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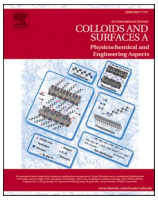

Effect of the interplay between lipid phase properties and ethanol concentration on the stability of model cream liqueurs

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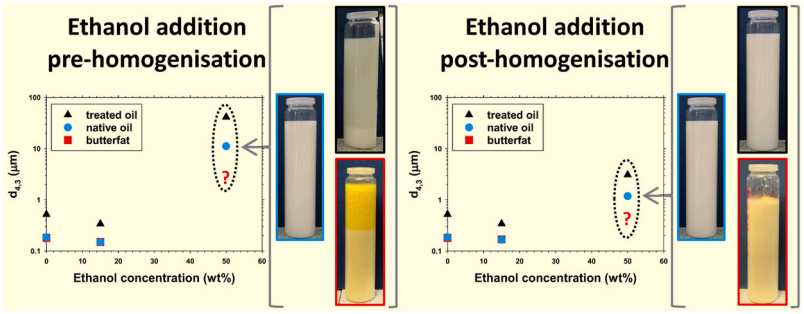
HIGHLIGHTS

- native sunflower oil and butterfat were partially soluble in aqueous ethanol.
- untreated lipid phases resulted in significantly smaller emulsion droplets.
- butterfat emulsions destabilised above a critical ethanol concentration via clumping.
- ethanol suppressed butterfat crystallisation for first day after emulsification.

GRAPHICAL ABSTRACT



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ABSTRACT

Cream liqueur formulations are limited to ethanol concentrations below 20 wt% due to clumping of the lipid phase at higher levels. However, it is not generally understood whether the properties of the dispersed phase, containing surface-active and crystallising lipids, or the detrimental impact of ethanol on the emulsifying properties of protein are responsible for this limitation. Here, model cream liqueurs were processed, containing solely ethanol (0, 15 or 50 wt%), water, sodium caseinate (3 wt%) and one of three lipid phases (10 wt%): sunflower oil stripped off surface-active molecules, native sunflower oil, or clarified butterfat. Ethanol was added either before or immediately after emulsion processing in a microfluidizer, and final emulsions were stored at 5 °C. At 0 and 15 wt% ethanol, independent of point of ethanol addition, native oil and butterfat resulted in up to three times smaller droplets than treated oil, and emulsions remained stable for at least nine months. The presence of ethanol (15 wt%) suppressed butterfat crystallisation at the selected storage temperature. At 50 wt% ethanol, the presence of a crystallising lipid fraction negatively affected emulsion stability, as such butterfat emulsions showed immediate clustering and creaming. Native oil-based emulsions were stable against creaming and coalescence at this ethanol level. The study concludes that the interplay of ethanol-compromised emulsifying properties of the protein and the crystallisation characteristics of the lipid inhibits the formulation of a commercially viable cream liqueur at elevated ethanol concentrations. For non-crystallising lipid phases however, the novel findings reported in this study are encouraging in view to formulating alternative product ranges.

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1. Introduction

Proteins are used as emulsifying agents in a wide range of applications, with pharmaceuticals, food and beverage products being just a few examples. When formulating such systems, it needs to be considered that proteins and their emulsifying properties are highly affected by fellow ingredients and their environment in general [47,77]. In the case of cream liqueurs, an emulsion-based alcoholic beverage, caseins are commonly used to stabilise the oil/water interface [70]. Therefore, an understanding of the interactions between other ingredients and these dairy proteins is of importance to ensure an efficient initial emulsion stabilisation as well as the long-term stability of such products. While additional casein may be added to cream liqueur formulations, it is mostly introduced as a naturally present component of the lipid phase ingredient – dairy cream. Dairy cream is a complex and naturally protein-stabilised emulsion, fluctuating in composition [34,55], which poses a challenge when designing a single universal formulation for cream liqueurs. Of the different components in dairy cream, salts – especially calcium ions – are the most problematic ones in terms of impacting protein properties and, hence, emulsion stability [24]. Sequestering agents such as trisodium citrate may be added to suppress calcium-induced destabilisation processes of casein micelles, which are further enhanced in the presence of ethanol [21,35].

Dairy cream comprises lipid fractions which undergo thermal transitions in the temperature range applied during the manufacturing process of cream liqueurs (0 °C – 90 °C, [62,76]) as well as during final product storage [8]. Storage conditions vary widely depending on geographical location and consumer practice between temperatures as high as roughly 40 °C and as low as 5 °C (refrigerated storage). Fat crystals are known to impact the (long-term) stability of emulsions, influenced by their wettability, their microstructure and their location within the emulsion [17,53,63]. Such crystals may be present at the interface, intraglobular within the lipid phase or simultaneously in both locations [63,73]. Generally, the higher the wettability of the crystals by the continuous phase, the more they are going to protrude from the interface. This arrangement might be beneficial in stabilising emulsions and preventing coalescence as the particles form a physical layer/barrier around the droplet [58]. On the other hand, the crystals may come into contact with neighbouring droplets and pierce their interface, forming a rigid droplet network stabilised by fat crystals [7]. This phenomenon is known as partial coalescence and a major source of emulsion destabilisation associated with fat crystals [20]. The thin film layering of casein sub-micelles between droplets or near fat crystal surfaces was reported to suppress partial coalescence [41,79]. Commercial food and beverage emulsion lipid phases often contain low molecular weight amphiphiles which are present alongside emulsifying agents which are added to the aqueous phase, leading to competitive adsorption at the interface [13,19]. It is well established that such amphiphiles, contained in the dispersed or continuous emulsion phase, may displace proteins from the interface, changing the mechanism of droplet stabilisation [18].

In the case of (model) cream liqueurs, emulsion stability is also affected by the presence of ethanol in the formulation. This effect has previously been studied for formulations comprising liquid oil [1,9,23,29,59] or crystallising fat, including dairy cream [4,5]. We have previously reported on the processing and stability of model cream liqueurs comprising water, ethanol, sunflower oil stripped of low molecular weight amphiphiles, and sodium caseinate [28]. Considering liquid food oil formulations at first, emulsion destabilisation via droplet coalescence and/or creaming was reported at compositions for which our previous study did not show any of these instability mechanisms [1,28,29]. Reasons for this discrepancy might be differences in processing sequence including changes to the point of ethanol addition. Motivated by the fact that as part of our previous study, stable emulsions at elevated ethanol concentrations could be produced utilising treated oil [28], the present study was designed. Native sunflower oil and clarified butterfat were included with the aim to unravel the contribution of compromised emulsifying properties of sodium caseinate alongside oil-soluble amphiphiles and lipid crystallisation in the emulsion droplet phase on the limits of ethanol in commercial cream liqueurs.

2. Experimental

2.1. Materials

Ethanol was obtained from Fisher Scientific (Analytical grade, Loughborough, United Kingdom) and sodium caseinate (CAS: 9005–46-3, lot no. BCBV4056) from Sigma-Aldrich Ltd (Gillingham, United Kingdom). Sunflower oil and clarified butterfat (pure butter ghee) were purchased from a local supermarket. In some cases, sunflower oil was treated with magnesium silicate (Florisil®) from Sigma-Aldrich Ltd (Gillingham, United Kingdom) to remove naturally present amphiphiles (described in 2.5). Throughout the entire sample preparation, milli-Q water from a reverse osmosis apparatus (Elix® Essential 5, Merck, Darmstadt, Germany) was utilised. Sodium hydroxide (1 M) and hydrochloric acid (1 M) solutions purchased from Sigma-Aldrich (Gillingham, United Kingdom) were used for pH adjustment.

2.2. Lipid analysis

The fatty acid profiles of the lipids applied in this study were analysed via thin layer chromatography by an external laboratory (Mylnefield Lipid Analysis, James Hutton Limited, Dundee, UK). The experimental protocol shared was as follows.

At first, a few milligrams of each lipid were dissolved in trichloromethane and placed on a thin layer chromatography plate alongside standards (TLC32 and TLC40). Consequently, the samples were separated into different lipid classes (mono-, di-, and triglycerides) using a solvent mixture of isohexane, diethyl ether and formic acid (78:20:2, by volume). The lipid class bands were then removed from the plate for analysis. This step was carried out by adding an internal standard (margaric acid) to the lipid sample and transesterification of the mixture. Lastly, the mix of standard and sample was separated into fatty acids by gas chromatography utilising a capillary column. The fatty acid profiles were obtained as methyl ester and results are reported as weight concentration based on the sample.

2.3. Ethanol solubility of lipid phases

The relative solubility of the three lipid phases (treated sunflower oil, native sunflower oil, butterfat) in aqueous ethanol and of aqueous ethanol in any of the three lipids were assessed for aqueous phase ethanol concentrations of up to 100 %. Initially, the lipid was mixed with the aqueous phase at the ratio of 1:9 by weight, as final emulsions contained 10 wt% lipid, for 60 min at 600 rpm on a magnetic stirrer. For the oil samples mixing took place at 20 °C and 60 °C, whereas mixing with butterfat was only carried out at 60 °C to ensure it was fully melted (see 3.5). This temperature also corresponded to the temperature during emulsion processing (see 2.5). Mixtures were then transferred into a separation funnel and allowed to bulk phase separate for three hours at 20 °C for oil and at 60 °C for butterfat samples. Next, roughly 3 ml were taken from both phases that had formed, transferred into glass vials and weighed. The vials were heated to 90 °C to remove any ethanol present within the samples and then transferred into a vacuum oven at 105 °C to remove any water. Each heating step was continued until the mass of the vials was constant, indicating complete evaporation of ethanol or water, respectively. Lastly, the final weight of each sample was recorded, and solubility values were calculated as defined in Eqs. (1) and (2).

$$\text{relative solubility of lipid in aqeuous } \textit{ethanol} = \frac{\text{dry weight of aqueous ethanol phase sample (g)}}{\text{initial weight of aqueous ethanol phase sample (g)}} \quad (1)$$

$$\text{relative solubility of aqueous ethanol in } \textit{lipid} = \frac{\text{initial weight of lipid phase } \textit{sample} \text{ (g)} - \text{dry weight of lipid phase } \textit{sample} \text{ (g)}}{\text{initial weight of lipid phase } \textit{sample} \text{ (g)}} \quad (2)$$

2.4. Interfacial tension

The interfacial tension at lipid/aqueous phase interfaces was determined with a profile analysis tensiometer (PAT-1 M, Sinterface Technologies, Berlin, Germany). In case of treated and native sunflower oil, data were acquired at 20 °C and 60 °C, whereas for butterfat data were only acquired at 60 °C. The temperature within the measurement chamber was controlled via a connected water bath. The ethanol concentration in the aqueous phase was selected at 0, 16.7 and 55.6 wt%, corresponding to the aqueous ethanol compositions in final emulsions. When present, sodium caseinate was added at 3.3 wt% (equivalent to 3 wt% in final emulsions). The equipment was fitted with a straight stainless-steel capillary (3 mm outer diameter) to create a pendant drop (27 mm^2 cross-sectional area) of the aqueous phase in the lipid phase which was contained in a quartz cuvette. Dynamic interfacial tension data were recorded until the standard deviation of the average interfacial tension of twenty consecutive data points was less than 0.01 mN/m, at a data point density of four points per minute. 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 and 60 °C.

2.5. Emulsion preparation

Oil-in-water emulsions containing 0, 15 or 50 wt% ethanol were prepared from 10 wt% oil (treated or native) or butterfat, 87 wt% water or aqueous ethanol and 3 wt% sodium caseinate. The ethanol levels correspond to approximately 18 % and 58 % ABV. Treated sunflower oil was prepared by stirring native oil with 4 wt% of magnesium silicate for 30 min at 600 rpm and 20 °C on a magnetic stirrer. The silicate was subsequently removed through centrifugation for 30 min at 8500 g and 20 °C (J2–21 floor model centrifuge, Beckman, Indianapolis, USA), as previously published [28,31]. Before the treated oil was used as the dispersed emulsion phase, it was verified that the interfacial tension at the oil/water interface was constant over time, confirming the absence of surface-active molecules. The continuous emulsion phase was prepared by initially dispersing the appropriate amount of sodium caseinate in water at 400 rpm and 60 °C on a magnetic stirrer for 30 min. The further procedure varied for the two sets of emulsions prepared in this study. The ethanol was added either before lipid phase addition or after emulsion processing.

2.5.1. Emulsion processing in the presence of ethanol

The required amount of ethanol was added into the vortex of the sodium caseinate dispersion during the first 30 s of a ten-minute mixing process on a magnetic stirrer at 400 rpm and 60 °C. In the case of preparing a zero-ethanol emulsion, this mixing step was omitted. Each sample was covered throughout mixing to avoid loss of liquid due to evaporation. In the meantime, the lipid phase was heated to 60 °C. Afterwards, the required amount of each phase was transferred into a jacketed vessel, heated to 60 °C. By processing the mixture with a high-shear overhead mixer (Silverson® L5M fitted with a fine emulsor screen, East Longmeadow, USA) for three minutes at 5000 rpm and 60 °C, a pre-emulsion was prepared which was immediately passed through a microfluidizer once at 1200 bar (Microfluidics M-110S, Newton, USA). The final emulsions were transferred into glass storage vials and kept at 5 °C to prevent microbial destabilisation until required for analysis.

2.5.2. Addition of ethanol after emulsion processing

In the second set of experiments, emulsions were processed as described above (see 2.5.1) but omitting the step of ethanol addition. Immediately after processing, the emulsions were stirred on a magnetic stirrer at 100 rpm and 60 °C for ten minutes. The appropriate amount of ethanol was added into the emulsion vortex within the first 30 s of this step. Final emulsions were stored at 5 °C.

2.6. Droplet size

Emulsion droplet size distributions were acquired at 20 °C and pH 7 using static light scattering (Mastersizer MS 2000 fitted with a Hydro SM manual small volume sample dispersion unit, Malvern Panalytical, Malvern, UK). Measurements were taken 90 min after emulsion processing and then every seven days for a total of four weeks. Refractive index values at 20 °C, required for data analysis, were selected as 1.33 for water, 1.47 for sunflower oil and 1.455 for butterfat, while the absorption was set to zero. The refractive indices for the continuous emulsion phases were measured with a refractometer (J 357 series, Rudolph Research Analytical, USA), at 20 °C, and used accordingly. As a representation of the average droplet size of the emulsions, the volume-weighted mean diameter, $d_{4,3}$, is reported together with the droplet size distribution (Figs. S2 – S6 in the supplementary material). The Span value, defined in Eq. (3), with $d_{10,3}$, $d_{50,3}$ and $d_{90,3}$ corresponding to the diameter below which 10 %, 50 % and 90 %, respectively, of the total volume of droplets were found, is reported as a measure of the distribution width.

$$\text{Span} = \frac{d_{90,3}(\mu m) - d_{10,3}(\mu m)}{d_{50,3}(\mu m)} \quad (3)$$

2.7. Zeta potential

The zeta (ζ-)potential of emulsion droplets was acquired at 20 °C and pH 7 utilising electrophoretic light scattering (Zetasizer Nano series, Malvern Panalytical, Malvern, UK). Prior to the measurement, emulsion samples were diluted with water or aqueous ethanol at ethanol concentrations corresponding to the ones in the final emulsion. The measurements were taken at the same time points as the ones outlined in 2.6. Additionally required values for dielectric constant and viscosity were taken from literature [2,39,45].

2.8. Differential scanning calorimetry

The crystallisation and melting profiles of butterfat and butterfat emulsions were assessed by differential scanning calorimetry (DSC25, TA Instruments, New Castle, USA). Approximately ten milligrams of sample were sealed in a Tzero hermetic pan (TA Instruments, New Castle, USA). The same mass of ethanol-water mixtures at an ethanol concentration corresponding to the level in the sample was used as reference for emulsion samples while an empty reference pan was used for lipid samples. The sample and the reference pan underwent the same heat treatment, which consisted of a four-step heating-cooling cycle. In the case of butterfat, the first step included an equilibration time of 3 min at −20 °C, the second step a heating ramp from −20 °C to 80 °C at a rate of 1 °C/min, the third step a three-minute hold period at 80 °C and the final step a cooling ramp to −20 °C at 1 °C/min. It should also be noted that the lipid was added to the pan in a fully melted state. For emulsion samples, the maximum temperature was set to 60 °C, whereas the minimum temperature was −5 °C for samples containing ethanol and 5 °C for emulsions without ethanol as at lower temperatures the profile would have been dominated by the peak associated with the crystallisation of water. The cycle was repeated twice to identify

potential hysteresis effects within the samples. In addition to the thermograms, the onset temperature, determined as the temperature of the first deviation from the baseline, the peak temperature relating to the temperature of maximum heat flow and the endset temperature are reported alongside changes in enthalpy (ΔH) during heating. Emulsion samples were analysed at different time points over a storage period of four weeks.

2.9. Cryogenic scanning electron microscopy

Cryogenic scanning electron microscopy was utilised to visualise the morphology of butterfat emulsion droplets. The analysis was carried out by an external laboratory (Reading Scientific Services Ltd, Reading, UK). The experimental protocol shared was as follows.

Emulsions were transferred onto a rivet to form a meniscus and cooled down by using liquid nitrogen. The rivet was mounted into the cryogenic preparation chamber and the top of the meniscus was fractured at −180 °C. The sample was then sublimated at −130 °C for ethanol-containing systems, or at −90 °C if ethanol was not part of the formulation. This process was continued for 60 s, before the sample was coated with gold-palladium for 50 s and imaged in a field emission electron microscope at −150 °C. The secondary electron detector was used for imaging at an accelerating voltage of 3 kV. At the end of the imaging, the samples were warmed to −90 °C and held for 15 min to rule out the presence of ice formation.

2.10. Statistical analysis

All measurements were performed in triplicate. Data plotted in figures represent the mean value plus/minus one standard deviation, shown as error bars. An analysis of variances (ANOVA) was carried out to analyse the statistical significance between average values of different sets of samples. The chosen level of significance was $p = 0.05$.

3. Results and discussion

3.1. Lipid composition

When analysing the results of the thin layer chromatography carried out for the three lipids applied in this research, it became apparent that native and treated sunflower oil differed only to a minor extent, whereas the butterfat showed a significantly different profile (see Fig. S1 in supplementary material). Especially the band indicating the triglycerides, which is the main part of each lipid profile, was much broader and more diffuse than the ones found for the oils. This indicated a more complex mixture of triglycerides, a finding which was confirmed by literature [32,38] and the data obtained through gas chromatography. When comparing the data for native and treated sunflower oil, it was found that the treatment of the oil resulted in a lower overall concentration of free fatty acids, mono- and diglycerides (43 mg/g vs. 50 mg/g for native oil). The main differences were identified in concentrations of the various lipid classes associated with oleic (18:1) and linoleic (18:2) acid. These two fatty acids were also the only ones exceeding a total concentration of 100 mg/g, which was in accordance with findings previously published [10,15,54]. Oleic acid was also found to be one of the main fatty acids in butterfat, but palmitic acid (16:0) was identified as the most predominant one at a concentration of 278 mg/g. Again, the results were in line with literature values [26,42,52]. In general, a larger number of different fatty acids was found to contribute to the lipid profile of butterfat, compared to the ones of sunflower oil, which can be attributed to the more complex and diverse lipid composition in dairy fats, including even- and odd-numbered fatty acid chains [32]. Especially fatty acids with shorter chains (down to C_{10}) were only identified when analysing butterfat. Previous studies reported the presence of fatty acids with even shorter hydrocarbon chains (down to C_4) in milk fat [32,38]. Curiously, such short-chain fatty acids were not found in the present study, even though previous publications reported individual concentrations of up to 35 mg/g. It should also be noted that only about 74 wt% of the butterfat sample analysed could be chemically identified. Fatty acids with a chain length of C_{24} were only found in sunflower oil. In total, butterfat showed the highest concentration of free fatty acids, but the lowest concentration of mono-, di- and triglycerides. In fact, monoglycerides were not identified in the butterfat sample analysed. The total concentration of non-triglyceride components was at 3.3 wt%, which was slightly above the range between 2 and 3 wt% previously reported in literature [52,6]. The numerical results of the fatty acid profile analysis are provided in the supplementary material (Tables S1 – S3).

3.2. Mutual solubility of lipid and aqueous ethanol

3.2.1. Solubility of lipid in aqueous ethanol

The relative solubility of treated sunflower oil, native sunflower oil and butterfat in aqueous ethanol is reported in Fig. 1. Lipid solubility in the aqueous phase increased with increasing ethanol concentration. This trend was caused by the molecular structure and properties of ethanol as it possesses a (short) hydrocarbon chain which increases the lipid solubility due to hydrophobic effects, as well as hydrophilic parts [64]. Hence, a positioning of lipid in the aqueous phase was energetically favourable and, thus, more likely if ethanol was present. Solubility was higher at elevated temperature (60 °C), as expected based on literature reports [27,60]. Comparing the three lipids at the same conditions (temperature, ethanol concentration), butterfat showed the highest solubility followed by native and treated oil. The presence of fatty acids with shorter hydrocarbon chains in butterfat (see 3.1) was the likely reason for the elevated solubility as it was previously reported that a shorter chain correlates with a higher solubility in water and aqueous ethanol [60]. The most likely explanation for the respective trend of the two oils is the difference in concentration of non-triglycerides, i.e., free fatty acids mono- and diglycerides (see 3.1) as those fractions are assumed to dissolve in aqueous ethanol more easily. The data sets for butterfat and native sunflower oil showed an almost linear relationship between the ethanol concentration and the relative solubility, which was indicated by high values for the coefficient of determination (r^2; $r^2 > 0.97$, see Table S6) obtained when applying linear regression. Utilising the slopes of such linear functions as a measure of general solubility revealed a 56 % higher value in butterfat compared to native oil (at 60 °C). An increase in temperature (from 20 °C to 60 °C) led to an increase in solubility of the oil by 42 %. The data for treated sunflower oil followed a quadratic relationship between ethanol concentration and

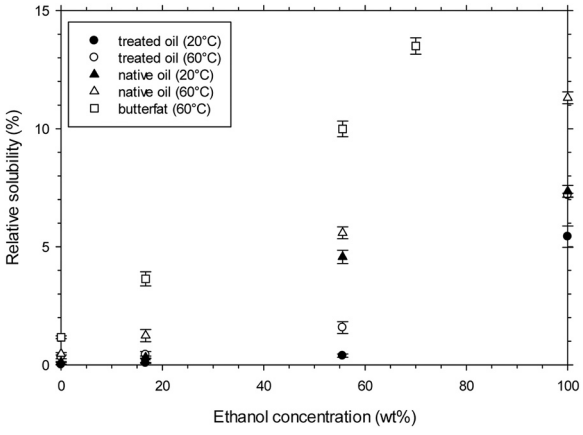

Fig. 1. Relative solubility of lipids in aqueous ethanol with differing ethanol concentration. The initial mixing took either place at 20 °C (filled markers) or at 60 °C (hollow markers). The data correspond to the average of three measurements with error bars representing one standard deviation. Where not visible error bars are smaller than the markers.

relative solubility and, hence, resulted in much lower values for the coefficient of determination when applying linear regression ($r^2 < 0.87$, see Table S6). Data for butterfat in pure ethanol are missing since this mixture was inseparable despite the density difference between the two phases (0.749 g/cm^3 for ethanol and 0.898 g/cm^3 for butterfat, 60 °C). The relative solubility of butterfat in an aqueous ethanol solution at 70 wt% ethanol is reported instead, resulting in a higher value than the one obtained for native sunflower oil at 100 wt% ethanol.

3.2.2. Solubility of ethanol in lipids

The relative solubility of aqueous ethanol in the three lipids is reported in Fig. 2. As for the reverse case (solubility of lipid in aqueous ethanol), solubility increased with increasing temperature and increasing ethanol concentration. Again, the lowest solubility was found for treated sunflower oil. However, compared to the data shown in Fig. 1, the relative behaviour of native oil and butterfat was different. At each ethanol concentration investigated, the relative solubilities for these two lipids showed no statistically significant difference ($p > 0.05$; see Tables S7 and S8), while again following a linear relationship as a function of ethanol level. In general, it was found that the solubility of aqueous ethanol in each of the lipids was consistently higher than the solubility of the respective lipid in aqueous ethanol (note the different y-axis scale between Fig. 1 and Fig. 2), with the difference increasing with ethanol concentration. This trend was most pronounced for native sunflower oil, where the slope of the linear regression function was 64 % higher than the one found for the function describing the relative solubility of that oil in aqueous ethanol (at 60 °C, see Table S9). The molecular structure (balance of hydrophilic and hydrophobic domains) as well as the size of ethanol molecules allow for a minimisation of surface free energy of the system, even when ethanol is positioned in the lipid phase. Lipid molecules, however, are significantly larger - the results of the lipid composition analysis revealed hydrocarbon chains with a length of up to 24 (see Tables S1 – S3) - and almost exclusively hydrophobic. These characteristics lead to larger energy constraints associated with a positioning of lipid molecules in the aqueous phase, resulting in a lower solubility of lipid in aqueous ethanol than vice versa.

3.3. Interfacial tension

The equilibrium data for the interfacial tension at the lipid/aqueous ethanol interface in the absence and presence of sodium caseinate in the aqueous phase are reported in Fig. 3. As expected based on previously reported results, the interfacial tension was lower in presence of sodium caseinate and decreased with increasing ethanol concentration [16,22,28,51] as well as increasing temperature [33,37]. In the presence of sodium caseinate and with increasing ethanol concentration (Fig. 3b), values became too low to be within measurement range of the selected technique (drop profile tensiometry). As expected, the highest interfacial tension values were recorded for treated oil, followed by native oil and butterfat. Comparing the two oils, the presence of a higher proportion of non-triglycerides, which are known to be surface-active [11], in native sunflower oil (see 3.1) explained the lower interfacial tension. In the case of butterfat, the shorter hydrocarbon chain of the predominant fatty acids resulted in the comparatively lowest interfacial tension value [80]. The difference in interfacial tension between the lipids decreased with increasing ethanol concentration but remained statistically significant throughout (see Tables S10 – S13). In agreement with published literature [3], interfacial tension correlated inversely with the solubility between the two phases, as a higher solubility (at a given temperature) generally resulted in a lower interfacial tension value between the lipids and aqueous ethanol (see 3.2). Overall, the interfacial tension values reported for butterfat/water interface were within the range expected based on literature [36,57], given the fact that butterfat is a biologically sourced dairy lipid and, hence, subject to natural variations in composition [26]. The data acquired at the native sunflower oil/water interface were in general accordance with literature values [25,30,57,79] and any deviations likely the result of different measurement techniques (Du Noüy ring or Wilhelmy plate versus drop profile).

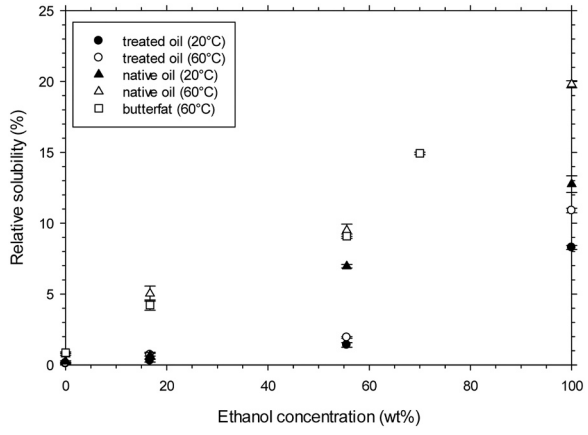

Fig. 2. Relative solubility of aqueous ethanol in different lipids. The initial mixing took either place at 20 °C (filled markers) or at 60 °C (hollow markers). The data correspond to the average of three measurements with error bars representing one standard deviation. Where not visible error bars are smaller than the markers.

3.4. Stability of processed emulsions

3.4.1. Visual appearance

It was not possible to prepare stable butterfat emulsions at 50 wt% ethanol for neither point of ethanol addition (pre- or post-processing). As depicted in Fig. 4, the visual appearance of the instability differed for the two processing protocols. When ethanol was added before the homogenisation, two distinct phases formed immediately upon leaving the microfluidizer and the top layer turned into a semi-solid fat crystal network within 30 min (Fig. 4a). The addition of ethanol after homogenisation resulted in a less distinct separation of clearly visible aggregates from the emulsion phase, see Fig. 4b and Fig. 4c. However, during the first two days of storage, the sample appeared to phase invert, resulting in solidification of about 90 vol% of the sample, resembling the texture of butter. Previous work showed that the emulsifying properties of sodium caseinate are compromised at 50 wt% ethanol [28]. Hence, for emulsion processing in the presence of ethanol, the interfacial stabilisation was not sufficient to result in small enough emulsion droplets to prevent creaming and, consequently, partial coalescence of butterfat crystals which formed upon cooling after the processing. An ethanol addition after the processing step resulted in changes of the protein properties, leading to the flocculation of protein and the formation of a network structure which comprised almost the entire sample.

The visual appearance of emulsions prepared with treated sunflower oil was similar to previous reports [28]. Emulsions containing 0 or 15 wt % ethanol showed no signs of gravitational separation, whereas at 50 wt % ethanol a sediment layer was clearly visible after one day if ethanol was added before homogenisation. In the case of native oil, none of the emulsions were visually unstable – not even the system at 50 wt% ethanol. Butterfat emulsions at 0 and 15 wt% ethanol were of slight yellow hue and showed no signs of gravitational separation.

Assessed by incrementally changing ethanol concentration and visual observation, the limit in stable final butterfat emulsions (at 10 wt% lipid) was 23 ± 0.5 wt% for ethanol addition before homogenisation and 30 ± 0.5 wt% for ethanol addition after emulsion processing. These concentrations were below the critical level for compromising protein properties [28], but apparently processing in the microfluidizer resulted in the formation of an insufficiently dense layer of protein to prevent interactions between ethanol and the lipid phase. In the case of butterfat, this led to changes in crystallisation behaviour, potentially

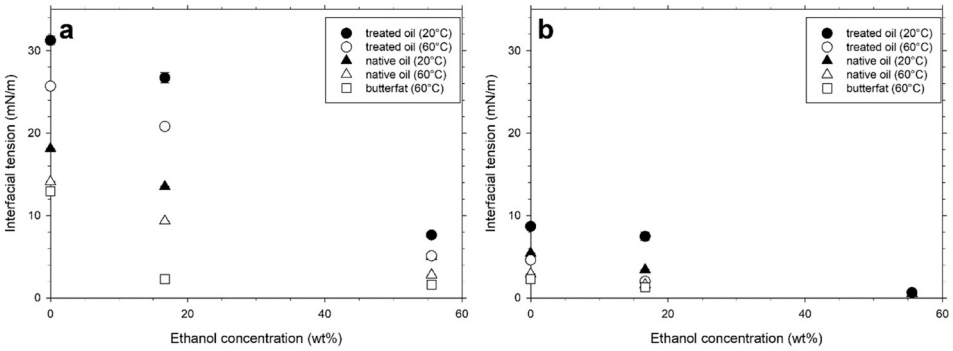

Fig. 3. Equilibrium interfacial tension of the lipid/aqueous ethanol interface, a) in the absence of sodium caseinate, and b) at 3.3 wt% sodium caseinate in the aqueous phase; data were acquired at 20 °C (filled markers) and at 60 °C (hollow markers). For sodium caseinate-containing systems at 50 wt% ethanol and at 60 °C, the equilibrium data approximated the detection limit of the equipment, hence, the lack of data in b). The data correspond to the average of three measurements with error bars representing one standard deviation. Where not visible error bars are smaller than the markers.

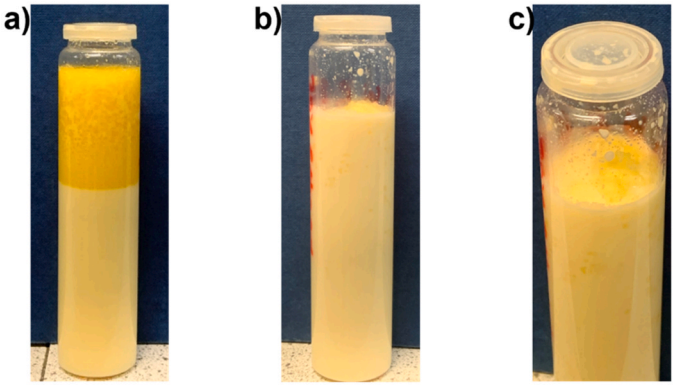

Fig. 4. Visual appearance of butterfat-in-water emulsions containing 50 wt% ethanol (a) addition of ethanol before homogenisation, b) and c) addition of ethanol immediately after homogenisation). b) and c) are showing the same sample, only from two different angles for a clearer view on the solid structure in the centre of the vial.

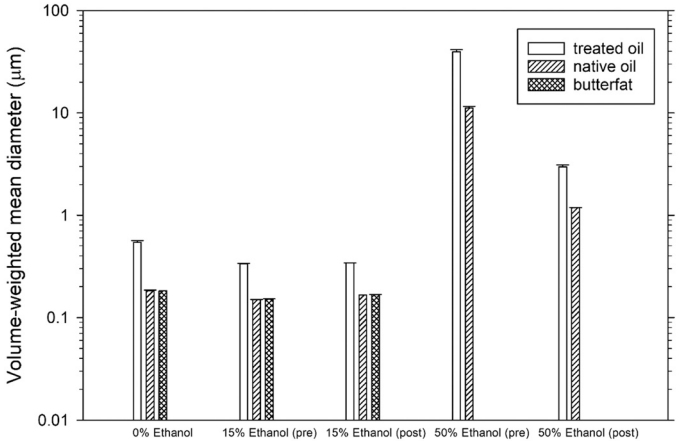

Fig. 5. Mean droplet diameter for three different lipids (treated sunflower oil, native sunflower oil and butterfat) and three different ethanol concentrations (0, 15 and 50 wt%). Ethanol addition took either place before (pre) or after (post) homogenisation. For butterfat emulsions no data at 50 wt% ethanol could be obtained (see 3.4.1). The data correspond to the average of five measurements over a storage time of four weeks, with error bars representing one standard deviation.

caused by the mutual solubilisation of lipid fractions and ethanol (see 3.2). Apparently, the crystallisation characteristics only changed if the solubility exceeded a certain level. If the emulsion was processed in the absence of ethanol, the critical ethanol concentration to trigger destabilisation due to aggregation of butterfat droplets was found to be higher than for processing in the presence of ethanol. Potentially, the protein layer on the interface was denser, limiting the interactions between ethanol and lipid more sufficiently.

3.4.2. Droplet size

The average values for the volume-weighted mean droplet diameter over a storage of four weeks are reported in Fig. 5 for the three different lipids and three different ethanol concentrations applied in this study. The droplet size data used to calculate those average values are presented in the supplementary material (Tables S14 – S18). Regardless of the ethanol concentrations, the mean droplet diameter was larger for treated oil as dispersed emulsion phase compared to native oil and butterfat (where applicable), which overlapped. The generally higher interfacial tension for treated oil (see 3.3) led to an increased resistance to droplet break-up during homogenisation [74], resulting in larger droplets. Over storage, only the emulsions formulated with treated sunflower oil showed statistically significant changes in droplet diameter within the four-week storage period (see Tables S14 – S18). Due to the surface-active nature of ethanol (see 3.3) and the emulsifying properties of sodium caseinate at 15 wt% ethanol [9,28], adding ethanol before homogenisation ("pre" in Fig. 5) resulted in smaller droplets compared to adding ethanol after droplet break-up ("post" in Fig. 5). However, the addition of ethanol after homogenisation to a final concentration of 15 wt% resulted in a decrease in mean emulsion droplet diameter compared to the 0 wt% ethanol system. It was hypothesised that phenomena like an ethanol-induced decrease in interfacial layer thickness of the protein and the solubilisation of lipid out of the droplet contributed to the decrease in droplet diameter. However, solubilisation of lipid into the continuous phase is known to increase the extent of Ostwald ripening in the system [65] – a destabilisation mechanism which was not found for the present emulsions, based on their droplet size distributions and stability upon storage. Consequently, solubilisation processes were concluded to only have a minor effect. The mean droplet diameters for native oil and butterfat emulsions were indiscernible, indicating that the presence of a crystallising lipid phase did not impact on the initial stabilisation of the emulsion. This was expected as the processing temperature was above the melting point of each butterfat fraction (see 3.5). Since butterfat was found to be mainly composed of triglycerides (see 3.1) which are known to not carry any charge [14], butterfat crystals were assumed to be neutral as well. Therefore, they would weaken the ζ-potential of the (charged) protein layer around the droplets if present on the droplet surface. The results obtained for the ζ-potential of the samples indicated that even after

storage for four weeks there were no butterfat crystals present on the droplet surface. Emulsions containing butterfat and native sunflower oil were found to have almost identical surface charges (−71.7 mV (oil) vs. −70.8 mV (butterfat)) which did not change significantly over time (see Table S19). Hence, it seemed likely that their interfacial arrangement and coverage with protein was similar. It is hypothesised that any butterfat crystals were only present within the emulsion droplets and not at the interface.

At 50 wt% ethanol, adding ethanol before homogenisation resulted in emulsion droplets one order of magnitude larger than for an addition after homogenisation. This was in line with previous results, as ethanol at such concentrations diminishes the emulsifying properties of sodium caseinate and the interfacial stabilisation [28]. However, an addition of ethanol after emulsion processing allows for initial droplet stabilisation by 'uncompromised' protein thus mitigating the effect of the change in solvent conditions on protein conformation [22]. Even for this system, the ethanol-induced rearrangement processes of interfacially-adsorbed protein at 50 wt% ethanol resulted in the formation of droplets one order of magnitude larger than in the presence of a lower level of ethanol (0 or 15 wt%).

Emulsions containing butterfat and native sunflower oil had a significantly smaller Span value at 0 and 15 wt% ethanol than the respective emulsions formulated with treated sunflower oil (as reported in Tables S14 – S16 in the supplementary material). In the case of the latter, the Span values were the same at 0 and 15 wt% ethanol. For the other two lipid phases (native sunflower oil and butterfat), the Span values were lower if ethanol was present, whether it was added before or after processing. It was previously reported that a lower interfacial tension decreases the droplet diameter as droplet break-up and interfacial stabilisation dominate recoalescence during the homogenisation process, which also leads to narrower droplet size distributions [50]. At 50 wt% ethanol, the data for the width of the droplet size distributions revealed different trends for the two oils. For native oil, emulsion processing with 'compromised' protein, i.e., in the presence of ethanol, resulted in a higher Span value compared to when ethanol was added after emulsion processing, i.e., droplet break-up and initial stabilisation was facilitated by 'uncompromised' protein. The opposite was observed for treated sunflower oil as dispersed emulsion phase. Apparently, the absence of a sufficient amount of emulsifying matter on the inside of the droplets alongside the diminished interfacial activity of the protein led to immediate droplet coalescence which resulted in one narrow population of larger droplet diameter if the emulsion was processed in the presence of ethanol.

3.5. Thermal properties of butterfat systems

Fig. 6 depicts the thermograms acquired on butterfat (Fig. 6a), emulsions containing no ethanol (Fig. 6b), and emulsions containing 15 wt% ethanol, added before and after homogenisation (Fig. 6c and Fig. 6d). All emulsions contained 10 wt% butterfat. Thermograms of the first and second heating-cooling cycle overlapped for all samples, hence, no hysteresis effects were identified and only the data for the first cycle are shown. The corresponding values for onset, peak and endset temperature as well as the changes in enthalpy are reported in the

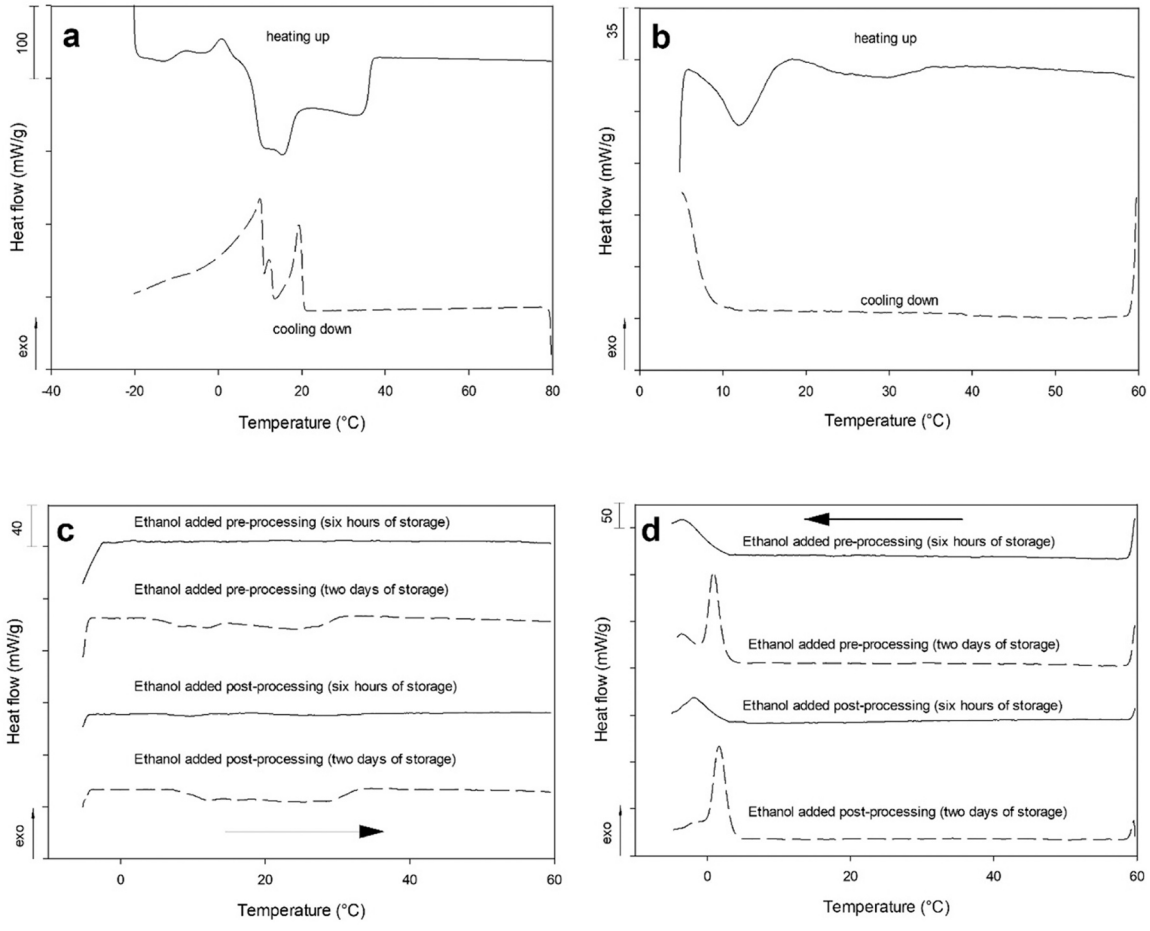

Fig. 6. a) Melting/crystallisation profile of pure butterfat; b) melting/crystallisation profile of a butterfat-in-water emulsion (10 wt% butterfat), after one day of storage. c) melting profile of ethanol-containing (15 wt% ethanol) butterfat emulsions (10 wt% butterfat), after six hours and two days of storage at 5 °C; d) crystallisation profile of ethanol-containing (15 wt% ethanol) butterfat emulsions (10 wt% butterfat), after six hours and two days of storage at 5 °C. The normalised curves were shifted along the ordinate for better visualisation. Note the different scales of the x- and y-axes throughout.

supplementary material (Tables S20 and S21).

Butterfat showed a broad melting temperature range similar to what has previously been reported in literature [46,66,69]. Previous studies reported three melting peaks, each ascribed to specific lipid fractions of milkfat [48,67]. However, only two endothermic peaks were found here, with peak temperatures of 15.4 °C and 32.4 °C between the onset and endset temperature of 7.6 °C and 38.3 °C, respectively. Such temperature values are in accordance with what is commonly described as the low- and medium-melting fraction of milkfat and can be attributed to the melting of lipid fractions containing short-chain or unsaturated fatty acids, such as oleic acid or palmitoleic acid [40,44]. As the butterfat applied in this study is an anhydrous milkfat, the lack of a high-melting fraction matches previous reports [48]. The cooling trace revealed multiple distinct peaks (see Table S21) indicating phase transitions within a temperature range between 20.7 °C and −20 °C (temperature limit of analysis). This behaviour was previously reported in literature [66,69] and is also caused by different thermal properties of the triglycerides composing butterfat.

The anomalous exothermic events identified between −12 °C and 5 °C upon heating were found to be reproducible but cannot be fully scientifically explained at this point. However, the authors would like to point out once more that with the compositional analysis (see Table S3) only about 74 wt% of the butterfat applied in this research could be chemically identified. It cannot be ruled out that some of the unidentified components caused such exothermic peaks.

The thermogram of the butterfat emulsion containing no ethanol (Fig. 6b) showed two melting peaks at 11.7 °C and 31.7 °C with onset temperatures of 6.7 °C and 18.5 °C. The corresponding endset temperatures were 17.7 °C and 35.0 °C, respectively, confirming that the overall melting temperature range was the same as for pure butterfat. However, it appeared that the confinement of butterfat to small droplet volumes affected the types of crystals formed during cooling. This hypothesis is congruent with the observation that the crystallisation onset temperature was decreased compared to the pure lipid phase (7.5 °C vs. 20.7 °C), a behaviour previously reported for milkfat emulsions [71]. It should be noted that the crystallisation onset temperature for the emulsion was close to the limit of the temperature range investigated as well as the freezing temperature of water. The distribution of butterfat in emulsion droplets also resulted in a decreased change in enthalpy associated with the thermal transitions during the heating process per mass of lipid (16.5 J/g vs. 20.4 J/g, see Table S20). Lastly, the presence of a viscoelastic layer of sodium caseinate on the droplet surface is the most likely reason why the butterfat crystallisation within the emulsion droplets did not result in destabilisation via partial coalescence (see 3.4.1).

The melting behaviour of the ethanol-containing emulsions (Fig. 6c) was more complex and depended on the time the samples were stored at 5 °C after emulsion processing. For either point of ethanol incorporation into the emulsion, the melting thermograms acquired after six hours of storage at 5 °C showed a peak at the lower limit of the temperature range analysed. It was previously shown that the presence of casein [61] and ethanol result in freezing point depression of water [43,45,72]. For the emulsions investigated in this study, the freezing point depression was found to be around 13 °C, thus, the peak aforementioned is assumed to be related to a lipid fraction with a low melting point in the system rather than the continuous emulsion phase. At this point in time (after six hours of storage at 5 °C), the low temperature thermal transition was the only one recorded for the emulsion processed in the presence of ethanol. Although barely visible in the thermogram (Fig. 6c), two additional peaks were recorded for ethanol addition immediately after processing. They were at 9.1 °C and 23.6 °C, within the broad temperature range of thermal transition between 7.1 °C and 30.8 °C. After two days of storage at 5 °C, the former emulsion (ethanol added before processing) showed a similar melting behaviour while the peak temperatures were slightly lower than for the emulsion to which ethanol was added after processing. For the latter emulsion, an increase in peak temperatures over storage was identified (see Table S20). It was also found that the peaks associated with the thermal transitions intensified over storage at 5 °C for both emulsions, resulting in higher melting enthalpies (see Table S20). However, the peak temperatures for both systems remained lower than the corresponding values for the emulsion processed and stored in the absence of ethanol. Such phenomena indicated that ethanol had an impact on lipid crystallisation, affecting the kinetics and polymorphism, a trend not previously reported in literature. So far, changes in lipid crystallisation were mostly linked back to the cooling rate applied [12,49] and changes in lipid content in emulsions [68].

The cooling thermograms (Fig. 6d) revealed onset temperatures of fat crystallisation (see Table S21) which were below the storage temperature of the processed emulsions (5 °C). The initial peak (around 60 °C) in these thermograms was not further considered as it was assumed to be the result of start-up effects of the measurement, given that pure butterfat did not show a thermal transition in this temperature range (see Fig. 6a). After two days of storage, the onset temperatures of the exothermic peaks were slightly higher than after six hours of storage, but still lower than 5 °C. The change in onset temperature was likely caused by ethanol-induced rearrangement processes of the interfacially-adsorbed protein as well as interactions between ethanol and the lipid within the emulsion droplets. The mutual solubility of ethanol and butterfat (see 3.2) seems to be the most likely explanation for the change in thermal behaviour of butterfat in the presence of ethanol. If ethanol penetrates both the interfacial layer of protein and the lipid inside the emulsion droplet, it is likely to affect the crystallisation behaviour by interacting with different lipid fractions. The calorimetry data in conjunction with the storage temperature selected in this research suggest that the butterfat emulsions containing 15 wt% ethanol had in fact an entirely liquid droplet phase, supporting the earlier presented argument that lipid crystallisation had no bearing on the stability of the ethanol-containing emulsions investigated (see 3.4.2). Finally, it should be noted that thermograms acquired after four weeks of storage at 5 °C coincided with those acquired after two days, for all three butterfat emulsions considered.

3.6. Microstructure

Micrographs obtained by cryogenic scanning electron microscopy are shown in Fig. 7. When analysing the emulsions investigated in the previous Sections (3.4 and 3.5), it was found that the resolution of the equipment utilised was not high enough to allow statements on the topography or the interfacial arrangement of the droplets. Nevertheless, the micrographs were in line with the findings for droplet size distributions of the systems investigated (butterfat and 0 wt% ethanol; butterfat and 15 wt% ethanol – added before homogenisation) discussed in 3.4.2, with the droplets in both samples being of similar diameter (Fig. 7a and b). In order to investigate the interface of the samples with the given resolution/magnification, the pre-emulsion (before processing in the microfluidizer) was imaged as well. As expected, the resulting droplets were considerably larger (in the range of 5 μm). It was found that the droplets in these systems were (again) highly spherical and showed a roughened and undulated surface (Fig. 7c). When imaging the cross-section of a fractured droplet, it could be seen that the inside of the droplet had a similar structure, which suggested that this was the general arrangement of the fat within the entire droplet (Fig. 7d). Since the dispersed emulsion phase was believed to only consist of butterfat, this finding matched the expectations. The micrographs of the pre-emulsion also showed fine irregularly shaped particles on the droplet surface as well as in the bulk of the emulsion (arrows in Fig. 7c). The position of such entities suggested that those might be casein micelles, which was supported by the fact that the size found experimentally was in good accordance with the size of casein micelles reported in literature (40 - 300 nm; [75]).

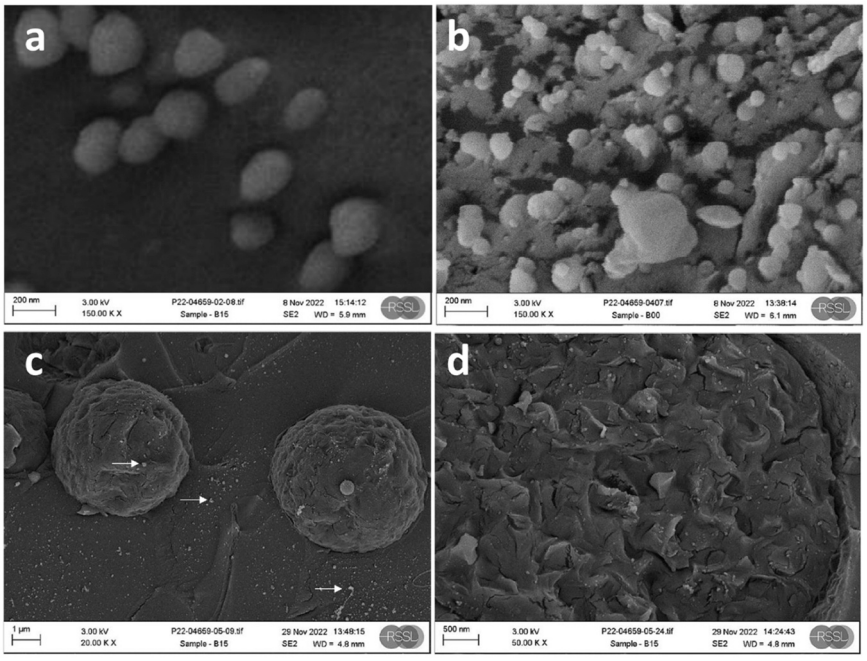

Fig. 7. Scanning electron microscopy micrographs of butterfat emulsions, a) containing 15 wt% ethanol – incorporated before homogenisation, processed in a microfluidizer; b) containing 0 wt% ethanol, processed in a microfluidizer; c and d) containing 15 wt% ethanol – incorporated before homogenisation, processed in a high- shear overhead mixer, with d) showing the cross-section of a butterfat globule. Arrows in c) point at structures believed to be casein micelles.

4. Conclusions

This study revealed that the detrimental impact of ethanol at elevated concentrations on the emulsifying properties of protein does not inhibit the manufacture of a commercially viable product, stable against creaming, if a non-crystallising lipid phase is utilised. While these novel findings do not offer solutions for traditional cream liqueur formulations, they pave the way for alternative product ranges. In agreement with existing literature, an upper limit of ethanol concentration for emulsions containing a crystallising lipid phase (butterfat) was found, above which creaming and fat globule clustering were observed. Notably, by adding ethanol after emulsion processing in the microfluidizer, this threshold could be increased from 23 wt% to 30 wt %. The critical concentrations themselves may be explained by the demonstrated mutual solubility of butterfat and ethanol, affecting the crystallisation behaviour. At a sub-critical ethanol level (15 wt%), butterfat crystallisation was suppressed and only detected below the storage temperature of 5 °C, precluding any contribution of crystals to the interfacial stabilisation.

Overall, it can be concluded that the instability of traditional cream liqueur formulations at elevated ethanol concentration is not exclusively caused by lipid crystallisation or the ethanol-compromised emulsifying capacity of the sodium caseinate, utilised for interfacial stabilisation, but by the interplay of the protein properties and the crystallisation characteristics of the lipid.

CRediT authorship contribution statement

Pelan Eddie: Methodology, Writing – review & editing. **Erxleben Stephan:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Wolf Bettina:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfa.2024.133233.

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