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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Experimental

2.1. Materials

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

2.2. Solubility assays

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

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

2.2.1. Solubility in ethanol-water mixtures

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

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

2.2.2. Ethanol-induced precipitation

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

2.3. Emulsion preparation

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

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

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

2.3.1. Emulsion processing in the presence of ethanol

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

2.3.2. Addition of ethanol after emulsion processing

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

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

2.4. Particle size

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

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

2.5. Zeta potential

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

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

2.6. Surface and interfacial tension

The surface tension of the various aqueous protein dispersions (after centrifugation), as well as the interfacial tension between the aqueous protein dispersions (without removal of insoluble material) and the treated sunflower oil as used for the emulsion preparation, were determined with a profile analysis tensiometer (PAT-1M, Sinterface Technologies, Berlin, Germany), at 20 °C. The equipment was fitted with a straight stainless-steel capillary (3mm outer diameter) to create a pendant drop (27 mm^2 cross-sectional area) of the aqueous phase in either an empty, or oil-containing quartz cuvette. Dynamic surface/interfacial tension data were recorded until a steady state was reached. It was assumed that equilibrium was obtained when the standard deviation of the average of twenty consecutive data points was smaller than 0.01 mN/m, corresponding to the resolution of the equipment. The data point density for the calculation of the standard deviation was four points per minutes, ergo one data point was recorded every 15 seconds. Liquid phase density values, required for the data analysis by the tensiometer software, were determined using a force tensiometer (K100, Krüss GmbH, Hamburg, Germany) and a solid measuring probe (2.33 g/cm^3 density), at 20 °C.

2.7. Statistical analysis

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

3. Results and discussion

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

3.1.1. Solubility in ethanol-water mixtures

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

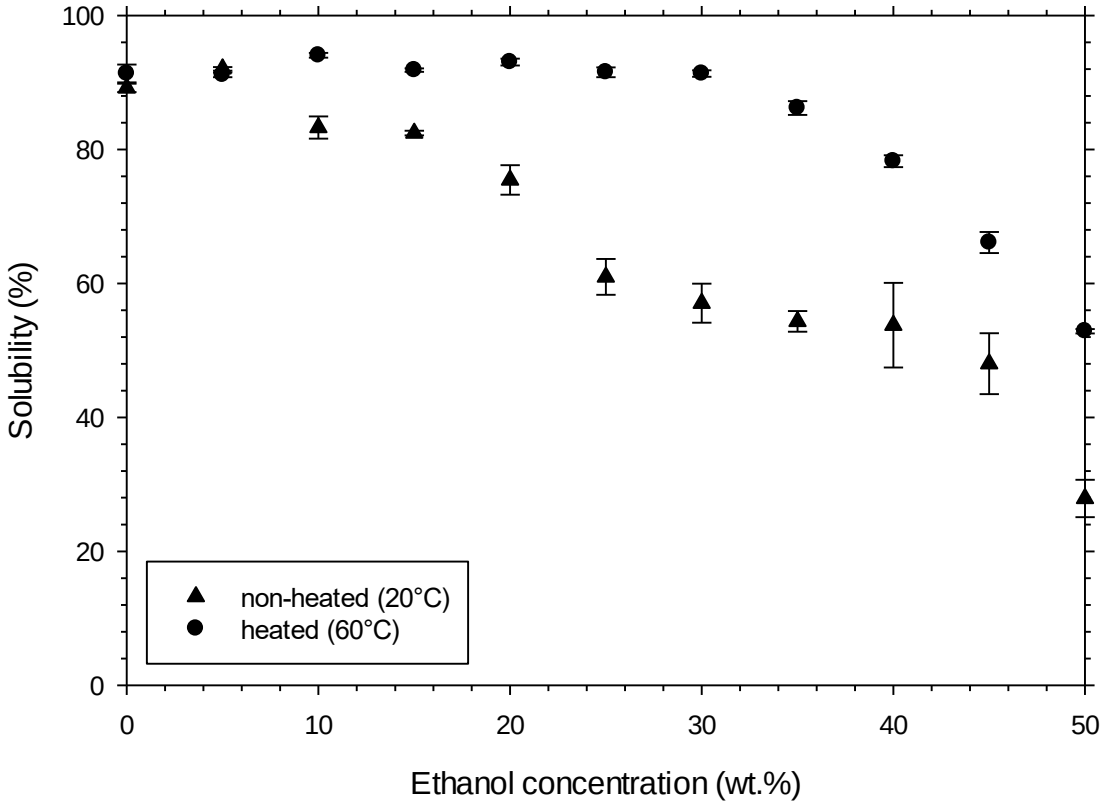


Figure 1: Solubility of sodium caseinate in aqueous dispersions with differing ethanol concentration. Samples were prepared either at 20 °C or 60 °C. The data correspond to the average of four measurements with error bars representing one standard deviation.

3.1.2. Soluble fraction following precipitation with ethanol

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

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

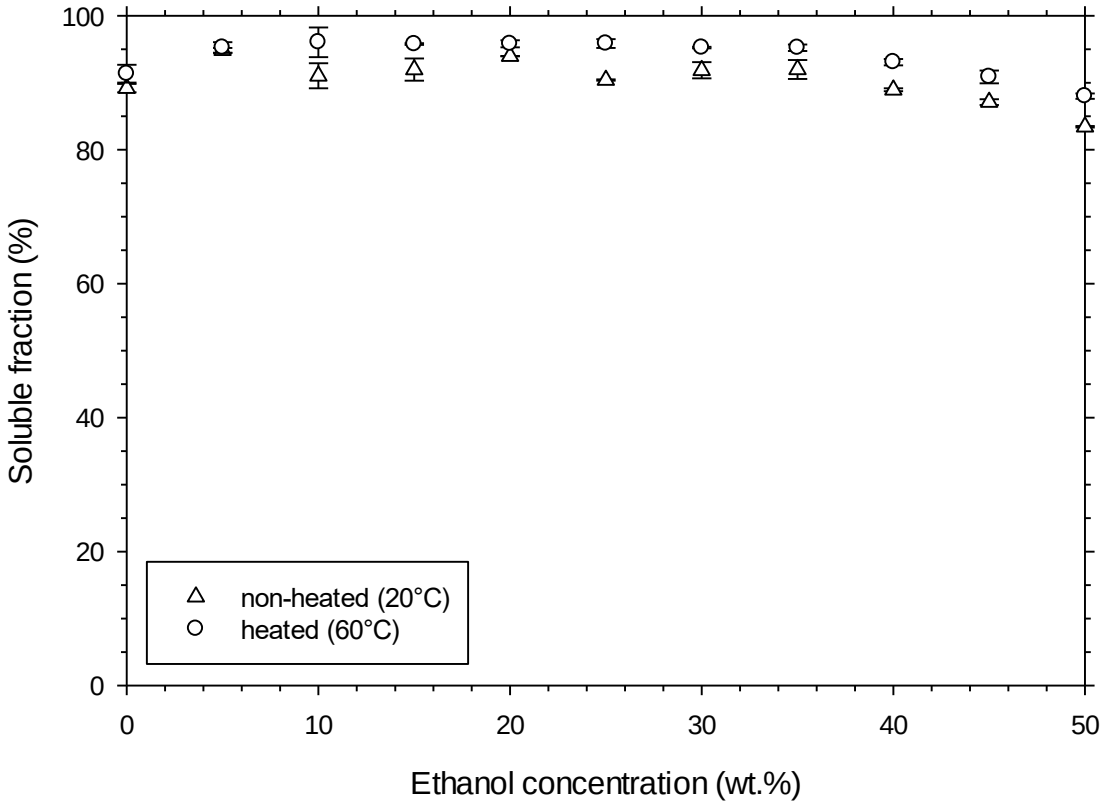


Figure 2: Soluble fraction of sodium caseinate in aqueous dispersions with differing ethanol concentration. Samples were prepared either at 20 °C or 60 °C and ethanol was incorporated after dispersion in water. The data correspond to the average of four measurements with the error bars representing one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

3.1.3. Aggregate size

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

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

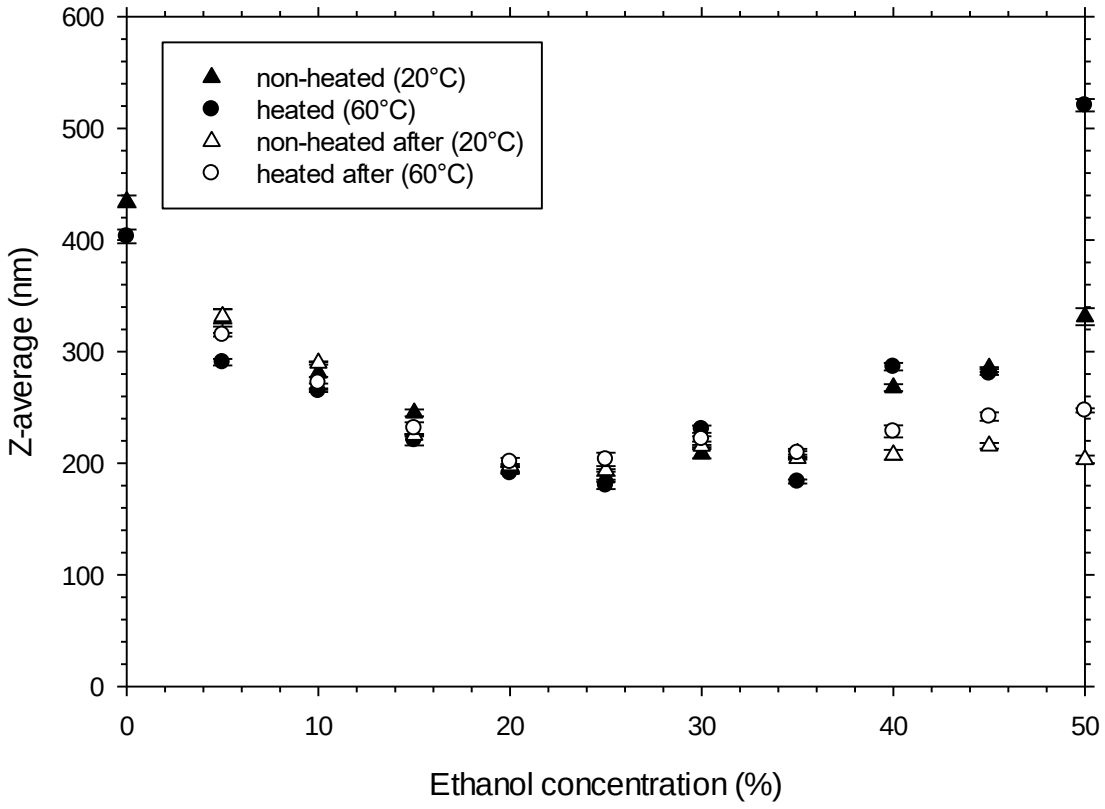


Figure 3: Z-average of protein aggregates in the soluble fraction of samples versus ethanol concentration, prepared either at 20 °C or at 60 °C with ethanol added before (filled markers) and after (hollow markers) the protein dispersion.

3.1.4. Zeta potential

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

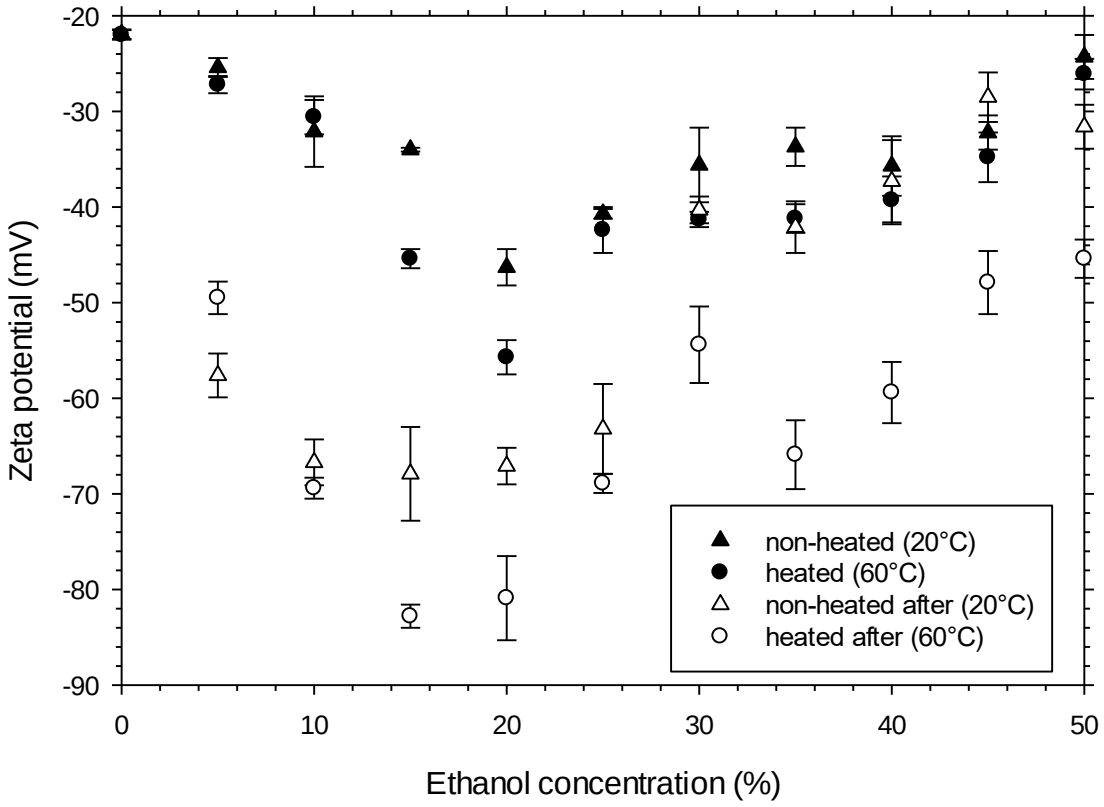


Figure 4: ζ-potential of protein aggregates in the soluble fraction of samples versus ethanol concentration, prepared either at 20 °C or at 60 °C with ethanol added before (filled markers) and after (hollow markers) the protein dispersion.

3.1.5. Surface tension

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

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

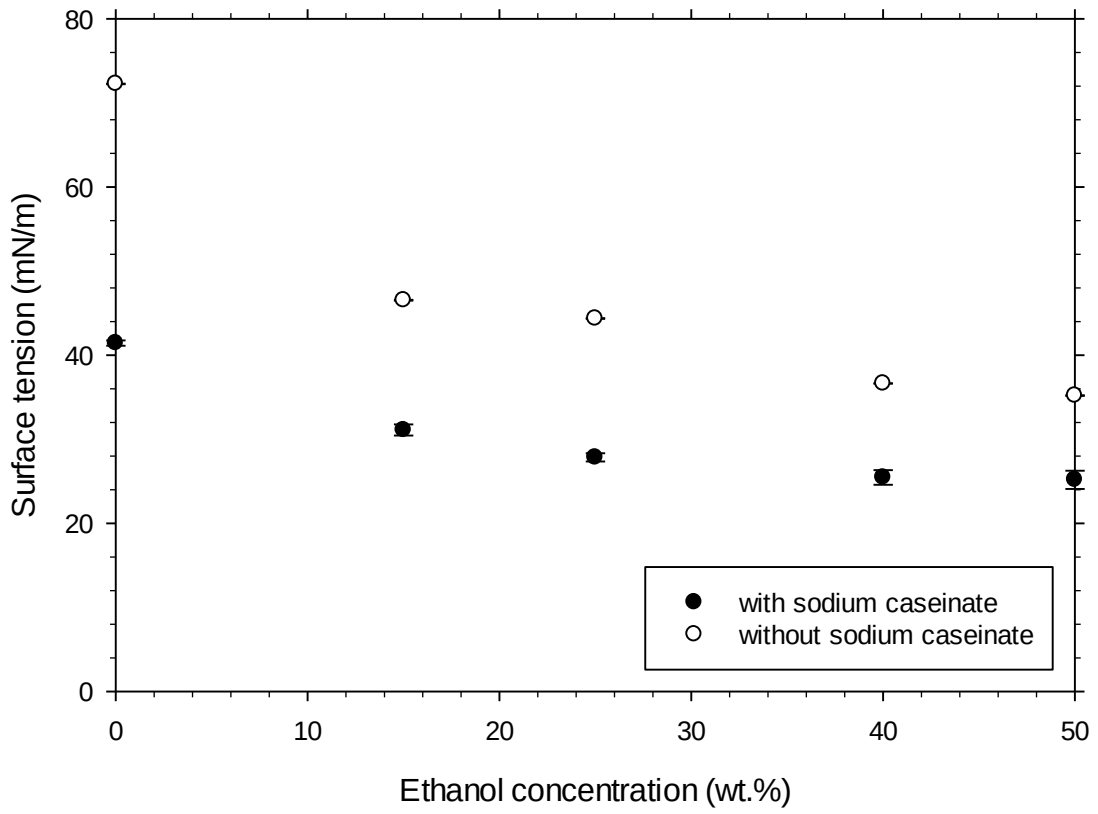


Figure 5: Surface tension of different water-ethanol mixtures with and without (as reference) 3 wt.% sodium caseinate at a steady state, data were acquired at 20 °C.

3.2. Emulsion stabilisation

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

3.2.1. Interfacial tension

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

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

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

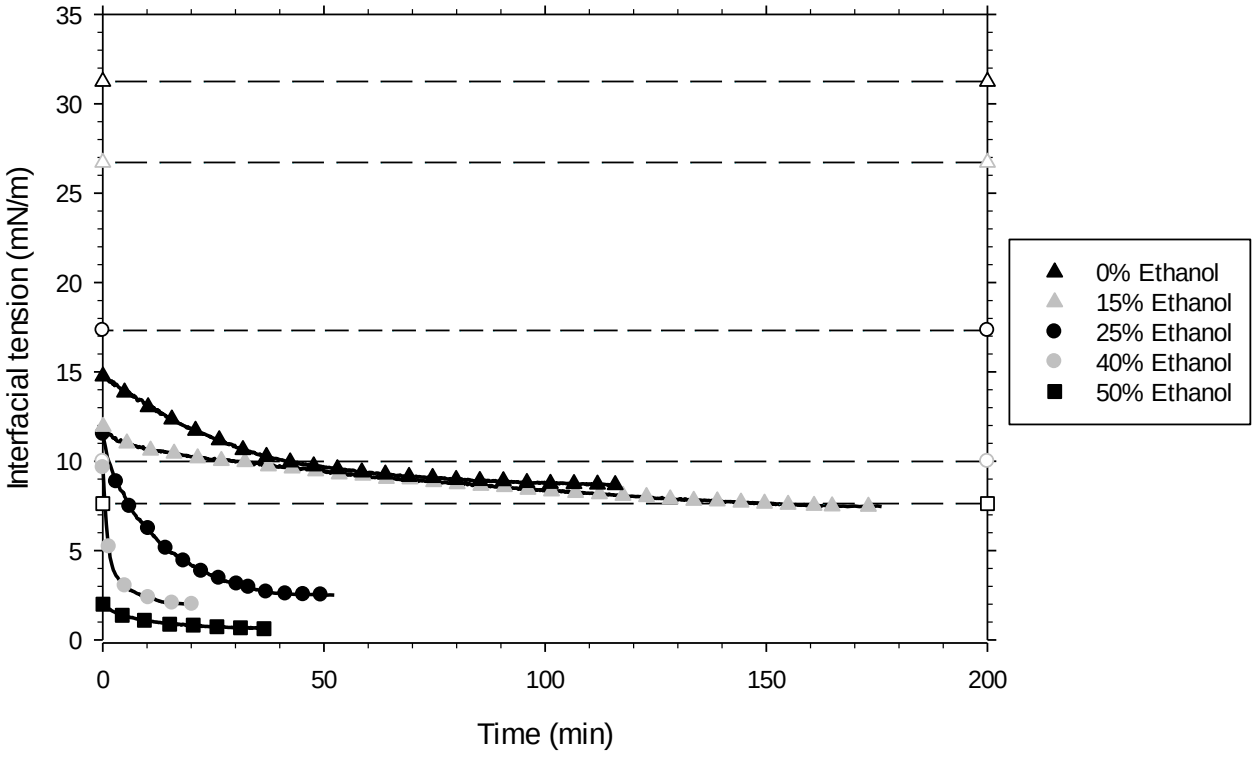


Figure 6: Dynamic interfacial tension between treated sunflower oil and ethanol-water mixtures containing 3 wt.% sodium caseinate (solid lines and filled markers) versus time until the respective steady state was reached; the dashed lines between the hollow markers represent the interfacial tension values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

3.2.2. Visual stability

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

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

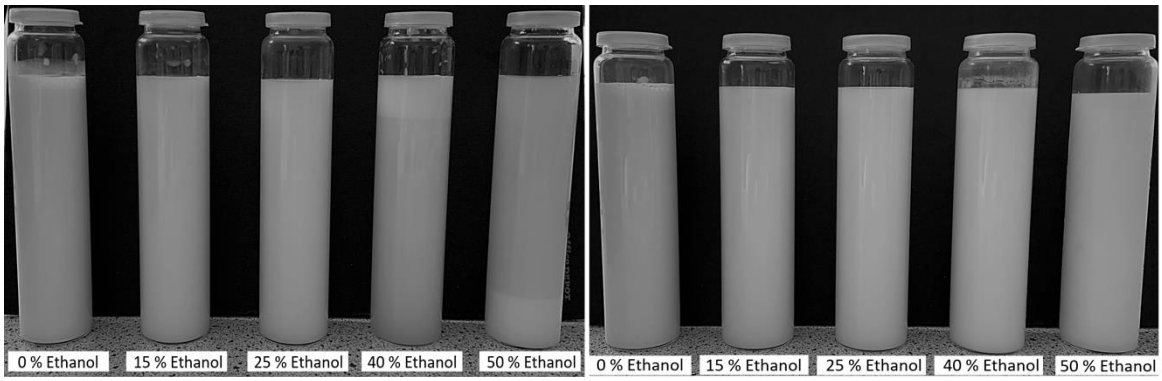


Figure 7: Emulsions containing different concentrations of ethanol - one day after preparation (no further change over nine months of storage); incorporation of ethanol took place a) before and b) after the homogenisation.

3.2.3. Droplet size

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

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

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

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

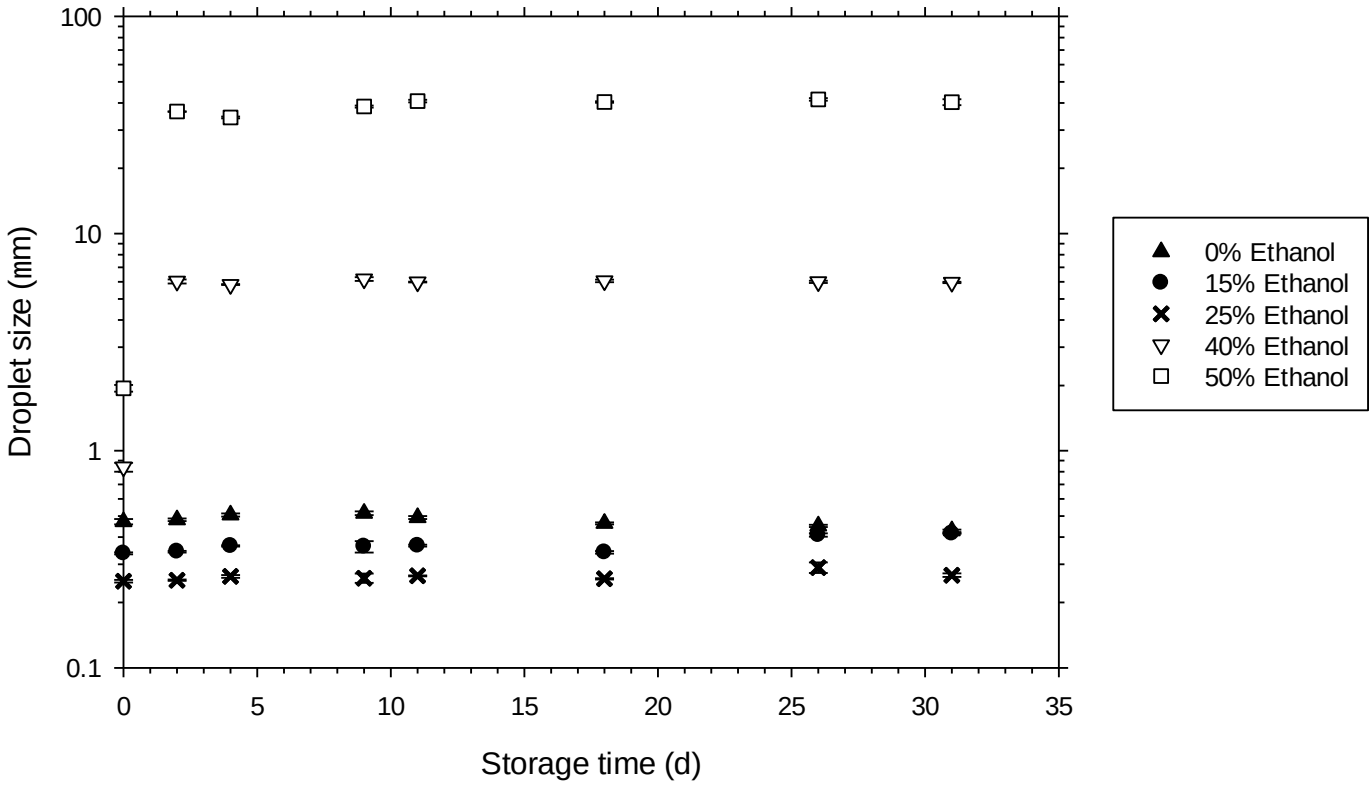


Figure 8: Droplet size versus time for five different systems (0, 15, 25, 40 and 50 wt.% ethanol – incorporated before homogenisation), data were acquired at 20 °C. Filled markers correspond to the z-average obtained by dynamic light scattering whereas hollow markers represent the $d_{4,3}$ acquired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

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

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

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

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

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

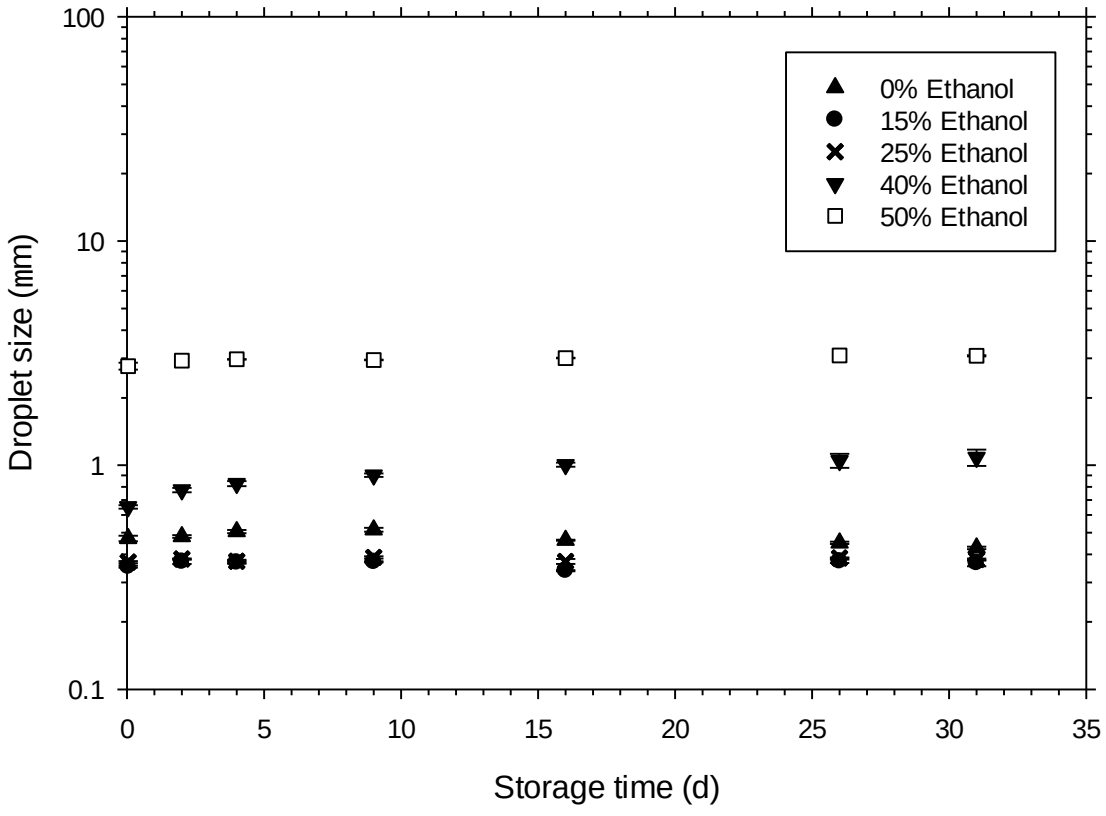
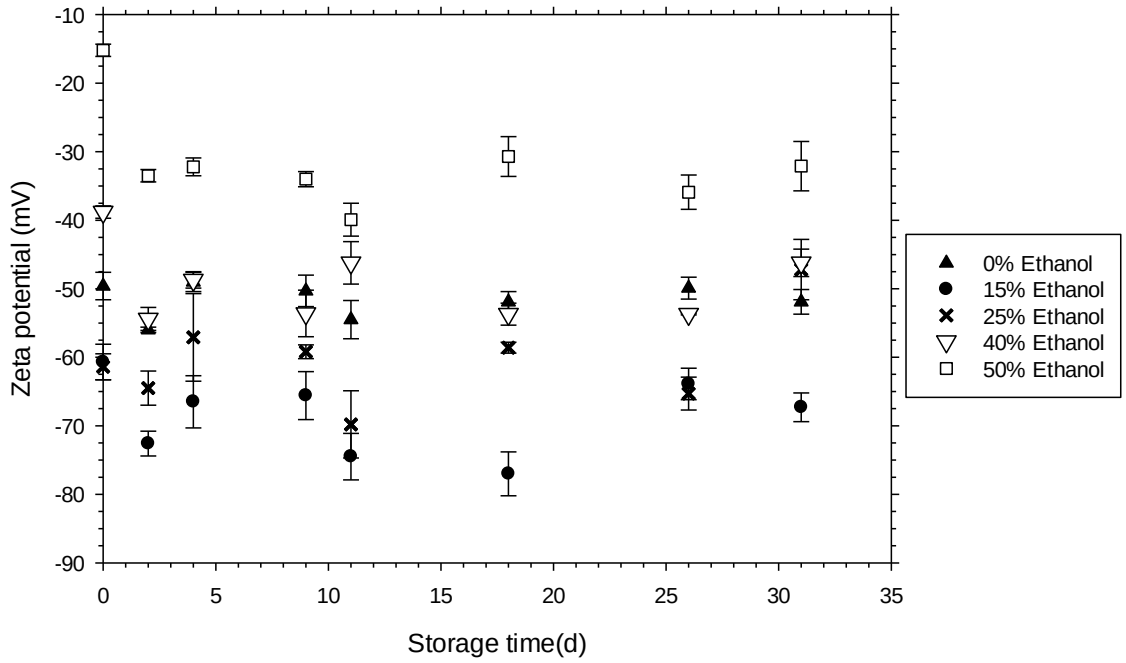


Figure 9: Droplet size versus time for five different systems (0, 15, 25, 40 and 50 wt.% ethanol – incorporated after homogenisation), data were acquired at 20 °C. Filled markers correspond to the z-average obtained by dynamic light scattering whereas hollow markers represent the $d_{4,3}$ acquired by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

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

3.2.4. Zeta potential

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

Table 2: ζ-potential versus oil concentration immediately after homogenisation and after addition of water or ethanol, data 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

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

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

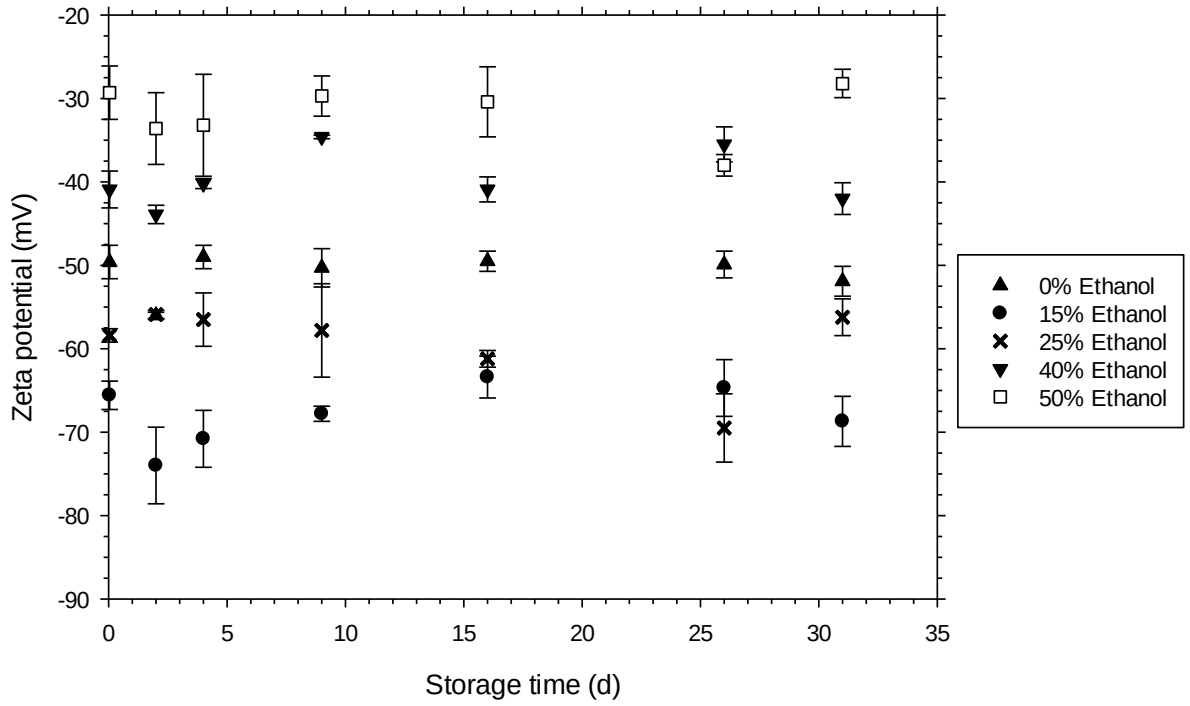


Figure 11: ζ-potential over time for five different systems (0, 15, 25, 40 and 50 wt.% ethanol – incorporated after homogenisation, data points for 0 and 25. wt.% ethanol overlap for day 2, data were acquired at 20 °C.

4. Conclusions

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

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

Acknowledgements: The authors express their gratitude to the School of Chemical Engineering for funding this studentship. We would like to thank Antonio Sullo, Scott Winston and Peter Lillford for discussion. Also, the input and support from Kilian Daffner is highly appreciated.

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