emulsification efficiency of the protein and, therefore, emulsion processing in the absence of this
15 solvent should lead to more stable oil-in-water emulsions. Sodium caseinate was either dispersed in
16 the presence or absence of heat and/or ethanol and multiple physicochemical properties of the
17 resulting protein aggregates were determined. Further, oil-in-water emulsions were created in a
18 microfluidizer at varying levels of ethanol in the aqueous phase and two different points of its addition
19 in the processing route. The resulting emulsion properties were investigated. Stable oil-in-water
20 emulsions could be prepared at each applied ethanol level (≤ 50 wt.%) and for both examined
21 processing routes. The point of ethanol addition only had an impact if the concentration of this solvent
22 was higher than 25 wt.% - with a processing in absence of ethanol being beneficial. This concentration
23 was also found to be a threshold for the interactions between ethanol and dispersed sodium caseinate
24 as higher ethanol levels resulted in significantly changed protein properties.

25

26 **Keywords:** sodium caseinate, ethanol, oil-in-water emulsion, cream liqueur, microfluidizer

27 1. Introduction

28 Proteins are commonly known as a fundamental part of a healthy and balanced diet (Li-Chan & Lacroix,
29 2018; O'Regan, Ennis, & Mulvihill, 2009). Thanks to their versatile properties, they are also used for a
30 wide range of applications within multiple industry sectors, such as food and pharma (Dawson &
31 Acton, 2018; Gill, 2009). One important role of proteins is to adsorb at the interface of oil-in-water
32 emulsions and, therefore, act as an emulsifying agent (Foegeding & Davis, 2011). This functionality is
33 facilitated by their amphiphilic structure, which results from the existence of hydrophilic and
34 hydrophobic parts within their molecules (Beverung, Radke, & Blanch, 1999). Typically, proteins are
35 polymers with a molecular weight in a range from ten up to a few thousand kilodaltons (Fox, 2003;
36 Ludescher, 1996), which appear as voluminous structures. Therefore, their adsorption kinetics at the
37 interface are not as fast as for low molecular weight surfactants, such as lecithin,
38 polyoxyethylene (20) sorbitan monostearate and sodium dodecyl sulfate, affecting the process of
39 emulsion formation, if proteins are selected as the sole stabilising agents (Pugnaroni, Dickinson,
40 Ettelaie, Mackie, & Wilde, 2004). After protein adsorption at the interface, the hydrophobic parts align
41 towards the oil phase and, hence, the conformation of their entire structure changes (Walstra, 2003).
42 As a result of the attainment of this thermodynamically favourable state of the protein, the interfacial
43 tension between the two phases is reduced and the emulsion is stabilised (Bos & van Vliet, 2001; Lam
44 & Nickerson, 2013). The rearrangement of proteins at the interface leading to an effective stabilisation
45 of the interface can take up to several days after adsorption, depending on the characteristics of the
46 interface and the protein chosen (Murray & Dickinson, 1996). Proteins tend to form viscoelastic
47 interfacial films, which are known to be effective in preventing coalescence by improving the steric as
48 well as the electrostatic stability of emulsion droplets (Tcholakova, Denkov, Ivanov, & Campbell, 2006).
49 Although the whole process of interfacial stabilisation is relatively slow, the formation of the described
50 stabilising film at the interface is the reason why proteins may be selected over low molecular weight
51 surfactants (Wilde, Mackie, Husband, Gunning, & Morris, 2004). It is common to use both types of
52 emulsifiers, which results in molecular interactions as well as competitive adsorption. The extent of
53 these phenomena depends on the type and the concentration of the low molecular weight surfactant
54 (Ananthapadmanabhan, 1992). In general, proteins are highly affected by the physicochemical
55 properties of their environment, such as temperature, pH, ionic strength and the presence of non-
56 aqueous solvents (McClements, 2004b). Especially for industrial applications, it is of central interest
57 to understand the impact of these factors on the functional properties of the proteins. This
58 understanding is crucial for both, new product development as well as the optimisation of production
59 processes, by potentially reducing the energy and water consumption.

60 Sodium caseinate is a soluble mixture of different caseins and widely used as a functional ingredient
61 in processed foods, such as meat and baked products, confectionery and cream liqueurs. (O'Connell
62 & Flynn, 2006). When sodium caseinate is brought into aqueous solution, it forms spherical micellar
63 structures. The integrity and functionality of the micelles are preserved by hydrophobic interactions
64 and calcium phosphate linkages (Walstra, 1990), which are known to be highly sensitive to the
65 addition or removal of calcium ions (Dickinson & Golding, 1997; Horne & Parker, 1981c; O'Regan et
66 al., 2009).

67 The exposure of proteins, including sodium caseinate, to ethanol results in conformational changes
68 and protein precipitation (Clark & Smith, 1989; Mezdour, Brulé, & Korolczuk, 2006; O'Kennedy,
69 Cribbin, & Kelly, 2001). These phenomena are triggered by a lowering of the dielectric properties of
70 the aqueous phase (Agboola & Dalgleish, 1996; Horne & Parker, 1981a, 1981c), causing a change in
71 the surface charge of the protein (Ye & Harte, 2013). The resulting changes of the energy barrier
72 around the protein molecules enhance coagulation processes (Davies & White, 1958; Horne & Parker,
73 1981a; Medina-Torres, Calderas, Gallegos-Infante, González-Laredo, & Rocha-Guzmán, 2009).
74 Previous research has shown that coagulation stability of aqueously dispersed sodium caseinate
75 towards the addition of ethanol is influenced by the concentration of ionic calcium (Abbott & Savage,
76 1985; Kaustinen & Bradley, 1987) - with an increasing concentration of these ions leading to a decrease
77 in protein stability. Hence, it is recommended to add a sequestration agent, such as sodium citrate, to
78 ensure the long-term stability of systems containing ethanol and sodium caseinate (Banks, Muir, &
79 Wilson, 1981; Davies & White, 1958). A similar dependency was observed between the solution pH
80 and the coagulation stability of sodium caseinate, since the pH value affects the equilibrium between
81 ionised and non-ionised calcium in the system (Banks et al., 1981; Horne & Parker, 1981b). In the case
82 of sodium caseinate-stabilised emulsions, the pH should be kept above 6.4 to avoid extensive
83 flocculation of the oil droplets caused by the reduction in electrostatic repulsion as the electrostatic
84 charges are negated close to the isoelectric point of the protein (Dickinson, Narhan, & Stainsby, 1989a,
85 1989b).

86 This research is concerned with the effect of ethanol on the physicochemical and functional properties
87 of sodium caseinate, the main dairy protein ingredient industrially applied in the formulation of cream
88 liqueurs. The stability of (model) cream liqueurs as a consequence of formulation parameters such as
89 ethanol concentration (Banks & Muir, 1985; Donnelly, 1987), the use of low molecular weight
90 surfactants (Dickinson, Narhan, & Stainsby, 1989c), and other ingredients such as sucrose (Banks,
91 Muir, & Wilson, 1982) or different dairy proteins (Kaustinen & Bradley, 1987; Lynch & Mulvihill, 1997),
92 as well as variations in the manufacturing process (Abbott & Savage, 1985; Heffernan, Kelly, &
93 Mulvihill, 2009; Heffernan, Kelly, Mulvihill, Lambrich, & Schuchmann, 2011; Muir & Banks, 1986) has

94 been examined previously. Phenomena including creaming and neck-plug formation (Dickinson et al.,
95 1989b; Espinosa & Scanlon, 2013), as well as overall shelf life stability (Banks et al., 1981; Power, 1996)
96 have been discussed. However, to the best of the authors' knowledge, a single comprehensive study
97 on the impact of ethanol on the protein properties and functionality, alongside the resulting emulsion
98 properties considering the point of ethanol addition during processing has not previously been
99 published. The present study was designed to close this gap and to establish a deeper understanding
100 of the phenomena occurring between sodium caseinate and ethanol, resulting in the destabilisation
101 of sodium caseinate-stabilised oil-in-water emulsions. Ethanol levels up to 50 wt.% (approximately
102 58% alcohol by volume (ABV)) were considered, as higher levels were deemed not relevant to cream
103 liqueur formulations and processes. A sequestration agent was not needed since, by solely using
104 sodium caseinate as the protein source, calcium ions were not brought into the system. Sunflower oil
105 freed from naturally present surface-active components was used as the model cream liqueur oil
106 phase to ensure interfacial phenomena could be linked to the behaviour of the protein alone. Since
107 emulsion processing in conventional top-down equipment, such as the microfluidizer used in this
108 study, goes along with energy dissipation, the impact of elevating the temperature close to the
109 approximate end temperature of the processing step on the properties of the sodium caseinate
110 dispersions is also reported. Finally, ethanol was either added before or after the homogenisation
111 step.

112 **2. Experimental**

113 **2.1. Materials**

114 Sodium caseinate (CAS: 9005-46-3, lot no. BCBV4056) was purchased from Sigma-Aldrich Ltd
115 (Gillingham, United Kingdom). The calcium content was determined via complexometric titration
116 (Nielsen, 2010) and found to be 0.05 wt.%. Ethanol was obtained from Fisher Scientific (Analytical
117 grade, Loughborough, United Kingdom). Sunflower oil was acquired from a local supermarket and
118 treated with magnesium silicate (Florisil®) from Sigma-Aldrich Ltd (Gillingham, United Kingdom) to
119 remove naturally present surface-active components as described below. For the preparation of all
120 samples, milli-Q water from a reverse osmosis apparatus (Elix® Essential 5, Merck, Darmstadt,
121 Germany) was used. The sample pH was adjusted with sodium hydroxide (1 M) and hydrochloric acid
122 (1 M) solutions purchased from Sigma-Aldrich (Gillingham, United Kingdom).

123 **2.2. Solubility assays**

124 The solubility of sodium caseinate was assessed in two ways to predict the most favourable processing
125 route for model cream liqueurs in terms of point of ethanol addition. Firstly, the effect of the presence
126 of ethanol on solubility during protein dispersion was quantified. In a second set of experiments, the

127 ethanol induced precipitation of sodium caseinate, which was previously dispersed in water, was
128 assessed.

129 **2.2.1. Solubility in ethanol-water mixtures**

130 The solubility of sodium caseinate in ethanol-water mixtures was quantified at a level of 3% wt./wt.,
131 based on the weight of the final sample. (in the following denoted as wt.%). This concentration was
132 chosen as it is close to the one used in commercial cream liqueurs (O'Kennedy & Donnelly, 2003). The
133 appropriate amount of sodium caseinate was added to ethanol-water mixtures, containing between
134 0 and 50 wt.% alcohol, based on total sample. This was followed by stirring for 30 minutes at 400 rpm
135 on a magnetic stirrer. The mixtures were either prepared at 20 °C or heated up to and kept at 60 °C
136 throughout the mixing process. The heated samples, which were covered throughout the whole
137 process to avoid any loss of liquid due to evaporation, were removed from the heat source and
138 allowed to cool to 20 °C for 90 minutes before further use. The undispersed material of either set was
139 separated from the soluble fraction via centrifugation at 2000 g for 30 minutes at 20 °C (J2-21 floor
140 model centrifuge, Beckman, Indianapolis, USA) approximately 2.5 hours after the initial sample
141 creation. Ultimately, the supernatant was removed and stored at 5 °C, while the pellet was dried for
142 one day at 40 °C in a drying cabinet. The results of this solubility assay are reported as follows:

$$\text{solubility} = \frac{\text{dry weight of sample (g)} - \text{dry weight of pellet (g)}}{\text{dry weight of sample (g)}} \quad (1)$$

143 **2.2.2. Ethanol-induced precipitation**

144 Initially, aqueous dispersios containing 3 wt.% of sodium caseinate were prepared at 20 °C as
145 previously described (see 2.2.1) and mixed on a magnetic stirrer at 400 rpm. After 30 minutes, ethanol
146 was added to obtain dispersions with a final ethanol concentration between 0 and 50 wt.% and the
147 samples were stirred for 30 minutes at 400 rpm, either at 20 °C or at 60 °C. Accordingly, the final
148 concentration of sodium caseinate in the sample sets varied between 1.5 and 3 wt.%. Further sample
149 treatment, i.e., cool down, separation and drying followed the same protocol (and timeline) as stated
150 in the above solubility assay. The results are reported as soluble fraction, which was calculated in the
151 same way as solubility in Equation (1). The alternative terminology was chosen to account for the
152 different ways of sample preparation.

153 **2.3. Emulsion preparation**

154 The prepared oil-in-water emulsions contained 10 wt.% of sunflower oil, 87 wt.% of water or an
155 aqueous ethanol solution and 3 wt.% of sodium caseinate – each concentration was based on total
156 emulsion. Final emulsions contained up to 50 wt.% of ethanol (approximately 58% ABV). It should be

157 noted that the changing ethanol concentration in the samples resulted in different densities of the
158 continuous emulsion phases (see Table S9 in the Appendix). Consequently, the final oil volume fraction
159 changed between 0.100 (for 50 wt.% ethanol) and 0.109 (for 0 wt.% ethanol). The sunflower oil was
160 initially treated with 4 wt.% of magnesium silicate by stirring for 30 minutes at 600 rpm and 20 °C. The
161 magnesium silicate was then removed through centrifugation for 30 minutes at 8500 g and 20 °C (J2-
162 21 floor model centrifuge, Beckman, Indianapolis, USA), following a published protocol (Gould & Wolf,
163 2018). Before the oil was used as the dispersed emulsion phase, it was verified that the interfacial
164 tension at the oil/water interface did not change significantly over an observation period of one hour
165 (31.2 ± 0.7 mN/m at 20 °C), indicative of the absence of low molecular weight surface-active
166 molecules. The interfacial tension method is described further below.

167 As the first step of the preparation of the continuous phase, the appropriate amount of sodium
168 caseinate was dispersed in water by stirring at 400 rpm and 20 °C on a magnetic stirrer for 30 minutes.
169 These dispersions were not further purified for practical reasons, justified by the fact that the
170 impurities present in the protein batch were not surface-active, see results section 3.1.5. The further
171 procedure varied for the two sets of emulsions prepared in this study. For one set, the ethanol was
172 added before the oil, while for the second set the ethanol was added after emulsion processing.

173 **2.3.1. Emulsion processing in the presence of ethanol**

174 The first step to process the emulsions in the presence of ethanol was the addition of the required
175 amount of ethanol into the sodium caseinate dispersion. The ethanol was carefully added into the
176 vortex of the protein dispersion during the first 30 seconds of a ten minute mixing process on a
177 magnetic stirrer at 400 rpm and 20 °C. In the case of preparing a zero-ethanol emulsion, this mixing
178 step was omitted. Afterwards, the appropriate amount of oil was added, and a coarse emulsion
179 prepared by processing with a high shear overhead mixer (Silverson® L5M fitted with a fine emulsor
180 screen, East Longmeadow, USA) for three minutes at 5000 rpm and 20 °C. This coarse emulsion was
181 then immediately passed once through a microfluidizer at 1200 bar (Microfluidics M-110S, Newton,
182 USA). The emulsions were transferred into glass storage vials and kept at 5 °C to prevent microbial
183 destabilisation until required for analysis.

184 **2.3.2. Addition of ethanol after emulsion processing**

185 All emulsions of the second sample set were processed in the absence of ethanol, following the
186 procedure outlined above but omitting the step of ethanol addition. Immediately after processing, the
187 emulsions were transferred into a glass beaker and stirred on a magnetic stirrer at 400 rpm and 20 °C
188 for ten minutes while adding the appropriate amount of ethanol into the emulsion vortex within the
189 first 30 seconds of mixing. The emulsions were then transferred into glass storage vials and also kept

190 at 5 °C. The initial analysis of these samples was carried out immediately after conclusion of the above
191 described mixing step.

192 **2.4. Particle size**

193 The size distributions of sodium caseinate dispersions and emulsions were measured at 20 °C and pH 7
194 using static (Mastersizer MS 2000 fitted with a Hydro SM manual small volume sample dispersion unit,
195 Malvern Panalytical, Malvern, UK) and dynamic light scattering (Zetasizer Nano series, Malvern
196 Panalytical, Malvern, UK) equipment. The analysis of the protein dispersions took place within three
197 hours after centrifugation, whereas the emulsion droplets were investigated at various time points.
198 Prior to measurement, all samples were diluted with their respective solvent mixture of ethanol and
199 water and their pH was adjusted with 1 M hydrochloric acid or sodium hydroxide as appropriate.
200 Refractive index values at 20 °C, required for data analysis, were selected in the instrument's software
201 as 1.33 for water, 1.47 for sunflower oil and 1.57 for sodium caseinate (Griffin & Griffin, 1985). The
202 refractive indices for the ethanol-water mixtures were determined with a refractometer (J 357 series,
203 Rudolph Research Analytical, USA), at 20 °C, and used accordingly. The absorption was set to zero, for
204 sodium caseinate as well as for emulsion samples. The droplet size of emulsions was primarily
205 determined as the intensity-weighted mean diameter, Z-average, obtained by dynamic light
206 scattering. If these results showed any sign of inadequateness due to droplets measured larger than
207 3 µm, the volume-weighted mean diameter, $d_{4,3}$, obtained by static light scattering was utilised. For
208 multimodal intensity-based size distributions the average size of the highest peak is reported.
209 Graphical representations of the size distributions are included in the electronic supplementary
210 material, Figures S1 to S3 and S9 to S11. As part of the analytical output of the dynamic light scattering
211 measurements, further information about the width of the respective distributions – the
212 polydispersity index (PDI_{DLS}) - was obtained. For the static light scattering experiments an equivalent
213 parameter was calculated as follows

$$PDI_{SLS} = \left(\frac{\text{standard deviation of the mean diameter } (\mu\text{m})}{\text{mean diameter } (\mu\text{m})} \right)^2 \quad (2)$$

214 **2.5. Zeta potential**

215 The zeta (ζ -)potential of the samples was determined via the method of electrophoretic light
216 scattering (Zetasizer Nano series, Malvern Panalytical, Malvern, UK), using the same measurement
217 conditions and sample preparation as for size. Furthermore, the respective time points of the
218 measurements were similar to the one described in the section above. The additionally required

219 dielectric constant and viscosity values for the data analysis were taken from literature (Åkerlöf, 1932;
220 Jouyban, Soltanpour, & Chan, 2004; Lide, 2006).

221 **2.6. Surface and interfacial tension**

222 The surface tension of the various aqueous protein dispersions (after centrifugation), as well as the
223 interfacial tension between the aqueous protein dispersions (without removal of insoluble material)
224 and the treated sunflower oil as used for the emulsion preparation, were determined with a profile
225 analysis tensiometer (PAT-1M, Sinterface Technologies, Berlin, Germany), at 20 °C. The equipment
226 was fitted with a straight stainless-steel capillary (3mm outer diameter) to create a pendant drop (27
227 mm² cross-sectional area) of the aqueous phase in either an empty, or oil-containing quartz cuvette.
228 Dynamic surface/interfacial tension data were recorded until a steady state was reached. It was
229 assumed that equilibrium was obtained when the standard deviation of the average of twenty
230 consecutive data points was smaller than 0.01 mN/m, corresponding to the resolution of the
231 equipment. The data point density for the calculation of the standard deviation was four points per
232 minutes, ergo one data point was recorded every 15 seconds. Liquid phase density values, required
233 for the data analysis by the tensiometer software, were determined using a force tensiometer (K100,
234 Krüss GmbH, Hamburg, Germany) and a solid measuring probe (2.33 g/cm³ density), at 20 °C.

235 **2.7. Statistical analysis**

236 All measurements were performed at least in triplicate. The plotted data represent the average
237 plus/minus one standard deviation, given as error bars. An analysis of variances (ANOVA) was carried
238 out to analyse the statistical significance between average values of different set of samples. The
239 chosen level of significance was $p = 0.05$. The results of ANOVA are presented in Tables S1 to S24
240 together with average and standard deviation of all data presented in figures.

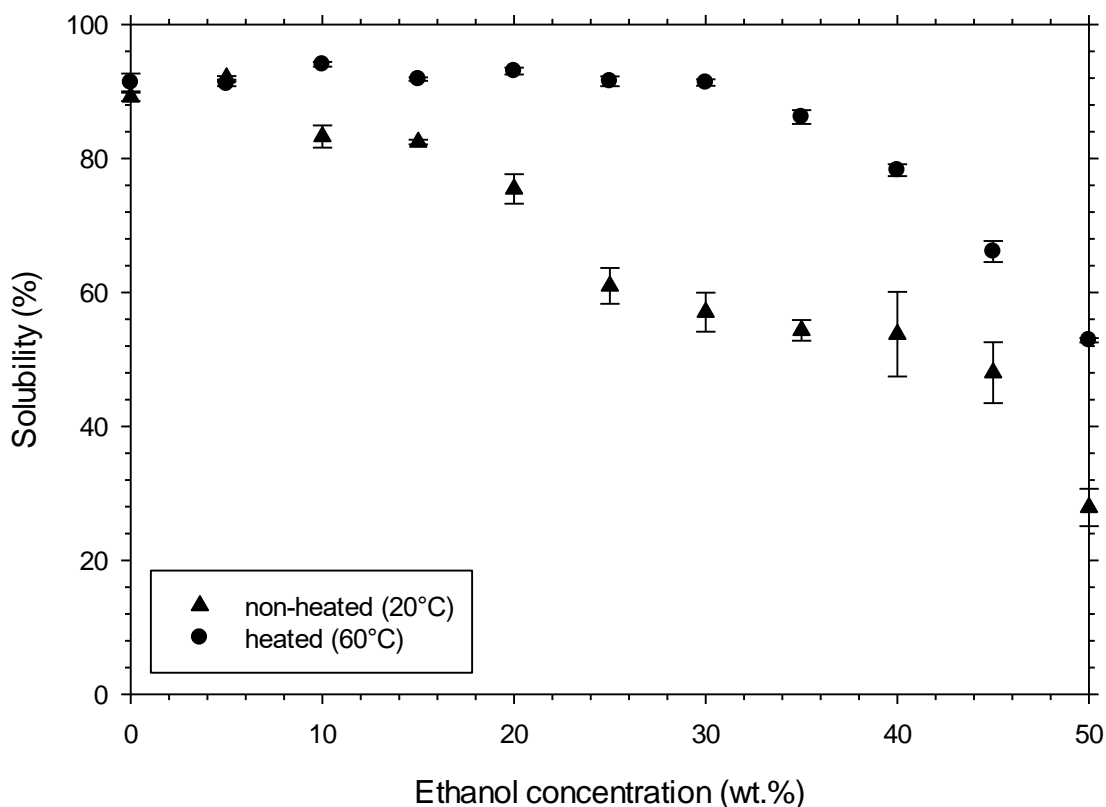
241

242 **3. Results and discussion**

243 **3.1. Material properties of sodium caseinate in ethanol-water mixtures**

244 **3.1.1. Solubility in ethanol-water mixtures**

245 The solubility of sodium caseinate in ethanol-water mixtures, containing up to 50 wt.% ethanol and
246 prepared in the presence (60 °C) or absence of heat, is presented in Figure 1. Heating the mixture
247 during sample preparation clearly increased solubility as the resulting values are higher for every
248 investigated ethanol concentration. Nevertheless, full dispersion was not achieved, indicating the
249 presence of insoluble material – most likely protein which was heat abused during the manufacturing
250 process of the sodium caseinate powder. For the heated samples, the solubility was between 90 and
251 95% for ethanol levels up to 30 wt.%. Above this, solubility decreased with further increasing
252 proportion of ethanol in the solvent, until it reached a value of around 53% for 50 wt.% ethanol. A
253 change in solubility of caseins at a “critical” ethanol concentration of approximately 30 wt.% in heated
254 systems has been reported in literature before (Dickinson, 2019; O'Connell, Kelly, Auty, Fox, & de Kruif,
255 2001). In the case of the non-heated samples, maximum solubility was achieved in the presence of 5
256 wt.% ethanol, being approximately 3% higher compared to the value for water as the sole solvent.
257 This initial increase in solubility indicates the presence of ethanol-soluble impurities, possibly in the
258 form of lipid contaminants (milk fats), within the sodium caseinate batch alongside the heat abused
259 protein aforementioned, as for levels of ethanol above 5 wt.%, solubility continuously decreased until
260 it reached a value of around 28% at 50 wt.% of ethanol, which is approximately half of the
261 corresponding value found for the heated sample. This overall trend has previously been reported
262 (Mezdour, Boyaval, & Korolczuk, 2008; Mezdour et al., 2006) and can be explained by the associated
263 decrease of the dielectric constant (Åkerlöf, 1932) of the solvent phase and, thus, the solvent quality
264 for protein. The structural conformation of the protein surface changes for varying ethanol
265 concentration, resulting in precipitation and the recording of decreased solubility values. The
266 combination of ethanol presence and heating, on the other hand, leads to an increased solubility due
267 to a change of the micellar structure, which results in dissociation of the casein micelles (O'Connell,
268 Kelly, Fox, & de Kruif, 2001), and a change of the hydrophobicity of the protein (Trejo & Harte, 2010).



269

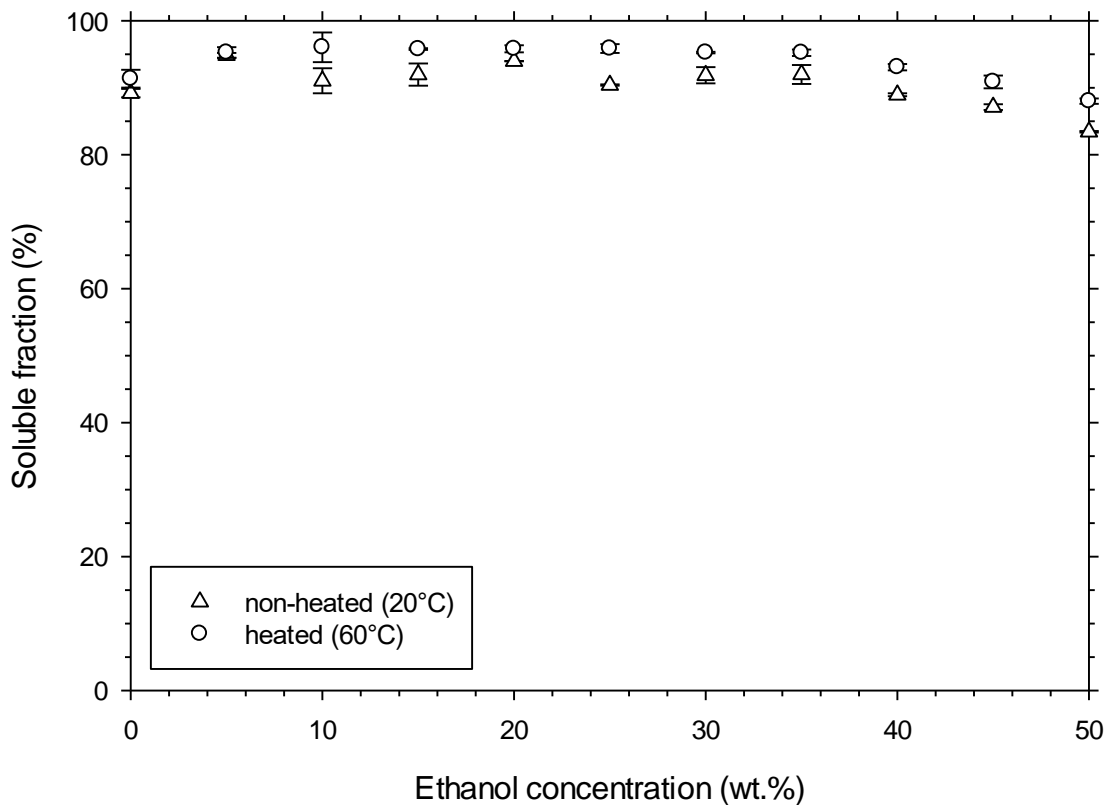
270 Figure 1: Solubility of sodium caseinate in aqueous dispersions with differing ethanol concentration. Samples were prepared
 271 either at 20 °C or 60 °C. The data correspond to the average of four measurements with error bars representing one standard
 272 deviation.

273

274 3.1.2. Soluble fraction following precipitation with ethanol

275 The soluble fraction of sodium caseinate dispersed in water, either at 20 °C or 60 °C, and then exposed
 276 to ethanol up to a level of 50 wt.% in the mixture, is reported in Figure 2. The behaviour was different
 277 compared to the dispersion in the presence of ethanol (see Figure 1) in that the soluble fraction hardly
 278 depended on the sample pre-treatment. It was found that the difference between heated and non-
 279 heated samples was less than 5% for the majority of the investigated ethanol concentrations. Also,
 280 the exposure of the samples to ethanol after dispersion in water rather than dispersion of the protein
 281 in ethanol-water mixtures led to higher values of solubility (soluble fraction), with the minimum values
 282 being 83% and 88% for non-heated and heated samples, respectively. Furthermore, the decrease in
 283 soluble fraction at ethanol levels above 30 wt.% was much less pronounced, although it was
 284 statistically significant ($p < 0.05$). Apparently, the main rearrangements of the protein took place
 285 during the dispersion in water, before ethanol was added. Therefore, the incorporation of the alcohol
 286 affected the protein solubility less than for protein dispersion in aqueous ethanol solutions. The
 287 conformational changes due to ethanol addition only had a significant impact on the protein solubility

288 for alcohol concentrations above 35 wt.% in the system. The reduction in solvent quality for the
289 protein at this alcohol level was high enough to result in an increasing extent of aggregation and,
290 eventually, of protein precipitation. *Dickinson et al. (1989c)* reported similar findings, as they observed
291 a greater rate of protein aggregation and precipitation for ethanol levels above 30 wt.%.



292

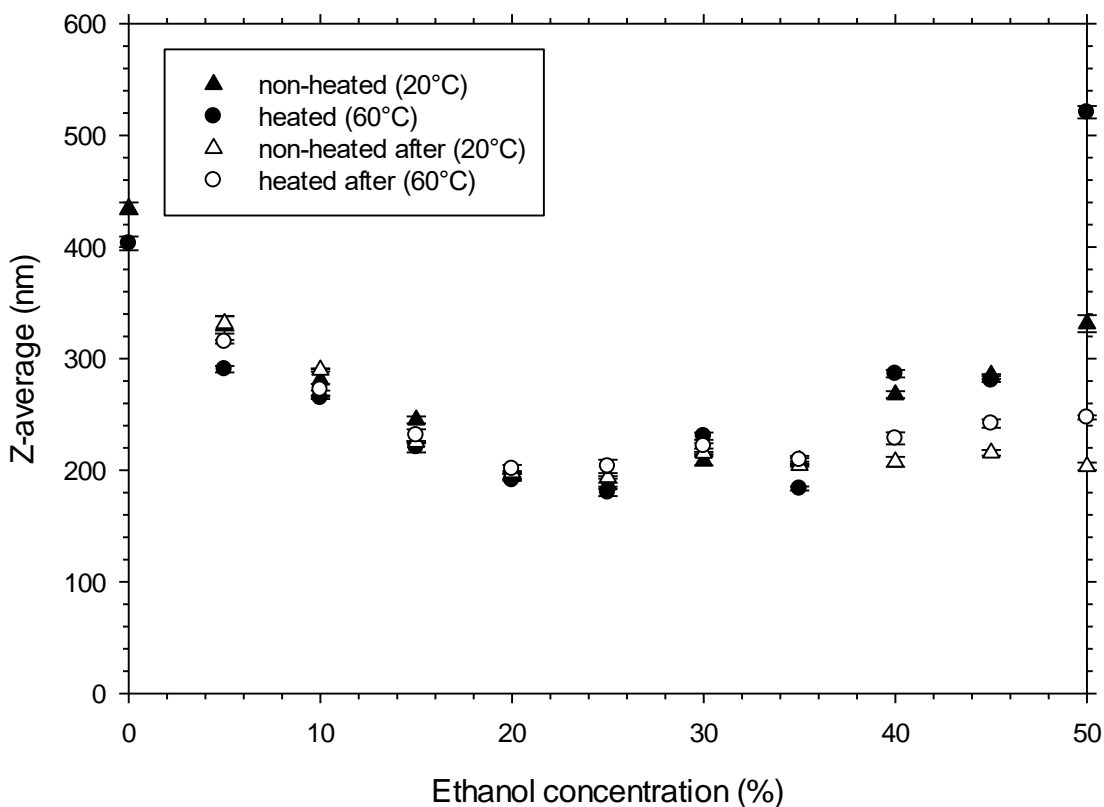
293 Figure 2: Soluble fraction of sodium caseinate in aqueous dispersions with differing ethanol concentration. Samples were
294 prepared either at 20 °C or 60 °C and ethanol was incorporated after dispersion in water. The data correspond to the average
295 of four measurements with the error bars representing one standard deviation.

296

297 3.1.3. Aggregate size

298 Examples for the size distributions acquired on the sodium caseinate dispersions are presented in
299 Figures S1 to S3, showing multimodal behaviour, which indicates aggregated systems. Previously
300 published work on the size of sodium caseinate dispersions also reported multiple populations
301 (Roullet, Clegg, & Frith, 2019; Srinivasan et al., 1996). Regardless of the existence of multiple peaks in
302 our data, the z-average of the distributions obtained is reported in Figure 3 for comparison to
303 published literature. All four sample sets showed an initial decrease in the z-average until a value of
304 about 190 nm was reached at 25 wt.% of ethanol in the mixture. A similar observation was reported
305 by *Horne (1984)* and explained with conformational changes of the protein molecule which

306 consequently also affect the structure of the micelles. Interestingly, the data of the present study
 307 suggest that the sample history (heat treatment and point of ethanol addition) had little influence on
 308 the z-average in this domain. At higher levels of ethanol (between 25 and 40 wt.%) in the final sample,
 309 the z-average initially remained approximately constant and then increased for ≥ 40 wt.% of ethanol.
 310 The increase in z-average is a sign of protein coagulation (Horne, 1984), promoted by the application
 311 of heat during protein dispersion. However, the impact of heat did not seem to make a difference at
 312 45 wt.% of ethanol for protein dispersion in the presence of ethanol, an observation which cannot be
 313 explained based on the set of analyses carried out in the study presented here. Still, for the same
 314 system at 50 wt.% of ethanol, the z-average of the heated sample was larger by a factor of 1.9 than
 315 the z-average of the unheated sample (and also the highest of all samples analysed) despite being
 316 more soluble at these conditions (see Figure 1). It appears that aggregate size is thermodynamically
 317 driven rather than by the processes determining protein solubility.



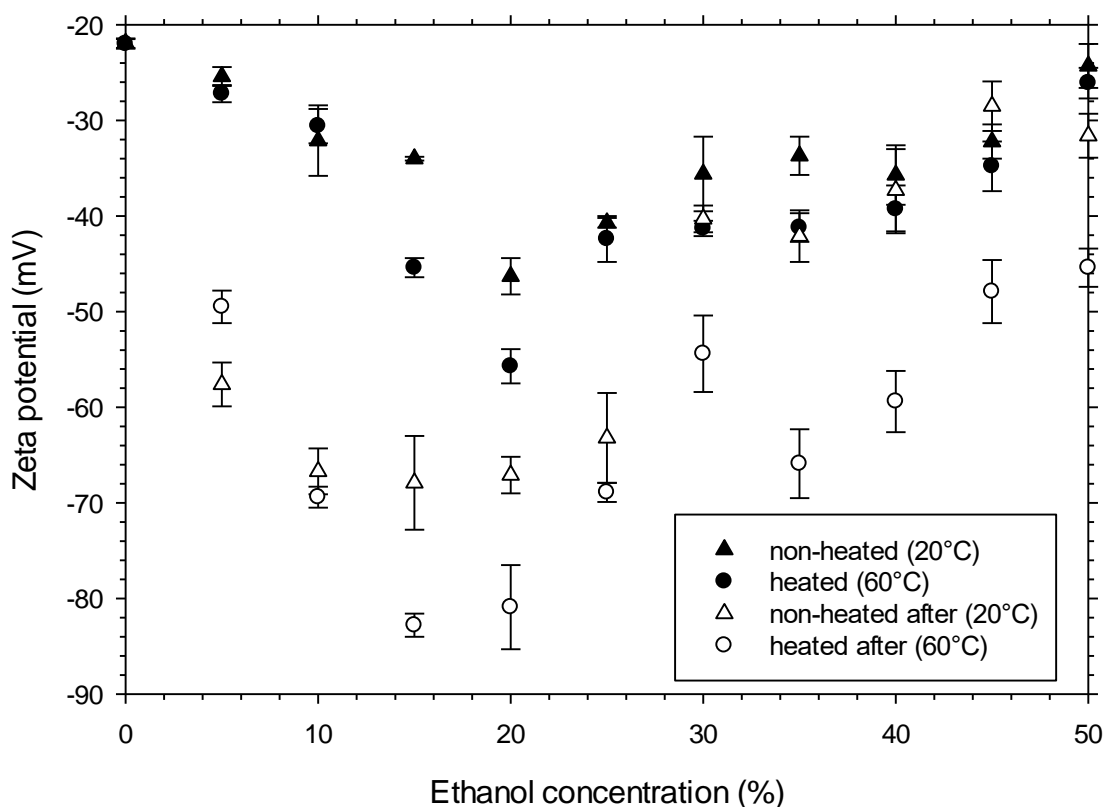
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319 Figure 3: Z-average of protein aggregates in the soluble fraction of samples versus ethanol concentration, prepared either at
 320 20 °C or at 60 °C with ethanol added before (filled markers) and after (hollow markers) the protein dispersion.

321

322 **3.1.4. Zeta potential**

323 Figure 4 shows the ζ -potential data acquired on the soluble fraction of sodium caseinate dispersions
324 prepared in this study. For ethanol concentrations of up to 20 wt.%, the ζ -potential decreased with
325 increasing alcohol level. Above this concentration, this trend was reversed. The general behaviour
326 mirrors the behaviour of the z-average (see Figure 3). A higher absolute ζ -potential means increased
327 electrostatic repulsion between the components of the systems, suppressing aggregation. Vice versa,
328 with decreasing absolute ζ -potential, aggregation is more likely to occur, especially at absolute values
329 below 30 mV (Gumustas, Sengel-Turk, Gumustas, Ozkan, & Uslu, 2017; Khosa, Reddi, & Saha, 2018),
330 at least for a system which is not sterically stabilised to an adequate extent. Since it was already not
331 possible to link the size data (see Figure 3) to the solubility (Figure 1) or soluble fraction (Figure 2)
332 data, the same applies to the ζ -potential data. The minimum in ζ -potential was more distinct when
333 the sodium caseinate was dispersed in water as opposed to the dispersion in an aqueous ethanol
334 solution. When sodium caseinate was dispersed in the presence of ethanol, heat treatment during
335 sample preparation had little impact on the ζ -potential of the final dispersion, with error bars
336 overlapping except for 15 and 20 wt.% of ethanol. While other data points were identified as
337 statistically significantly different ($p < 0.05$; see Tables S5 and S6), the relatively large error bars for ζ -
338 potential data call for caution in data interpretation. When ethanol was added after protein dispersion
339 in water, the ζ -potential of the final dispersion was clearly affected by the temperature history of the
340 sample. Heating of the samples led to higher absolute values of the ζ -potential.



341

342 Figure 4: ζ -potential of protein aggregates in the soluble fraction of samples versus ethanol concentration, prepared either
 343 at 20 °C or at 60 °C with ethanol added before (filled markers) and after (hollow markers) the protein dispersion.

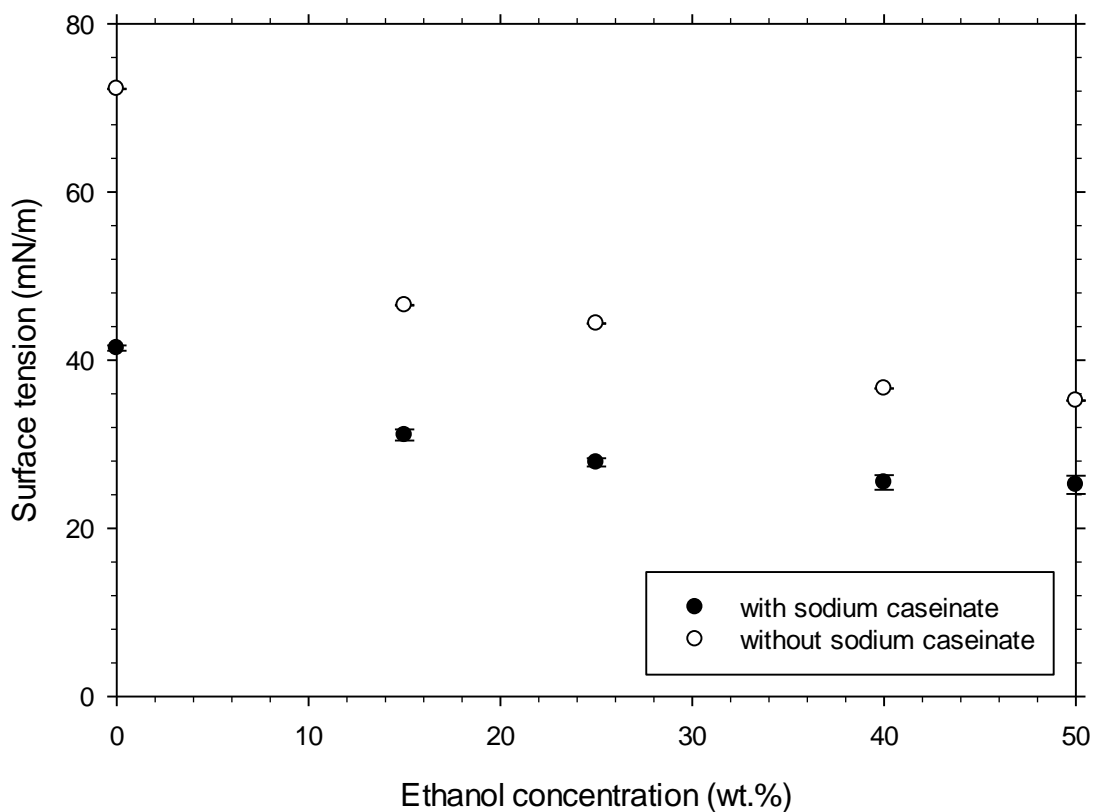
344

345 3.1.5. Surface tension

346 Surface tension data were acquired for sodium caseinate samples prepared by each of the four
 347 preparation routes (two different points of ethanol addition, two temperature histories). Steady state
 348 surface tension values showed very little impact of the sample history, as verified by the small error
 349 bars in Figure 5 reporting the surface tension data averaged over all four preparation methods. The
 350 time-dependent data are reported in the supplementary material (Figures S4 to S8 and Table S8). Only
 351 at 40 and 50 wt.% of ethanol, the data showed some statistically significant differences ($p < 0.05$, see
 352 Table S8). As reference, Figure 5 includes data obtained for pure ethanol-water mixtures (hollow
 353 markers) which are in good agreement with literature (Khattab, Bandarkar, Fakhree, & Jouyban, 2012).
 354 Overall, the steady state surface tension of the protein dispersions decreased with increasing amount
 355 of alcohol, as previously reported in literature (Abascal & Gracia-Fadrique, 2009; Abbott & Savage,
 356 1985; Mulvihill & Murphy, 1991). Heated samples required more time to reach steady state compared
 357 to non-heated samples (see Figures S1 to S5), indicative of structural changes and rearrangements of
 358 the proteins due to heating that are not reflected in the result of the size measurement (Figure 3). For

359 the protein containing systems, the surface tension only changed slightly above an ethanol
360 concentration of 40 wt.%, leading to a marginally reduced value for 50 wt.%, which is in contrast to
361 the findings for pure ethanol-water mixtures at these alcohol levels. However, this observation is in
362 line with the solubility data (see 3.1.1 and 3.1.2), which indicate that above an ethanol level of 35
363 wt.%, the effective amount of protein present in the system decreases continuously. The described
364 phenomenon and the surface tension-reducing effect of ethanol appear to compensate each other,
365 resulting in similar values for both systems. The surface tension data presented in Figure 5 were not
366 statistically significantly different to equivalent data obtained for samples in which the insoluble
367 material was not removed (data not shown), indicating that the impurities within the sodium caseinate
368 batch were not surface-active.

369



370

371 Figure 5: Surface tension of different water-ethanol mixtures with and without (as reference) 3 wt.% sodium caseinate at a
372 steady state, data were acquired at 20 °C.

373

374

375 **3.2. Emulsion stabilisation**

376 The material properties of sodium caseinate dispersed in aqueous ethanol solution, and as influenced
377 by the point of ethanol addition and heating during sample preparation, were investigated in order to
378 advance the understanding of the stability and processing of model cream liqueurs. These were
379 formulated as sunflower oil-in-water emulsions. Naturally present surface-active components of the
380 sunflower oil were removed to be able to interpret interfacial processes in these systems purely based
381 on the contributions by ethanol and sodium caseinate. Our primary definition of stable emulsions
382 relates to stability against droplet coalescence, although observations of creaming and sedimentation
383 phenomena are also reported. Based on the findings on the protein dispersions, it was hypothesised
384 that processing in the absence of ethanol will result in more stable emulsions, due to higher solubility
385 expressed as soluble fraction (see Figure 2 cf. Figure 1) and higher absolute ζ -potential (see Figure 4).
386 It was further hypothesised that at an ethanol concentration above 25 wt.%, a change in the
387 emulsification properties of the applied protein will be observed, due to a significant decrease in
388 sodium caseinate solubility. Testing this hypothesis, also for emulsions processed in the presence of
389 ethanol, sodium caseinate was always initially dispersed in water alone, and no heat supplied. The
390 insoluble fraction of the sodium caseinate was not removed as it was previously established that those
391 impurities were not surface-active (see section 3.1.5). All final emulsion samples contained 10 wt.%
392 oil, 3 wt.% sodium caseinate and between 0 and 50 wt.% of ethanol. The oil fraction during processing
393 was higher when the ethanol fraction of the final emulsion was added after emulsion processing.
394 Depending on the amount of ethanol added, it varied between 11.8 and 20 wt.% for 15 and 50 wt.%
395 of ethanol in the final sample.

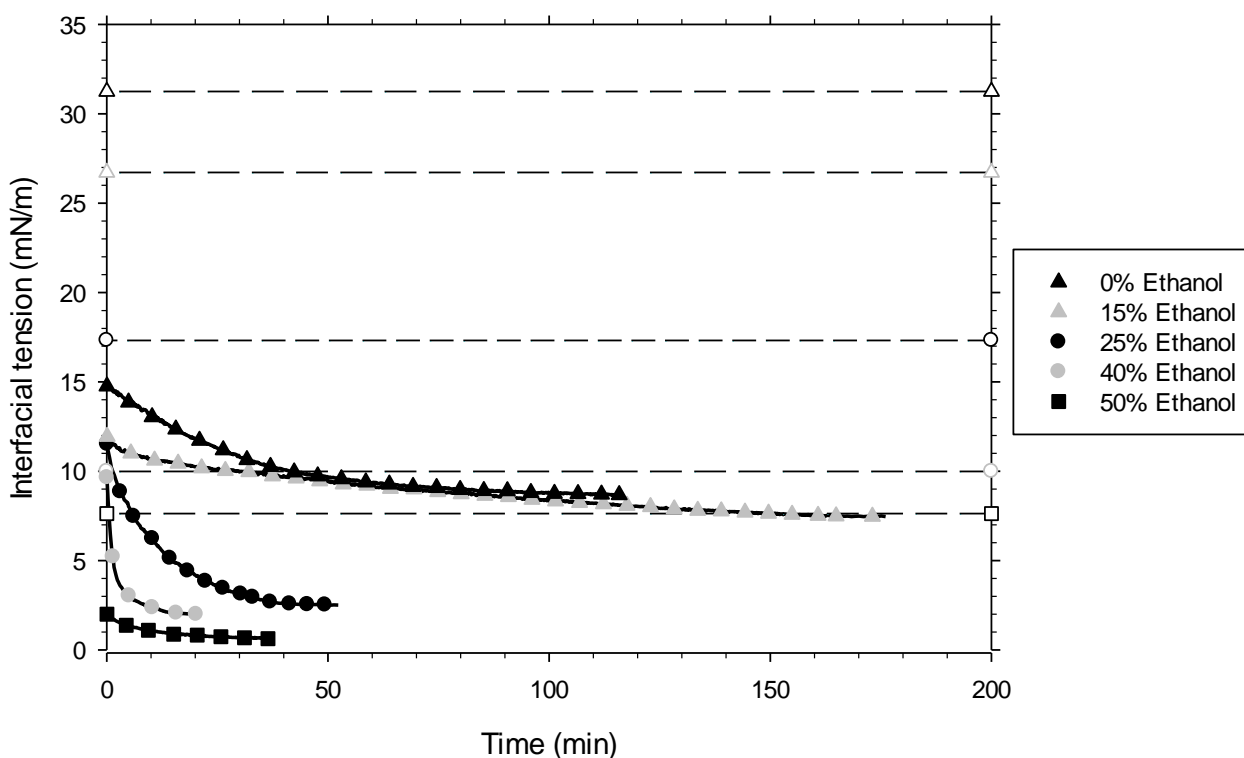
396

397 **3.2.1. Interfacial tension**

398 Figure 6 shows the kinetic data for the interfacial tension between ethanol-water mixtures containing
399 3 wt.% sodium caseinate and treated sunflower oil (filled markers), i.e., naturally present surface-
400 active components were removed. Both liquid phases applied to interfacial tension analysis
401 corresponded to the emulsion phases introduced below. Reference interfacial tension values acquired
402 in the absence of added protein did not show significant changes over time and, hence, are presented
403 as dashed lines between hollow markers.

404 Overall, an increasing ethanol content in the sodium caseinate dispersion led to a decreasing
405 interfacial tension as ethanol itself is surface-active and, additionally, reduces the dielectric constant
406 of the solvent which further enhances the surface activity of the protein. This trend was noticeable
407 for the initial interfacial tension as well as for the steady state values. These tendencies are in

408 accordance with the data reported above for surface tension (see Figure 5) and with findings by
409 *Dickinson & Woskett (1988)*. However, the differences in interfacial tension at concentrations above
410 25 wt.% are larger than for the surface tension, which is most likely caused by differences in the
411 properties of the hydrophobic phase, i.e., oil compared to air. The nature of the hydrophobic phase is
412 of importance as ethanol is causing rearrangements of the protein, resulting in an increased surface
413 hydrophobicity of the sodium caseinate (*Dickinson, 1992; Srinivasan, Singh, & Munro, 1996*). The
414 equilibrium time varied for the analysed ethanol concentrations, with the maximum found for 15 wt.%
415 ethanol in the aqueous phase (approximately three hours). For higher alcohol concentrations, this
416 time did not exceed one hour, with the systems containing 40 wt.% ethanol reaching the steady state
417 fastest. Since the preparation of the aqueous phase took place at least one hour before the
418 measurements were conducted, it was assumed that the phenomena observed during the interfacial
419 tension measurements were exclusively caused by the creation of the interface. This assumption was
420 supported by the existence of only minor differences between the repetitions of the measurement,
421 regardless of the further aging of the samples for up to three hours before the repeat measurements
422 were conducted. As the results for the solubility of sodium caseinate indicated (see 3.1.1 and 3.1.2),
423 an increasing ethanol level in the aqueous phase decreases the solvent quality for the protein. Hence,
424 a protein placement at the interface is energetically favourable over one in the bulk solution,
425 consequently, a steady state is reached within a shorter time frame for higher ethanol fractions in the
426 aqueous phase. Previously published studies reported the existence of casein micelles and smaller
427 protein entities in sodium caseinate dispersions (*Roullet et al., 2019; Srinivasan et al., 1996*) and found
428 that the fraction of each form of appearance was dependent on a range of parameters. Assuming that
429 the presence of ethanol is one of such parameters, a change in the ratio between micelles and smaller
430 protein entities would serve as an explanation for the faster adsorption of sodium caseinate at the oil-
431 water interface for higher ethanol concentrations, in combination with the rearrangements of the
432 protein aforementioned (*Walstra & van Vliet, 2003*).



433

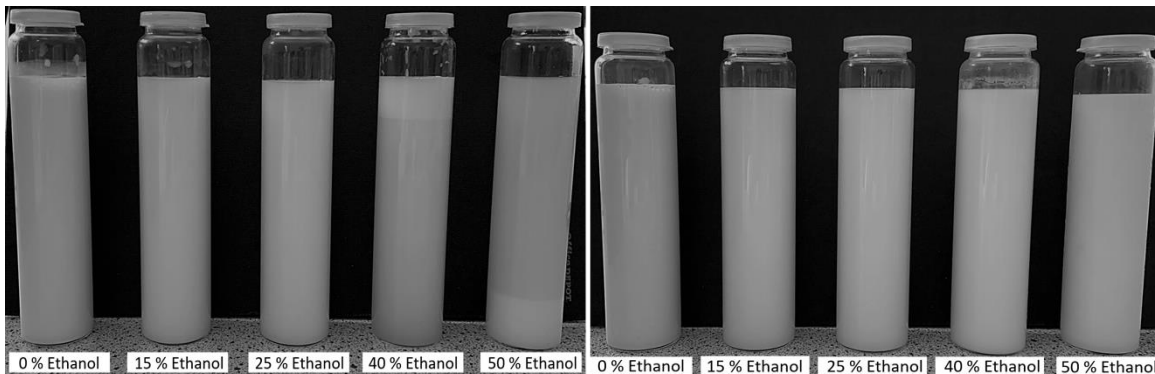
434 Figure 6: Dynamic interfacial tension between treated sunflower oil and ethanol-water mixtures containing 3 wt.% sodium
 435 caseinate (solid lines and filled markers) versus time until the respective steady state was reached; the dashed lines between
 436 the hollow markers represent the interfacial tension values between treated sunflower oil and ethanol-water mixtures (at
 437 the same ethanol concentration as the protein containing systems represented by the same marker). The data correspond
 438 to the average of three measurements and were acquired at 20 °C; due to existence of only minor differences between the
 439 repetitions of the measurement, no standard deviations are provided.

440

441 3.2.2. Visual stability

442 The visual appearance of all samples one day after processing is shown in Figure 7. For each system,
 443 the visual appearance did not change any further over prolonged storage of several months. When
 444 the samples were processed in the presence of ethanol (see Figure 7a), clear signs of gravitational
 445 instability were seen at 40 and 50 wt.% ethanol. Before discussing this finding in more detail, it is
 446 worth mentioning that the addition of ethanol after processing led to visually stable emulsions (see
 447 Figure 7b). While creaming of around 1 vol.% of the overall emulsion is considered as non-problematic
 448 and is in some cases actually desired (Dickinson et al., 1989c), the cream layer observed at 40 wt.%
 449 ethanol was well above this limit with about 17 vol.% (see Figure 7a, height of the cream layer was
 450 measured). The emulsion processed in the presence of 50 wt.% ethanol developed a sediment layer,
 451 rather than a cream layer, which was about 14 vol.% of the overall system. It appears that the density
 452 difference between dispersed and continuous emulsion phase changed sign. Data obtained for the
 453 density difference between the two phases showed a decrease of this difference with an increasing

454 ethanol content, but not the predicted change of sign (see Table S9). Therefore, it was concluded that
455 the adsorption of sodium caseinate at the surface of the oil droplets weighed the droplets down to
456 cause the oil phase to sediment rather than cream (see Figure 7).



457 Figure 7: Emulsions containing different concentrations of ethanol - one day after preparation (no further change over nine
458 months of storage); incorporation of ethanol took place a) before and b) after the homogenisation.

459

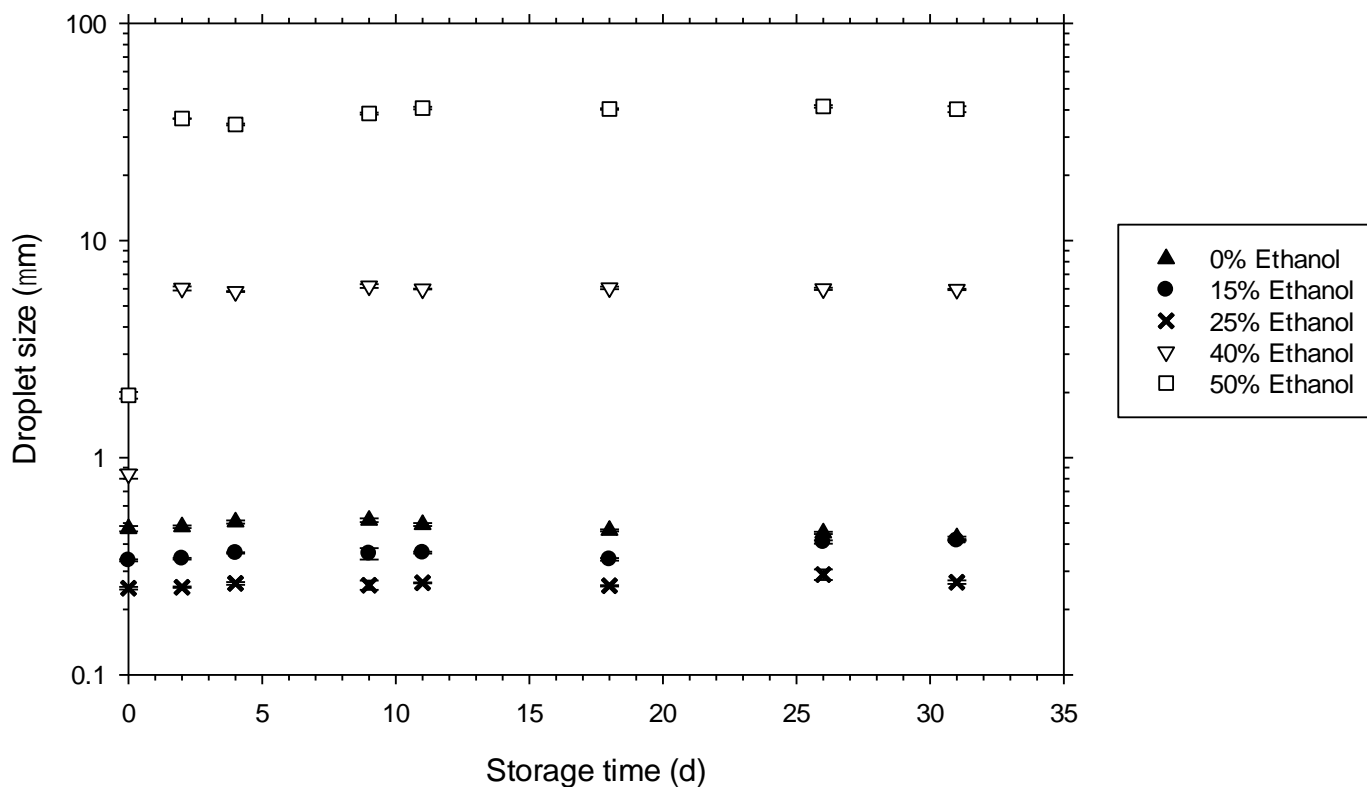
460 3.2.3. Droplet size

461 Figure 8 shows the mean droplet size data, measured either as z-average via dynamic light scattering
462 or as $d_{4,3}$ via static light scattering, acquired on emulsions processed in the presence of ethanol – the
463 ethanol was added roughly fifteen minutes prior to passing through the microfluidizer. The data are
464 shown as a function of sample age (up to one month). Corresponding droplet size data for adding the
465 ethanol after emulsion processing are reported in Figure 9. Where droplets were too large for the
466 dynamic light scattering method, static light scattering was used and $d_{4,3}$ values are reported. Z-
467 average and $d_{4,3}$ values were within 15% of each other for emulsions with droplet size ranges suitable
468 for both techniques. Before discussing the data acquired on the emulsions, it is worth noting that the
469 emulsion processing conditions also affected the size of the sodium caseinate. Processing sodium
470 caseinate alone revealed a reduction in size from what is reported in section 3.1.3 for unprocessed
471 sodium caseinate down to 100 to 150 nm. Therefore, it is possible to clearly differentiate between
472 protein and protein stabilised droplets in the size distributions (see Figures S9 to S11 in the Appendix).

473 As Figure 8 reveals, emulsion processing in the presence of ethanol showed two types of behaviour as
474 a function of ethanol content. Up to a level of 25 wt.% of ethanol the mean droplet size decreased,
475 whereas this trend was reversed at higher concentrations, resulting in average droplet sizes that were
476 more than one order of magnitude larger for 40 and 50 wt.% ethanol. The first trend observed was in
477 accordance with the data published by *Burgaud & Dickinson (1990)* and most likely caused by the
478 corresponding decrease in interfacial tension (see 3.2.1). However, as reported by *Burgaud &*
479 *Dickinson (1990)*, these trends are inverted when higher concentrations of ethanol (≥ 30 wt.%) are
480 present in the system, resulting in emulsions, which were subject to creaming (or sedimentation)

481 within the first day after preparation (see 3.2.2). The droplet size data obtained for these systems
482 revealed that the existence of such a concentrated and compacted phase with close contact between
483 oil droplets resulted in an enhancement of coalescence within the first two days of storage. Since the
484 protein to oil ratio in the emulsions was relatively high, almost certainly unabsorbed sodium caseinate
485 remained and acted as a driving force for depletion flocculation which further enhanced the formation
486 of a cream/sediment layer. The excess caseinate undergoes self-association processes, forming so
487 called “casein nano-particles”, which cause depletion flocculation of the oil droplets in a similar way
488 to micelles of low molecular weight surfactants (Dickinson & Golding, 1997; Radford & Dickinson,
489 2004; Radford, Dickinson, & Golding, 2004). The observations described above for emulsions
490 containing more than 25 wt.% ethanol indicate changes in the properties of the aqueous phase, which
491 is in accordance with the findings for protein solubility (see 3.1.2). The emulsion destabilisation is likely
492 caused by a decrease of the dielectric properties due to the presence of ethanol in the aqueous phase.
493 As a consequence, the steric layer on the surface of the protein covered droplets collapses, reducing
494 the steric stabilisation of the emulsions, leading to droplet aggregation or even coalescence (Medina-
495 Torres et al., 2009; Radford et al., 2004). The impact of these phenomena appeared to be highest
496 within the first 48 hours of storage as the droplet size distributions of the systems only changed slightly
497 after this time frame. While the samples containing 25 wt.% ethanol did not show much significant
498 change ($p < 0.05$) within the first month of storage, for samples at an ethanol level of 15 wt.%, the
499 droplet size stayed almost constant at around 360 nm for the first ten days and, subsequently,
500 increased consistently until a maximum of approximately 420 nm was reached after one month.
501 Microbial growth as a reason for this increase could be ruled out as the presence of 15 wt.% ethanol,
502 and more, suppresses microbial activity (O’Kennedy & Donnelly, 2003). In the case of non-ethanol
503 containing samples, the absence of any changes in the droplet size distributions over the time frame
504 of this study negated concerns about microbial instability. In their study, *Dickinson & Golding (1998)*
505 linked a gradual increase in mean droplet size over prolonged storage of ethanol-containing oil-in-
506 water emulsions, similar to the one observed here, to the occurrence of Ostwald ripening. However,
507 in the absence of additional experimental insight, this possible explanation could not be corroborated
508 any further. For the non-alcohol samples an initial settling phase of two days was observed, before
509 the droplet size first increased over the period of one week, and continuously decreased for the rest
510 of the storage time considered. The changes in mean droplet size were both, significant ($p < 0.05$, see
511 Table S10) and reproducible, but at present this phenomenon observed cannot be fully scientifically
512 explained. Each of the three systems aforementioned had monomodal droplet size distributions (see
513 representative graphs in the supplementary material, Figures S9 to S11). For samples up to ethanol
514 concentrations of 25 wt.%, the values of the PDI_{DLS} of all systems were similar, around 0.25, indicative

515 of similarly shaped droplet size distributions. Therefore, the samples were neither described by
 516 narrow ($PDI < 0.05$) nor broad size distributions ($PDI > 0.7$) (Danaei et al., 2018). For the two highest
 517 ethanol concentrations investigated, the size data were obtained using static light scattering and,
 518 hence, the PDI_{SLS} was used to describe their distributions. The sole significant changes occurred within
 519 the first two days after emulsion preparation. While initially PDI_{SLS} values of 0.59 and 1.71 were
 520 recorded for the samples containing 40 and 50 wt.% ethanol, respectively, those values decreased to
 521 about 0.42 and 0.18 after two days and showed no further change over a time period of one month.
 522 Therefore, it can be noted that the emulsions at the highest level of ethanol were subject to more
 523 distinct changes within the first 48 hours but were characterised by narrower distributions than the
 524 samples at 40 wt.% of ethanol.



525

526 Figure 8: Droplet size versus time for five different systems (0, 15, 25, 40 and 50 wt.% ethanol – incorporated before
 527 homogenisation), data were acquired at 20 °C. Filled markers correspond to the z-average obtained by dynamic light
 528 scattering whereas hollow markers represent the $d_{4,3}$ acquired by static light scattering.

529 If the solvent is added immediately after the creation of the oil-in-water emulsions, the properties of
 530 the resulting dispersions are affected by two simultaneous phenomena. On the one hand, the addition
 531 of solvent to the samples, i.e., the dilution of the samples until an oil concentration of 10 wt.% is
 532 reached, and on the other hand, the changes of the continuous phase due to the properties of the
 533 added solvent. In order to quantify both effects, the droplet size of each sample was measured right

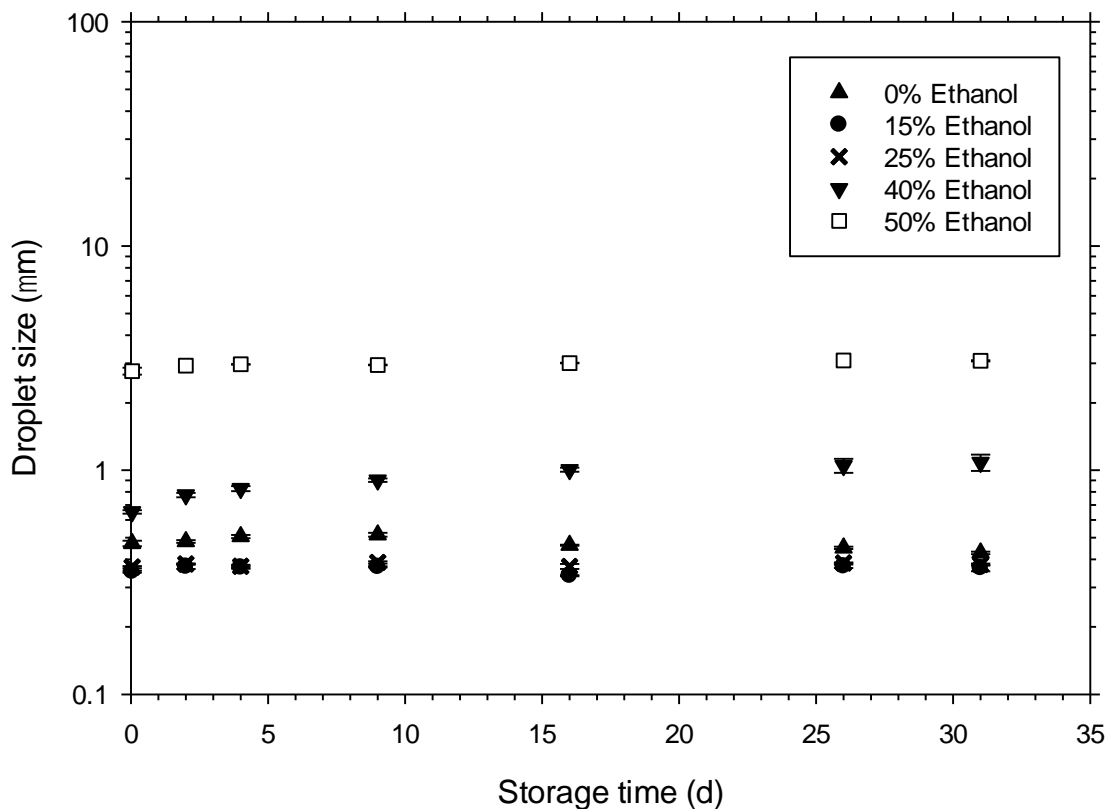
534 after homogenisation, i.e., before dilution with either water or ethanol (Hour 0). The samples that
 535 were diluted with ethanol corresponded to the final emulsion samples. Their droplet size behaviour
 536 over an observation period of one month is discussed later on. The results of the initial measurements
 537 are presented alongside the obtained sizes immediately after solvent incorporation in Table 1. A
 538 higher oil concentration during processing led to higher initial sizes and multimodal droplet size
 539 distributions – a clear indicator for the occurrence of aggregation. The addition of solvent, either
 540 ethanol or water, caused a decrease in both droplet size and PDI, which can be explained by
 541 deagglomeration processes as a consequence of the dilution. As a result, the size distributions of the
 542 systems after the dilution showed much sharper peaks. Hence, aggregates of multiple oil droplets,
 543 which formed due to the higher oil fraction during emulsification, were broken down to individual oil
 544 globules. Since the droplet size data obtained for water and ethanol dispersion showed notable
 545 differences, it can be concluded that interfacial processes such as protein rearrangement and
 546 displacement were still ongoing when the solvent addition took place. Below a solvent concentration
 547 of 25 wt.%, ethanol led to smaller droplets than water, which was most likely caused by the
 548 combination of an increased protein solubility (see 3.1.2) and a lower interfacial tension (see 3.2.1)
 549 (McClements, 2004a). For higher ethanol concentrations on the other hand, the effects of changes in
 550 dielectric properties of the continuous phase and protein solubility seemed to exceed the interfacial
 551 tension trends, causing larger droplets. The droplet sizes of the samples, which were diluted with
 552 water did not change significantly during a storage time of one month (data not shown).

553 Table 1: Droplet size versus oil concentration immediately after homogenisation and after addition of water or ethanol, data
 554 presented in lines 1 to 4 correspond to final ethanol concentrations of 15, 25, 40 and 50 wt.%, respectively, data were
 555 acquired at 20 °C. The asterisk indicates the use of $d_{4,3}$ which was acquired by static light scattering, whereas every other
 556 value corresponds to the z-average obtained by dynamic light scattering.

before solvent addition		after solvent addition		
		Water		Ethanol
Oil concentration (wt.%)	Droplet size (nm)	Oil concentration (wt.%)	Droplet size (nm)	Droplet size (nm)
11.8	601 ± 19	10	492 ± 14	353 ± 3
13.3	712 ± 33	10	549 ± 13	369 ± 4
16.7	1468 ± 37	10	476 ± 16	652 ± 12
20	3429 ± 53*	10	456 ± 10	2766 ± 97*

557
 558 In general, Figure 9 shows similar trends with increasing ethanol content for a post-processing
 559 addition of ethanol than for the pre-processing addition aforementioned. However, the increase in
 560 mean droplet size for concentrations above 25 wt.% of ethanol was not as distinct in the case of an
 561 emulsification in the absence of the solvent. For alcohol levels of 15 and 25 wt.%, the droplet size did

562 not change significantly ($p < 0.05$) during the observation period, except for a slight increase of about
 563 20 nm within the first two days. If 40 wt.% ethanol was present in the system, the average droplet size
 564 increased consistently within the first month of storage, leading to droplets with a diameter of
 565 approximately one micron. This droplet growth slowed down over time, as the differences between
 566 the obtained mean droplet sizes decreased towards the end of the observation period. The
 567 corresponding size distributions indicate that this increase was caused by coalescence as the peak
 568 became broader and shifted towards larger droplet sizes, with the PDI_{DLS} increasing from
 569 approximately 0.20 to 0.25 within this time frame. Overall, the values for the PDI_{DLS} showed only minor
 570 differences compared to the ones measured for the emulsions at an ethanol level of 15 and 25 wt.%
 571 (see Tables S17 to S19). For samples containing 50 wt.% of ethanol, the droplet size increased within
 572 the first month of storage to a value of slightly above 3 microns. The increase in droplet size was
 573 accompanied by a reduction in dispersity, seen by a decrease in PDI_{SLS} from 1.09 to 0.31. This trend
 574 alongside a decreasing rate of droplet growth over time is likely to indicate that the system was
 575 approaching a steady state with a successful long-term droplet stabilisation.



576

577 Figure 9: Droplet size versus time for five different systems (0, 15, 25, 40 and 50 wt.% ethanol –
 578 incorporated after homogenisation), data were acquired at 20 °C. Filled markers correspond to the z-average obtained by
 579 dynamic light scattering whereas hollow markers represent the $d_{4,3}$ acquired by static light scattering.

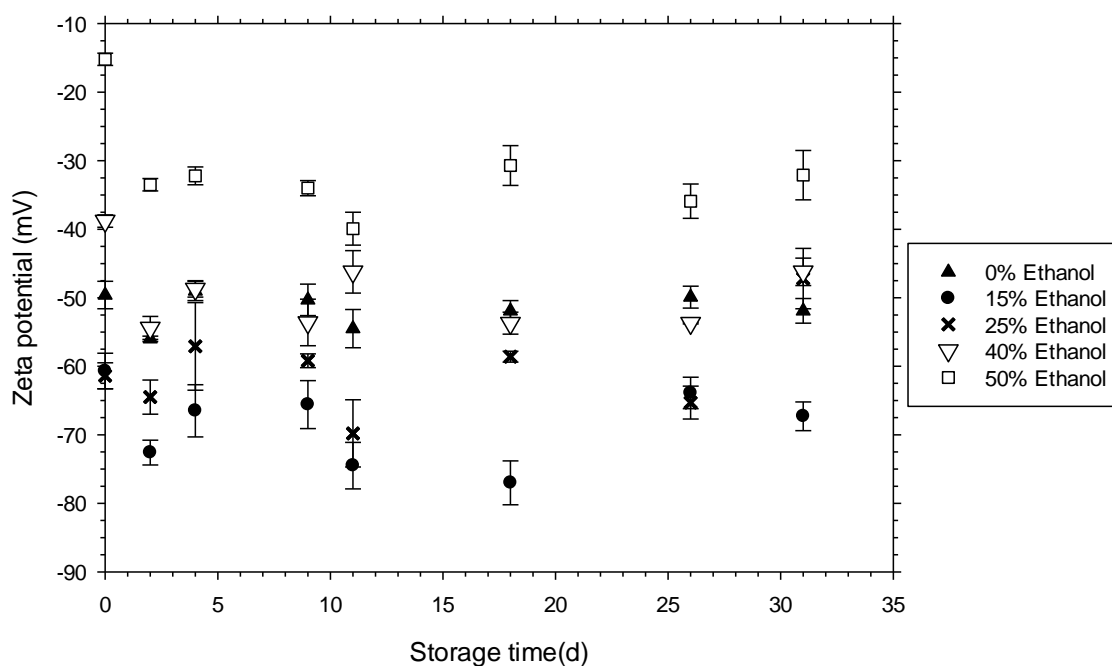
580

581 The data for the mean droplet sizes indicate a slower destabilisation of emulsions containing at least
582 40 wt.% of ethanol, if the alcohol was added after the homogenisation step. This resulted in smaller
583 droplets, which were not subject to gravitational destabilisation (see 3.2.2). If the ethanol addition
584 took place before homogenisation, two contrary phenomena determined the resulting droplet sizes.
585 Firstly, the incorporation of the solvent before the emulsification decreased the interfacial tension
586 between the two processed phases prior to this step, resulting in smaller droplets. However, the
587 exposure to alcohol also led to conformational changes of the protein, which for levels above 25 wt.%
588 of ethanol caused the formation of larger droplets, as the surface coverage depends on the state of
589 the protein during/before emulsification (Dickinson & Woskett, 1988; Srinivasan et al., 1996).
590 Generally, the average droplet size only changed slightly over the storage of one month, if the ethanol
591 level was below 25 wt.%. For such alcohol concentrations, the effects of a decreasing dielectric
592 constant in the aqueous phase as well as changes in the hydrophobicity of the protein due to the
593 ethanol addition and a reduced interfacial tension between the emulsion phases seemed to
594 compensate one another, ensuring a sufficient droplet stabilisation in the first place.

595

596 **3.2.4. Zeta potential**

597 The ζ -potential is a widely assessed emulsion property to infer emulsion stability (Bhatt, Prasad, Singh,
598 & Panpalia, 2010). At first, the findings for the emulsions processed in the presence of ethanol are
599 reported in Figure 10, for an observation period of one month. It is obvious that there is a lot of data
600 scatter and no clear trend over time. However, comparing the initial values recorded for the emulsions
601 with the values found for the sodium caseinate system on its own (filled triangles in Figure 4) reveals
602 that the same trend is seen as a function of ethanol concentration. Consequently, the value for
603 15 wt.% ethanol is lower than the one for 0 wt.% ethanol but similar to the system with an ethanol
604 level of 25 wt.%. With the exception of the first data point, the values of the ζ -potential for 50 wt.%
605 ethanol sample are lower than -30 mV, indicative of an electrostatically stabilised emulsion (Lowry et
606 al., 2016). Each system experienced a drop in ζ -potential over the first two days of storage, although
607 for ethanol levels of up to 25 wt.% it was less steep. The overall trends are in line with the droplet size
608 data (see Figure 8) and suggest that the higher extent of destabilisation phenomena (coalescence,
609 creaming/sedimentation) shown by the samples with the highest ethanol concentrations (see Figure
610 7) were enhanced by unfavourable electrostatic properties of the oil droplets for the emulsions with
611 the highest ethanol contents investigated.



612

613 Figure 10: ζ -potential versus time for five different systems (0, 15, 25, 40 and 50 wt.% ethanol – incorporated before
614 homogenisation), data were acquired at 20 °C.

615 In a second set of experiments, the emulsions were created without any ethanol and the incorporation
616 of the ethanol took place after the homogenisation process. As for the droplet size (see 3.2.3), the
617 influence of the dilution itself, by diluting with water or ethanol, and of the added ethanol, over the
618 observation period of one month, on the ζ -potential was assessed. In Table 2, the values for the ζ -
619 potential of the samples before and immediately after the addition of solvent are presented. Prior to
620 the incorporation of any solvent, the ζ -potential of all four samples was around -58 mV. The addition
621 of water only led to slight changes in ζ -potential, both initially and within a storage period of one
622 month (data not shown). While the dilution with water was seen to result in the deagglomeration of
623 the oil droplets (see 3.2.3), it does not appear to have affected the interfacial properties of the protein-
624 coated oil droplets. In contrast, the dilution with ethanol up to a level above 15 wt.% caused a
625 decrease in the absolute value of the ζ -potential. At such concentrations, the alcohol-induced lowering
626 of the dielectric constant and the solvent quality in the aqueous phase results in a decreased solubility
627 and conformational changes of the protein utilised (Kruzel et al., 2004; Walstra & van Vliet, 2003). As
628 a consequence, the functional hydrophobicity of the sodium caseinate changes and, thus, the
629 formation of the adsorption layer on the droplet surface is affected (Trejo & Harte, 2010). The
630 reported trend for values of ζ -potential is potentially caused by such described phenomena as they
631 are likely to influence the surface charge of the oil droplets. Following the results for the droplet size
632 (see Table 1), the overall trend for the ζ -potential as a function of ethanol concentration met the
633 expectations, as an inversely proportional correlation between these two data sets was found.

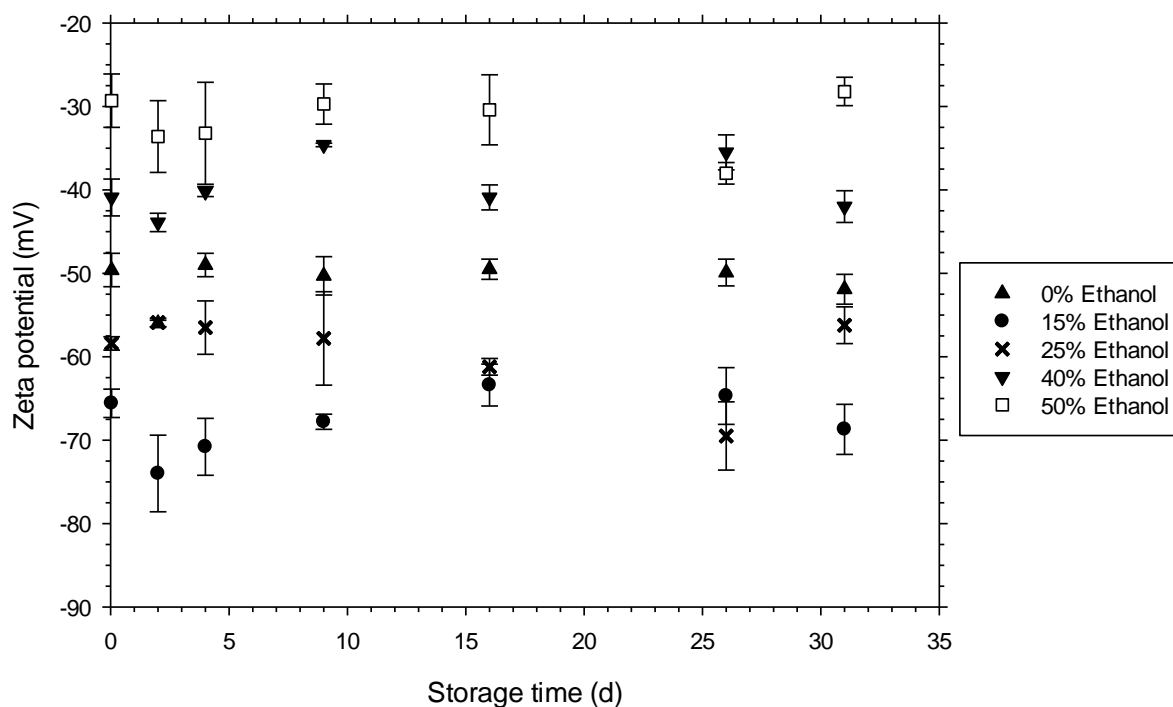
634 Table 2: ζ -potential versus oil concentration immediately after homogenisation and after addition of water or ethanol, data
 635 presented in lines 1 to 4 correspond to final ethanol concentrations of 15, 25, 40 and 50 wt.%, data were acquired at 20 °C.

before solvent addition		after solvent addition		
		Water	Ethanol	
Oil concentration (wt.%)	ζ -potential (mV)	Oil concentration (wt.%)	ζ -potential (mV)	ζ -potential (mV)
11.8	-58.2 ± 0.9	10	-61.1 ± 3.6	-65.6 ± 1.7
13.3	-59.0 ± 0.7	10	-54.6 ± 0.1	-58.4 ± 0.8
16.7	-58.0 ± 1.3	10	-54.1 ± 1.1	-40.9 ± 2.2
20	-58.5 ± 0.2	10	-52.1 ± 2.8	-29.3 ± 3.2

636

637 The trend of the ζ -potential over time for these emulsions is shown in Figure 11. As for the emulsions
 638 processed in the presence of ethanol, the values of ζ -potential as function of ethanol concentration
 639 followed the same trend as reported for the systems solely containing sodium caseinate (see open
 640 triangles in Figure 4). Similarly to Figure 10, there is extensive data scatter and various fluctuations
 641 over time. However, for the systems reported in Figure 11, the initial decrease in ζ -potential for
 642 ethanol concentrations above 25 wt.% was not as steep as the one reported for the systems processed
 643 in the presence of ethanol, resulting in a value of approximately -30 mV for the emulsions containing
 644 50 wt.%. Subsequently, the surface charge did not decrease any further over one month of storage,
 645 staying close to the stability limit for emulsion droplets (-30 mV, (Lowry et al., 2016)) and resulting in
 646 emulsions, which were subject to only slow (electrostatic) destabilisation, as indicated by only slight
 647 increases in droplet size over time (see Figure 9).

648 Apparently, an addition of ethanol after the processing step led to a more preferable initial
 649 electrostatic stabilisation of the emulsion droplets, resulting in only minor changes of the droplet size
 650 over the first month of storage (see 3.2.3). Hence, an incorporation of ethanol after the emulsification
 651 step, generally, led to more stable emulsions, which matched the findings of *Banks & Muir* (1985). In
 652 their publication, *Banks & Muir* stated that an oil-in-water emulsion with high ethanol concentrations
 653 could only be reached, if the addition of ethanol takes place after the homogenisation process.
 654 Otherwise, the main destabilising interactions between protein and alcohol will already occur during
 655 the emulsification step.



656

657 Figure 11: ζ -potential over time for five different systems (0, 15, 25, 40 and 50 wt.% ethanol –
 658 incorporated after homogenisation, data points for 0 and 25. wt.% ethanol overlap for day 2, data were acquired at 20 °C.

659

660 4. Conclusions

661 In the first part of this study, it was found that the presence of ethanol and/or heat during the
 662 dispersion of sodium caseinate highly affected the protein solubility but not physical parameters of
 663 aggregates of this protein, such as size and surface charge. Further, the protein solubility was
 664 significantly higher if sodium caseinate was dispersed in water alone and ethanol added afterwards.
 665 The second part of this study revealed that the point of ethanol addition is critical at ethanol
 666 concentrations above 25 wt.% (31.7% ABV) if sodium caseinate is used as the sole emulsifying agent
 667 for oil-in-water systems. These emulsions were subject to slower destabilisation mechanisms when
 668 the ethanol was added after the processing step, which was in accordance with findings from
 669 *Banks & Muir* (1985). For the emulsion preparation an oil phase free of low molecular weight
 670 surfactants was deployed, which was then used to create samples with an ethanol concentration of
 671 up to 50 wt.% (58% ABV). Below an ethanol level of 25 wt.%, the point of addition hardly made any
 672 difference in emulsion properties, with an increasing ethanol concentration in this range enhancing
 673 emulsion stability. To conclude, both, the investigation of the properties of sodium caseinate in
 674 presence of ethanol, and the determination of the emulsification properties of this protein indicated
 675 the existence of a critical concentration of 25 wt.% of ethanol, with levels above this limit leading to

676 changed protein properties and a decrease in emulsion stability. Similar trends have been reported
677 before (Burgaud & Dickinson, 1990; Dickinson, 2019) and are most likely the result of the interplay
678 between a decreased dielectric constant of the aqueous phase and the limited solubility of the protein
679 for an increasing concentration of ethanol. Further research should focus on emulsions made from
680 oil phases containing natural present surface-active components, such as cream, and their impact on
681 the (long-term) stability of these systems. This would mean to investigate competitive phenomena
682 between sodium caseinate and the other ingredients at the interface and would help to fully
683 understand their role in emulsion stabilisation.

684

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