# The formation of double emulsions in skim milk using minimal food-grade emulsifiers – A comparison between ultrasonic and high pressure homogenisation efficiencies

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## Abstract

Double emulsions of W1/O/W2-type were formed in skim milk. Skim milk (W1) was emulsified within sunflower oil (O) using ultrasonication that was in turn emulsified within an external skim milk phase (W2) using ultrasonication or high pressure homogenisation (HPH). The internalised aqueous phase was stabilised within the oil phase using food-grade surfactants: polyglycerol polyricinoleate (PGPR) and/or lecithin. Encapsulation yields of the W1/O emulsion into the double emulsion were between 30-100%, with increased yields achieved with reduced sonication time or HPH pressure, or increased PGPR or lecithin concentration. Ultrasonication was found to form relatively better monodisperse emulsions that showed greater stability to coalescence than those produced by HPH. Ultrasonication and HPH were found to be translatable in the sense that at a similar specific energy density (~ 20 J/g) emulsion droplet sizes with a similar size distribution between 1-10 μm and encapsulation yield (*ca* 37 wt%) could be achieved.

## Keywords

Double emulsion; skim milk; ultrasonication; high pressure homogenisation; lecithin; PGPR

## 1. Introduction

The development of reduced-fat food products that retain the same sensory properties as those of full fat products is of high interest to the food industry. One promising method by which this can be achieved in foods such as sauces and cheese, and in various beverages is by creation of what are known as double emulsions (Muschiolik and Dickinson, 2017). A double emulsion is, in simple terms, an emulsion that is dispersed within another emulsion. For reduced fat products, the double emulsions of interest are typically water-in-oil-in-water type (W1/O/W2). That is, water droplets are emulsified within an oil phase that is emulsified as droplets within an external aqueous phase. The result is an emulsion containing oil droplets partially occupied by an internalised water phase. Fat reduction can be achieved without compromising sensory properties if the emulsion occupies a similar fat phase-volume as a full fat emulsion. The strategy of employing double emulsions in foods for fat reduction has been patented for products such as salad dressings (Gaonkar, 1994), low fat spreads (Okonogi et al., 1994) and previously reported for potential application in reduced fat cheese (Felfoul et al., 2015; Lobato-Calleros et al., 2007; Lobato-Calleros et al., 2006; Lobato-Calleros et al., 2008) and meat (Serdaroğlu et al., 2016). Other promising double emulsion applications in food can be found in a recent comprehensive review (Muschiolik and Dickinson, 2017).

The uptake of double emulsions in the food industry has been limited to date. Emulsions are inherently unstable thermodynamically, and double emulsions are further complicated by having multiple phases that require stabilisation. Large amounts of surfactants are typically required to stabilise both the inner and outer phases of the formed emulsions (Muschiolik and Dickinson, 2017). There is however, growing interest to replacing or reducing the use of synthetic surfactants in emulsions with natural biopolymer emulsifiers such as polysaccharides and milk proteins (Benichou et al., 2002; Muschiolik and Dickinson, 2017; Shanmugam and Ashokkumar, 2014). Some studies have also reported that the interaction of biopolymers with synthetic monomeric emulsifiers, can improve the stability of double emulsions by creating a gel-like barrier that retards water transport across the internal and external aqueous phases (Dalgleish, 2006; Garti, 1997; Oppermann et al., 2015).

Recently, Leong et al. (Leong et al., 2016) have shown that double emulsions of W1/O/W2 type could be produced from sunflower oil and skim milk using the synthetic surfactant Span80 to stabilise the inner emulsion, and the milk proteins alone to stabilise the outer emulsion. This has particular promise for application in the dairy industry. The double emulsions were demonstrated to be sufficiently stable to avoid coalescence for up to 7 days. However, due to sub-optimal stabilisation of the W1/O emulsion, a maximum encapsulation yield of only ~ 35% was achieved, even with 20% Span 80 in the oil phase. Reducing the surfactant requirements and replacing Span 80 with fully approved food grade surfactants would represent an improvement to this formulation. Polyglycerol polyricinoleate (PGPR) and soy lecithin, are highly effective food grade lipophilic emulsifiers commonly used in the food industry, and have been successfully used in the formation of double emulsions (Altuntas et al., 2017; Knoth et al., 2005; Muschiolik, 2007; Scherze et al., 2006) with high stability and encapsulation yield. Their usage has yet to be evaluated in the context of forming double emulsions combined with native skim milk proteins in the inner and outer aqueous phases, particularly using high-shear processes that can produce small emulsion droplets.

46
47 In addition to the surfactants used to stabilise the water-oil interfaces, the size of both the inner
48 and outer emulsion droplets is an important factor in double emulsion stability. The creation of
49 smaller emulsified droplets by high-shear processing can improve the stability of a double
50 emulsion by increasing the kinetic stability. The preparation of small-sized primary (inner)
51 droplets, has been shown to be important for providing stability to the system as a whole (Guan et
52 al., 2010; Kanouni et al., 2002), whilst the formation of smaller secondary (outer) emulsion
53 droplets, will reduce the rate of creaming and phase separation as well as improving the 'mouth-
54 feel' of the emulsion (Kentish et al., 2008).
55
56 Various high-shear devices are available that can be used to produce the primary and secondary
57 emulsions including ultrasonication, high pressure homogenisers, high-shear mixers,
58 Microfluidisers, and membrane systems (Leong, 2016). Ultrasonication is of particular interest for
59 the production of the primary emulsion, as it can produce small droplets with a narrow size
60 distribution, and be implemented as a reasonably simple and robust unit operation (Jafari et al.,
61 2007). The second emulsification step is a considerable challenge in double emulsion production,
62 as there is a fine balance between creating smaller emulsion droplets that are beneficial to stability
63 and sensory properties, without causing excessive droplet breakup that will result in loss of
64 encapsulated material. In addition, the second emulsification stage involves a much greater
65 volume of material than the first step (typically 5-10 times greater depending on the level of
66 encapsulation), which makes reducing the energy and capital investment associated with this step
67 important for large scale applications. While ultrasound has the potential to be operated at large
68 scale, it is still considered a reasonably new technology. High pressure homogenisation is another
69 effective technology for creating emulsions for which there are commercially available off-the-
70 shelf units already in established large scale operation in the food and beverage industry. The
71 relative effectiveness of ultrasonication and high pressure homogenisation in the second
72 emulsification stage is yet to be evaluated.
73
74 To compare the effectiveness of both systems, the properties of the resultant double emulsions
75 need to be compared in relation to the amount of energy used in their formation. A suitable basis
76 for comparison is the delivered energy density. For ultrasonication, a typical measurement is the
77 calorimetric power delivered to the fluid (Kimura et al., 1996; O'Sullivan et al., 2017), which is
78 the product of energy intensity and processing time. For high pressure homogenisation, the
79 pressure that the fluid is subject to provides a direct indication of the energy density applied to the
80 system. The units for pressure can be directly converted to energy/unit volume i.e., 1 MPa is
81 equivalent to an energy density of 1 J/mL. There is a concern that using high shearing techniques
82 such as ultrasonics and high pressure homogenisation for double emulsion formation may lead to
83 excessive loss of encapsulated material (Lamba et al., 2015). However, this can be minimised
84 provided suitable formulations and operating conditions are used. For example, the use of
85 ultrasonication in the production of stable double emulsions for encapsulation of aspirin has been
86 reported, achieving entrapment yields of up to 99% (Tang and Sivakumar, 2012; Tang et al., 2013).
87 It is yet to be established how to best produce stable, high-encapsulation-yield double emulsions
88 in skim milk while minimising the amount of energy and food grade surfactant used.
89
90 In the present study, the creation of double emulsions using the food grade lipophilic surfactants
91 PGPR and/or lecithin in the oil phase, and natural skim milk proteins alone as the main stabiliser

of the secondary droplets in the external phase, is investigated. The use of different high shear techniques, namely ultrasonication and high pressure homogenisation in the second emulsification step are also investigated and compared on the basis of energy applied to the system, to determine their viability for double emulsion production directly in skim milk.

## 2. Materials and Methods

### *2.1 Materials*

The oil phase used in this study was sunflower oil (Woolworths Homebrand, Australia) purchased off the shelf. To promote and stabilise the inner W1/O emulsion, the surfactants polyglycerol polyricinoleate (PGPR) and soy lecithin were used. These emulsifiers were kindly provided by a confectionery company located in Australia. Pasteurised and homogenised skim milk (Paul's brand, Australia) with <0.1% w/v fat and a total protein content of 4.2% w/v, purchased from a supermarket, was used for all trials as the basis for both the inner and outer aqueous phase. Sodium azide (Chem Supply, 99 %, Australia) was added at ~0.02 wt% to each batch of milk to limit microbial growth during refrigerated storage.

### *2.2 Primary emulsification using ultrasound*

A two-step emulsification process adapted from Leong et al. (Leong et al., 2016) was employed for the preparation of the double emulsions. In the first step, the inner aqueous phase (skim milk containing 4% w/w sodium chloride as an entrapment marker) was loaded at a concentration of 30% w/w into a sunflower oil/PGPR/lecithin mixture and emulsified using a 20 kHz 3 mm microtip ultrasonic horn (Branson Ultrasonics, USA) inside a 15 mL test tube. The concentration of PGPR used was 1 wt% of the oil phase unless otherwise specified. The concentration of lecithin used varied between 0 to 10 wt% of the oil phase as specified in the text. The total mass of the W1/O emulsion formed was 7.5 g. Sonication was performed at 10 W calorimetric power (an amplitude setting of 30%) and a duration of 90 s (specific energy = 120 J/g), until the emulsion formed was homogenous in appearance without obvious pooled regions of unemulsified aqueous phase. The horn tip was positioned at a fixed position approximately 40-50 mm from the bottom of the test tube, so that it was located above the oil/water interface.

### *2.3 Secondary emulsification using ultrasound*

In the second emulsification step, 0.375 g of the pre-formed W1/O emulsion was emulsified into skim milk using ultrasound to create a double emulsion with a total mass of 7.5 g (i.e., 5 % w/w final W1/O loading concentration). Ultrasound was applied at a fixed calorimetric power level of 6 W (i.e. 20% amplitude setting) for varying durations as specified in the text. The horn tip was positioned at a fixed location near the top of the tube, between 3 to 5 mm from the surface of the sample near the oil/water interface. All emulsions were prepared in triplicate.

### *2.4 Secondary emulsification using high pressure homogenisation*

Emulsions (100 mL) containing a 5 wt% W1/O loading were formulated. The W1/O emulsion consisted of a fixed formulation of 1 wt% PGPR, 2 wt% lecithin and 30 wt% skim milk (containing 4 wt% NaCl). The bulk 100 mL emulsions were first pre-emulsified using an Ultraturrax stirrer (4500 rpm, 2 min) prior to loading into the sample hopper of the high pressure homogeniser (GEA Niro Soavi homogeniser, Panda). Samples were passed once through the 1$^{st}$ stage of the

137 homogeniser at selected pressures between 30-200 bar with a constant volumetric flow rate of 10
138 L/hour. Emulsions were prepared in triplicate.
139

140 *2.5 Conductivity measurements*
141 Sodium chloride (4% w/v) was included in the inner aqueous phase as an entrapment marker, with
142 the release of inner phase into outer phase resulting in an increased conductivity associated with
143 the increased salt concentration. To quantitatively relate changes in conductivity to the release of
144 salt from the emulsions after preparation, standard solutions representing 0, 50 and 100% NaCl
145 release were prepared. Standards for each specific formulation used in the W1/O/W2 emulsion
146 were prepared that included the same concentrations of each component, and which were
147 sonicated with 20 kHz ultrasound for 2 minutes at 50% amplitude using an 11 mm horn (82 J/g
148 specific energy based on calorimetry). The total energy delivered was sufficient to ensure
149 complete homogenisation of the fat droplets and limit phase separation and creaming in the
150 standards. Conductivity was measured in the standard solutions and samples after equilibration to
151 room temperature (~23 °C) for 2 hours, using a k=1.0 laboratory conductivity sensor (TPS,
152 Australia) connected to TPS LabCHEM-Cond conductivity meter (TPS, Australia). The
153 conductivity probe was calibrated using a 2.76 mS standard solution.
154

155 *2.6 Scanning electron microscopy*
156 Cryo-scanning electron microscopy (Cryo SEM, FEI Qanta) was used to investigate the surface
157 and internal morphology of the oil-milk double emulsion system. The sample was first transferred
158 into a glass tube (1.3 mm × 1.3 mm × 5 mm in size) and then mounted on a copper holder. The
159 sample and copper holder were quickly immersed into liquid nitrogen slush at -210 °C. After
160 freezing, the frozen sample was immediately transferred into an attached cryo preparation chamber
161 using a vacuum transfer device. The sample was fractured using a chilled scalpel blade within the
162 chamber at -140 °C under high vacuum conditions. The fractured sample was then coated with
163 sputtered gold (6 nm) after etching at -95 °C for 20 min to remove the ice from the surface of the
164 fractured sample. The sample was then transferred under vacuum onto a nitrogen gas-cooled
165 module at -140 °C. The detector used for the SEM observation was a solid state backscattered
166 electron detector (SSD).

167

168 *2.7 Particle size measurements*
169 The particle size of the double emulsion droplets was measured using a Malvern Mastersizer 3000
170 (Malvern Instruments, UK) with Hydro-G3000 accessory. Distilled water was used for dilution.
171 A refractive index of 1.462 and absorption of 0.001 were used by the software to determine the
172 size of the droplets. The particle size of the primary water-in-oil emulsions was determined using
173 a Zetasizer Nano ZS (Malvern Instruments, UK), with sunflower oil used for dilution.
174

175 *2.8 Viscosity measurements*
176 The viscosity of emulsion samples was measured using an AR-G2 rheometer (TA instruments,
177 USA) using a cone and plate configuration with a cone diameter of 40 mm, angle of 2 ° 50" and a
178 truncation gap of 52 mm (TA instruments, serial number 988134). Approximately 0.5 mL of
179 sample was loaded into the geometry. A flow procedure was employed, where the shear rate was
180 increased from 1 to 100 $s^{-1}$ step-wise. The samples were maintained at a temperature of 30 °C
181 throughout the measurement.

### *2.9 Statistical analysis*

All emulsions were prepared in triplicate unless otherwise specified. The statistical significance of results were assessed using the Student's t-test (de Winter, 2013) in Minitab 17 (Minitab Pty. Ltd.) where required. A 95% confidence interval was used to assess statistical significance.

## 3. Results and discussion

### 3.1 Morphology of primary and secondary emulsion droplets

Lecithin and PGPR were chosen as the food grade lipophilic surfactants to stabilise the inner W1/O emulsion. The effect of using these emulsifiers in combination with the skim milk proteins on the morphology and stability of the primary and secondary emulsions resulting from ultrasonication, is of novel interest. To investigate the performance of the surfactants individually and in combination with each other, W1/O and W1/O/W2 emulsions were formed by ultrasonication using PGPR (1 wt% concentration in oil phase), lecithin (2 wt % in oil phase) and combined PGPR with lecithin (1 wt% PGPR, 2 wt% lecithin in oil phase). Microscopic images of these emulsions are shown in Fig. 1 a-c. The W1/O/W2 emulsions here were formed using 30 s ultrasonication at 6 W power.

The W1/O emulsions, formed using lecithin (Fig 1b), display a crystalline gel-like network, which indicates a strong interaction between the emulsified aqueous phase droplets in the emulsion. This behaviour is consistent with that reported in the study by Knoth et al. (Knoth et al., 2005). The PGPR only W1/O emulsion lacks this structure (Fig 1a), instead consisting of what appears to be nano-sized droplets dispersed throughout, and with a noticeable absence of larger droplets. The W1/O emulsions formed containing PGPR were found to be very stable, undergoing minimal observable phase separation for several weeks when stored in the refrigerator. This is likely due to the smaller nano-sized droplets conferring increased kinetic stability (see section 3.2). The W1/O emulsion formed using lecithin alone however, did result in some observed phase separation after several days, likely due to separation of oil from the gel network. Issues were also reported in the use of lecithin alone to stabilize the W1/O emulsion in the recent study by Altuntas et al. (Altuntas et al., 2017).

In each case, the internal morphology of the formed W1/O/W2 double emulsion droplets, resembled the dispersed phase in the W1/O emulsions. The two emulsions containing lecithin are characterised by the same gel-like network dispersed through the oil droplets. Interestingly, the emulsions where only lecithin was employed (Fig 1b) appear to have incomplete distribution of aqueous phase in the oil phase droplets of the W1/O/W2 emulsion despite the morphology of the W1/O emulsion looking reasonably homogenous. This may be connected with the observation that the W1/O emulsion formed using lecithin alone displayed some separation of oil from the gel network during storage. As reported by Scherze et al. (Scherze et al., 2006), the presence of salt with lecithin may cause coalescence and phase separation in formed emulsions. Another possible contributing factor to the incomplete distribution is the increased viscosity of the W1/O emulsion phase resulting from the presence of lecithin (see section 3.2). By contrast, the internalised water phase for emulsions containing PGPR with lecithin are more thoroughly dispersed in the oil droplets (Fig 1a). Also reported by Scherze et al. (Scherze et al., 2006), salt is noted to promote

225 the coalescence stability of emulsions formed with PGPR. This may counteract the
226 coalescence/phase separation problems with lecithin in the presence of salt. The presence of PGPR
227 has also been reported to drastically reduce the yield stress of emulsions (Schantz and Rohm,
228 2005). The implication is that the W1/O emulsions formed with PGPR are able to flow more easily
229 (i.e. have a less rigid structure), and hence the gel-like aqueous phase is more readily able to
230 disperse throughout the oil phase of the W1/O/W2 droplets.

231 The external and internal morphology of individual double emulsions droplets stabilised by milk
232 proteins in the outer phase, and a combination of milk proteins, lecithin and PGPR in the internal
233 phase, was further characterised using cryo-SEM. Micrographs of these emulsions are depicted in
234 Fig. 2.

235 The external morphology is consistent with that previously reported for double emulsions formed
236 in skim milk (Leong et al., 2016). In the interior, small aqueous phase droplets can be seen
237 distributed through a rough network, consistent with the gel-like oil phase seen in the light
238 microscopy images in Fig. 1c. It is speculated that this gel-like network could improve stability in
239 regards to loss of the encapsulated water phase, similar to that as reported previously by studies
240 whereby the internal water phase was gelled using whey proteins (Balcaen et al., 2016; Dalgleish,
241 2006; Oppermann et al., 2015).

242 A general observation of the morphology from light microscopy shows that PGPR facilitates the
243 production of small W1/O droplets and lecithin is able to produce gel-like W1/O structures (Fig.
244 1 a and b). However, the PGPR-only emulsions (at 1 wt% concentration of the oil phase here) did
245 not appear to encapsulate a large amount of aqueous phase within the W1/O/W2 droplets, whilst
246 the double emulsions produced with only lecithin did not appear to be stable due to observable oil
247 separation from the gel-like network and incomplete distribution of the network in the W1/O/W2
248 droplets. By using the two emulsifiers in combination, the gel-like W1/O emulsions could be
249 entrapped within small secondary emulsion droplets, which appeared to be more stable and
250 dispersed more uniformly throughout the oil phase. The encapsulation efficiency of these
251 combined systems is discussed further in the following section.

252 3.2 Effect of lecithin concentration on encapsulation yield, droplet size and W1/O viscosity

253 As the lecithin on its own was found to be ineffective at creating stable double emulsions,
254 combinations of PGPR and lecithin were investigated further. The effect of lecithin concentration
255 on the encapsulation yield of PGPR/lecithin double emulsions was evaluated. In Fig. 3, the
256 encapsulation yields are shown for emulsions containing a fixed amount of PGPR (1 wt% of the
257 oil phase) and varying concentrations of lecithin (0-10 wt% of the oil phase) and formed using a
258 constant ultrasonication duration of 30 s, 6 W (24 J/g) are shown. With no lecithin the W1/O
259 emulsion was seen to consist of small droplets (Fig 1a), and the encapsulation yield was only ca
260 20 wt% (Fig 3). At 2 wt% lecithin the W1/O was seen to consist of a gel-like material (Fig 1c)
261 that was able to be encapsulated to a great extent (ca 35%). Further increases in the amount of
262 lecithin in the presence of PGPR resulted in further increases in the encapsulation yield (and hence
263 displacement of the oil phase with water).

264 To investigate the reasons for the enhanced entrapment resulting from increased lecithin
265 concentration, viscosity of the W1/O emulsions formed with varying concentration of lecithin in
266 the oil phase was measured as a function of the shear rate (see Supplementary Information Fig.
267 S1). There is a clear trend of increased viscosity as a function of increasing lecithin concentration

268 (at 30 °C). The viscosity of the W1/O with 2% lecithin was approximately 4-times greater than
269 that of the PGPR-only W1/O emulsion, consistent with the gel-like structure that was observed
270 (Fig. 1c).

271

272 The implications of increased viscosity in the context of the second emulsification step are as
273 follows. During emulsification of the W1/O into the W2 phase the resulting droplets will be larger
274 for the more viscous W1/O emulsions. This is confirmed from the particle size distribution of the
275 W1/O/W2 emulsions (Fig. 4 a). Note that the peak at 0.1 µm corresponds to the size of casein
276 micelles (Farkye and Shah, 2014) (see Supplementary Fig. S2). The size distribution data show
277 that the emulsions containing a higher lecithin concentration in the oil phase consist of somewhat
278 larger particles, particularly the emulsion containing 10% lecithin. There is a noticeable decline in
279 the volume fraction occupied by sub-micron sized droplets, and a corresponding increase in the
280 droplets >1 µm in diameter with increasing lecithin concentration. This apparent decline in the
281 casein micelle peak at 0.1 µm with increasing lecithin concentration is an artefact of the
282 measurement, due to the larger sized droplets diffracting more light, and hence contributing
283 disproportionately to the signal.

284 The size distributions of the aqueous phase droplets loaded within the corresponding W1/O
285 emulsions formed also display a trend of increasing size with increasing lecithin concentration
286 (Fig. 4 b). At 5% and 10% lecithin these distributions were bi-modal, presumably a consequence
287 of the highly viscous gel-like interaction. For all lecithin concentrations the peak diameter of the
288 W1/O/W2 droplets (Fig. 1a) was considerably greater than that of the corresponding W1/O
289 droplets (Fig. 1b), suggesting that these W1/O/W2 droplets are sufficiently large to accommodate
290 the W1/O.

291 There is not a completely clear relationship between W1/O viscosity, W1/O droplet size, and
292 W1/O/W2 encapsulation rate as the increase in encapsulation rate is reasonably linear as a function
293 of lecithin concentration (Fig. 3), whereas there is relatively minor difference in viscosity between
294 the 5% and 10% lecithin W/O1 emulsion and a much more apparent difference in the W1/O and
295 W1/O/W2 droplet size (Fig. 4). The exact mechanisms are likely quite complex, but it is clear
296 from these results that encapsulation yields can be improved by using lecithin in combination with
297 PGPR. Also, the increased encapsulation yield with higher lecithin concentration, would also need
298 to be considered in practical terms. The high viscosity of W1/O emulsion containing 10wt%
299 lecithin would likely present challenges for production in terms of increasing pumping and
300 cleaning requirements.

301

302 3.3 Effect of secondary emulsification method on encapsulation yield and droplet size
303 distributions

304 The dispersion of W1/O emulsions into skim milk was further investigated by comparing the
305 effectiveness of ultrasonication and high pressure homogenisation (HPH) on the basis of specific
306 energy density delivered to the fluid. W1/O emulsions (formulated with 1 wt % PGPR and 2 wt
307 % lecithin in the oil phase) were emulsified into skim milk using either ultrasonication for varying
308 durations (5-60 s) or a HPH operated at varying pressures between 30 to 200 bar. The size
309 distributions of the resulting W1/O/W2 emulsions formed using ultrasonication or HPH at select

310 conditions are shown in Fig. 5b. Prior to entering the HPH, a coarse emulsion was formed using
311 an Ultraturrax mixer (4500 RPM, 5 min) (for reference, the size distribution of this pre-emulsion
312 is also shown in Fig. 5b). Note that the peak between 0.01 to 1 μm is present in every sample
313 measured and as noted earlier, represents contribution from casein micelles present in the milk
314 and possibly some smaller sub-micron droplets that are formed in the emulsification process. It
315 can be seen that the Ultraturrax (rotor-stator) mixing generated mostly large droplets in the range
316 between 10-100 μm. These droplets were not stable to phase separation. These coarse emulsions
317 were further processed using HPH to yield emulsion droplets in the range between 1 to 50 μm. An
318 increase to the pressure in the HPH, or increase in sonication duration, both resulted in smaller
319 diameter droplets (i.e. a shift to the left in the size distribution), as expected.

320 A size range between 1 to 10 μm, similar to native fat droplets in whole milk, was achievable
321 using a homogenisation pressure of 150 bar, or sonication duration of 30 s (6 W power). For these
322 two processing conditions, the shape of the size distributions and the encapsulation yield (38% for
323 HPH and 37% for US) were statistically the same (P=0.69). The specific energy required for the
324 two treatments was also similar: ~15 J/g for the HPH and ~24 J/g for the ultrasonication. The
325 results therefore indicate that double emulsions with droplets of similar size, and with similar
326 extents of encapsulation can be formed using either ultrasound or HPH for a given energy load.

327 At lower specific energy inputs, the size of the double emulsion droplets increased (Fig. 5b),
328 however the encapsulation yield was seen to decrease for HPH and increase for US (Fig. 5a). The
329 higher encapsulation yield achieved with US at low specific energy (e.g. 4 J/g; 5 s) could be due
330 to a high degree of encapsulation occurring in the large droplets (i.e. >20 micron) that were present
331 in the double emulsions from low-specific energy US but not in the double emulsions produced
332 using HPH at a low specific energy (5 J/g, 50 bar) (Fig. 5b). The HPH is more efficient at creating
333 exclusively small emulsion droplets (Schultz et al., 2004), since the mechanism of the HPH is such
334 that droplets larger than the valve gap in the disruption chamber should not be produced. By
335 comparison, the mechanism of ultrasonication is primarily acoustic cavitation (Kentish et al.,
336 2008; Leong et al., 2009), which is due to the formation and collapse of microbubbles in the fluid.
337 The size reduction of droplets caused by exposure to these collapsing bubbles is inherently
338 stochastic, since the high shear is confined to a localised region near the horn tip and near the
339 surface of collapsing bubbles, resulting in a broader size distribution. In this case the presence of
340 larger droplets may have resulted in higher encapsulation yields, but this may also reduce the
341 stability of these double emulsions.

342

343 ### 3.3 Stability of encapsulation over time

344 To investigate the issue of stability, emulsions prepared using both shearing methods were
345 assessed and compared in regards to the loss of salt encapsulation as well as their propensity to
346 coalesce/phase separate over 7 days (i.e. typical shelf life of a pasteurised milk).

347 Particle size measurements of the emulsions formed using selected ultrasonication or HPH
348 conditions can be found in Supplementary Information (Fig. S3). For the ultrasonically prepared
349 samples, coalescence was detected only in the size distributions for emulsions processed using 5
350 s sonication time (6 W power) where the presence of large droplets in the range 10-100 μm were
351 formed. This coalescence was indicated by an increase in the particle size distribution at day 7
352 compared with day 1. Emulsions sonicated for longer duration at the same power displayed no

353 coalescence. Instead, the emulsions generally resulted in a small decline in the measured size,
354 indicated by a small shift to the left after day 7. This shrinkage of droplet size would suggest loss
355 of encapsulated material with time, consistent with observations made previously by Leong et al.
356 for emulsions created by ultrasonication in skim milk (Leong et al., 2016).

357 Similar to the double emulsions produced using US for 5s, the larger droplets produced using HPH
358 displayed coalescence instability with storage. The size distributions of the droplets formed in the
359 size range < 10μm however, were found to shift to the left, similar to the shrinkage of droplets as
360 observed in the case of the ultrasonically produced emulsions. The reason could be the loss of
361 encapsulated material that leads to shrinkage. However, the combination of these smaller emulsion
362 droplets with the larger droplets (i.e. Ostwald ripening) present in the emulsions cannot be ruled
363 out as it is another potential source of instability. Another possible reason for this increase in size
364 is swelling of the droplets due to osmotic pressure.

365 To evaluate further, microscope observations were made over the 7 day storage period to visually
366 check for loss of encapsulated material over time. Selected images for emulsions prepared using
367 10 s ultrasonication duration and HPH pressure of 150 bar are presented in Fig. 6. An obvious
368 trend that can be observed is that the HPH emulsions have a more poly-disperse range of droplets
369 (especially a lot more sub-micron sized droplets), consistent with the size distributions obtained.
370 It can also be observed that there is a visual decline in the number of encapsulated droplets within
371 the secondary W1/O/W2 droplets that is more prominent in the US produced samples compared
372 with HPH. However as can be observed in the salt-release measurements with time (Fig. 7 a and
373 b), the HPH produced emulsion (150 bar) had a significantly greater ($P<0.05$) release of salt
374 compared with US produced using 10s, 6 W power in the initial few days of storage. The HPH
375 sample also appears to approach a plateau in salt loss (~65%) after the 2$^{nd}$ day, as compared with
376 the US samples which do not reach an equivalent degree of salt loss until the 7$^{th}$ day. Note that the
377 salt released on day 1 for these samples are different to those presented in Fig. 5, as there were
378 made using a different batch of milk.

379 The release of salt with time has some limitation in regards to quantifying the retainment of
380 encapsulated water phase, which should be addressed. This is because the NaCl used as an
381 entrapment marker can diffuse both in and out of the oil droplets with storage time and so the
382 conductivity measured will provide either an overestimate or underestimate to the degree of
383 aqueous phase entrapment. In general, water will diffuse across a semi-permeable membrane (in
384 this case the oil and surfactant boundaries) faster than the $Na^+$ or $Cl^-$ ions, which tend to diffuse
385 together in order to maintain charge neutrality (Hancock and Cath, 2009). In this case, as the
386 osmotic pressure is higher in the internal phase due to salt loading, the tendency is for water to
387 transfer into the internal water droplets with time. This will lead to some swelling of the internal
388 droplets until they can no longer be retained in the oil droplets, which will collapse, resulting in a
389 reduction in the size of the secondary emulsion droplets (Wen and Papadopoulos, 2001). As
390 mentioned above, it appears that for these samples, the encapsulation yield of salt plateaus once it
391 reaches a release of ~65%, indicating possibly a balance of the osmotic pressure such that transport
392 of salt comes to an equilibrium. This is supported by the microscopy images that indicate minimal
393 change in the encapsulation morphology of the HPH samples after day 2, suggesting encapsulation
394 stability.

One of the motivations in this present work was to limit the amount of surfactants used to stabilise the double emulsions. It is possible to significantly improve the encapsulation stability by increasing the amount of surfactant used. An increase of PGPR from 1 wt% to 5 wt% of the oil phase (without lecithin), produced emulsions (formed using US 10 s, 6W) that were able to retain ~ 100% of the salt marker, even after 7 days of storage (Fig. 7c). These results show there is a trade-off between surfactant use and encapsulation stability that will need to be considered when formulating these double emulsions for particular applications.

## 4. Conclusion

Double emulsions were formulated in skim milk using ultrasonication or high pressure homogenisation in the secondary emulsification stage. Displacement yields were found to be dependent on the viscosity of the internalised W1/O phase, which in this study was controlled by increasing the relative amount of lecithin in the oil phase. Fat displacement yields of between 15 to 30 % (i.e. 50 to 100 % encapsulation) could be achieved, using minimal amounts of surfactant in the inner oil phase and no additional surfactants in the external aqueous phase. Encapsulation stability with storage can be improved by adding more emulsifier to the oil phase. The use of 5% PGPR in the oil phase maintained an encapsulation yield of ~ 100% in the emulsion over 7 days.


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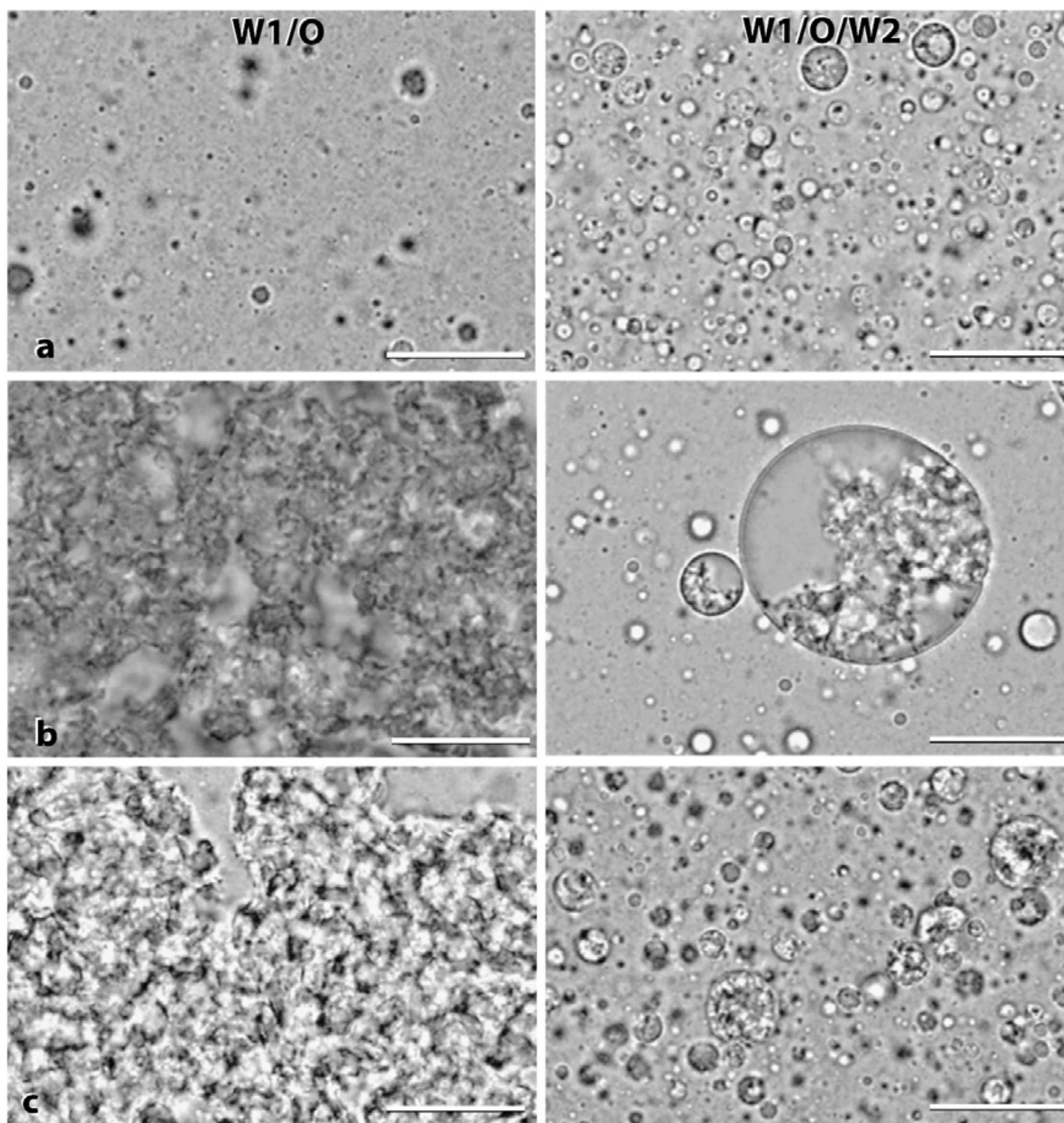


518 Fig. 1. Micrographs for W1/O and W1/O/W2 emulsions formed using a) PGPR (1 wt% of oil
519 phase) b) lecithin (2 wt% of oil phase) and c) PGPR (1 wt% of oil phase) and lecithin (2 wt% of
520 oil phase) combined. The scale bars represent 20 μm.

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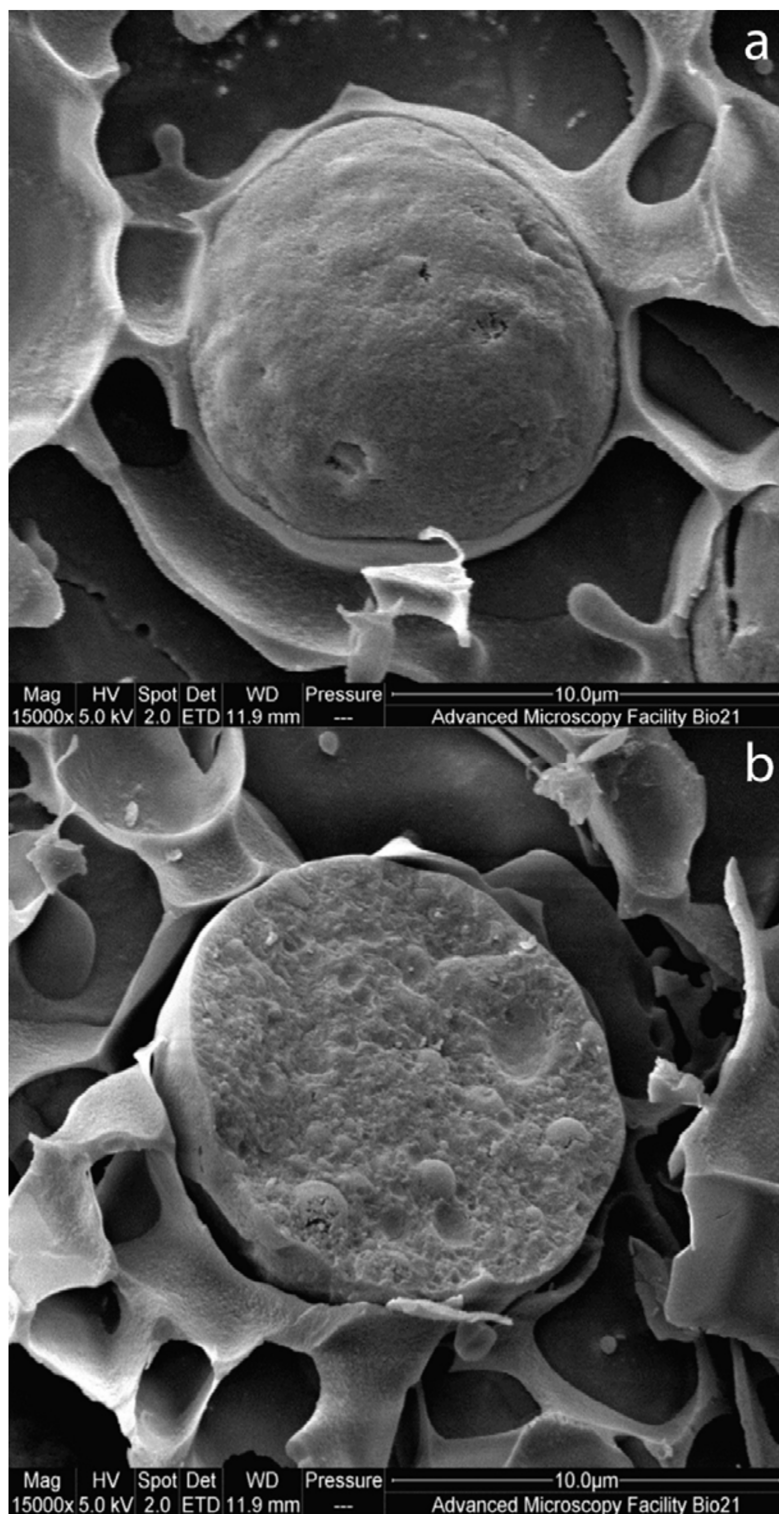
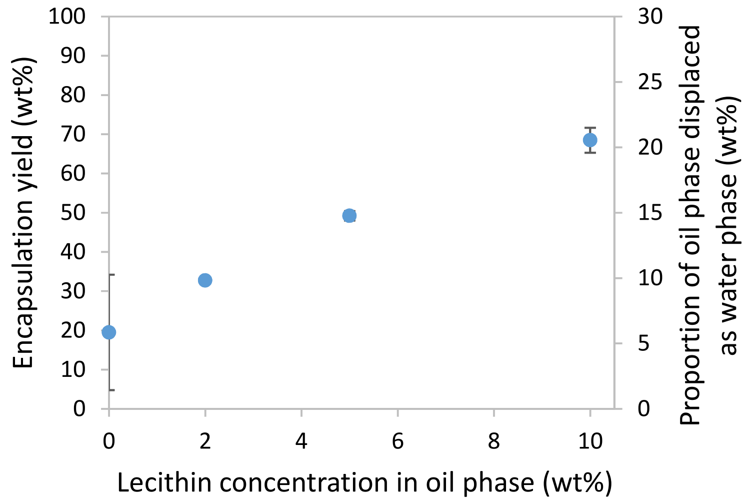



522
523 Fig. 2. Cryo-SEM images of the a) external and b) internal morphology of a double emulsion
524 droplet stabilised by milk proteins in the exterior and milk proteins/PGPR/lecithin surfactant in
525 the interior. The 'flake-like' masses surrounding the oil droplet is the frozen liquid milk phase of
526 the emulsion.
527




528
529 Fig. 3. Encapsulation yield and proportion of oil phase displaced as water phase as a function of
530 lecithin concentration in the oil phase. The emulsions also contain PGPR at a concentration of 1
531 wt% of the oil phase. All emulsions were processed at ultrasonic amplitude of 20% for 30s
532 duration (24 J/g). Error bars represent the standard deviation for the encapsulation yield of
533 triplicate emulsions.
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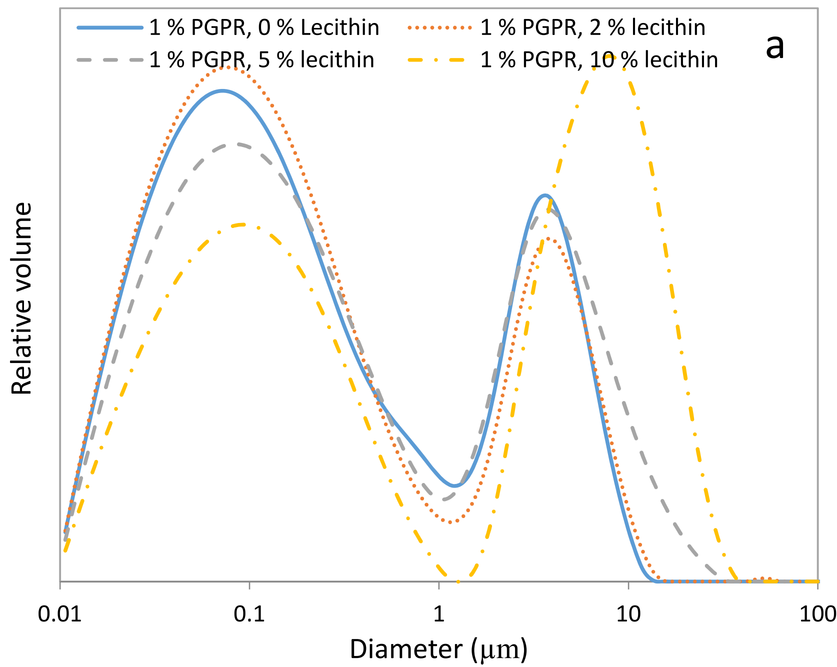


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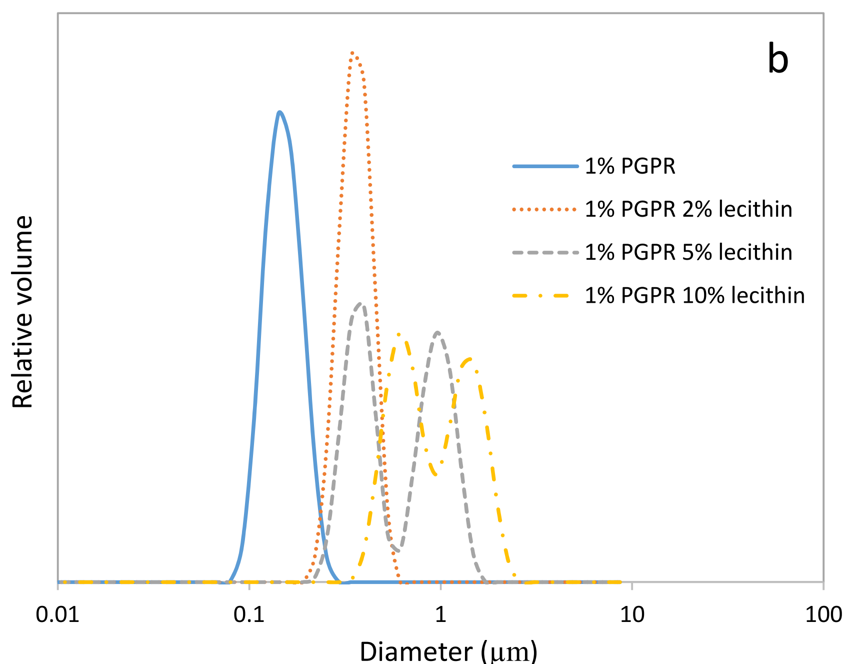


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538 Fig. 4. Size distributions of the a) secondary W1/O/W2 emulsion and b) primary W1/O emulsion
539 droplets as measured by a Malvern Mastersizer. The W1/O/W2 droplets were all formed using 30
540 s ultrasonication at 6 W. The primary W1/O droplets were all formed using 90 s ultrasonication at
541 10 W. The data are the average of triplicate measurements for each emulsion and are representative
542 of the trends from triplicate emulsion samples.

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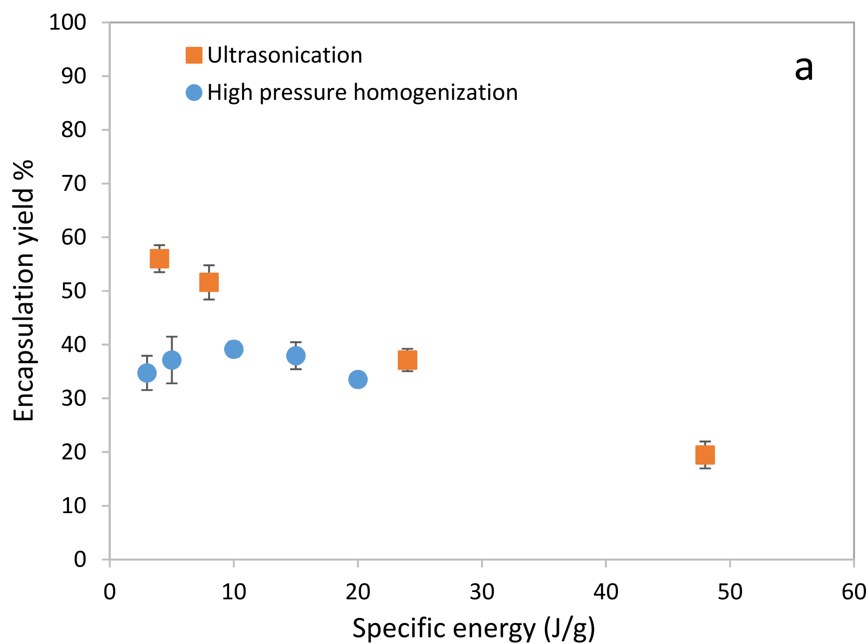


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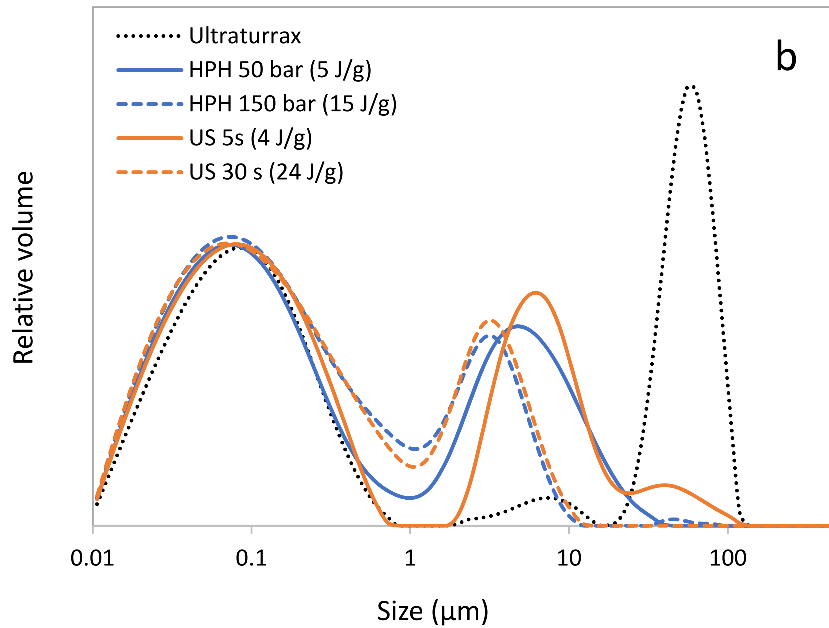


545

546 Fig. 5. a) Encapsulation yield as a function of specific energy for ultrasonication and high pressure
547 homogenisation. Error bars represent the standard deviation of measurements of triplicate
548 emulsions. b) Particle size distributions of W1/O/W2 droplets at select ultrasonication and HPH
549 processing conditions. The data are the average of triplicate measurements for each emulsion and
550 are representative of the trends from triplicate emulsion samples. All emulsions are formulated
551 with emulsifier concentrations of 1 wt % PGPR and 2 wt % lecithin in the oil phase.

552

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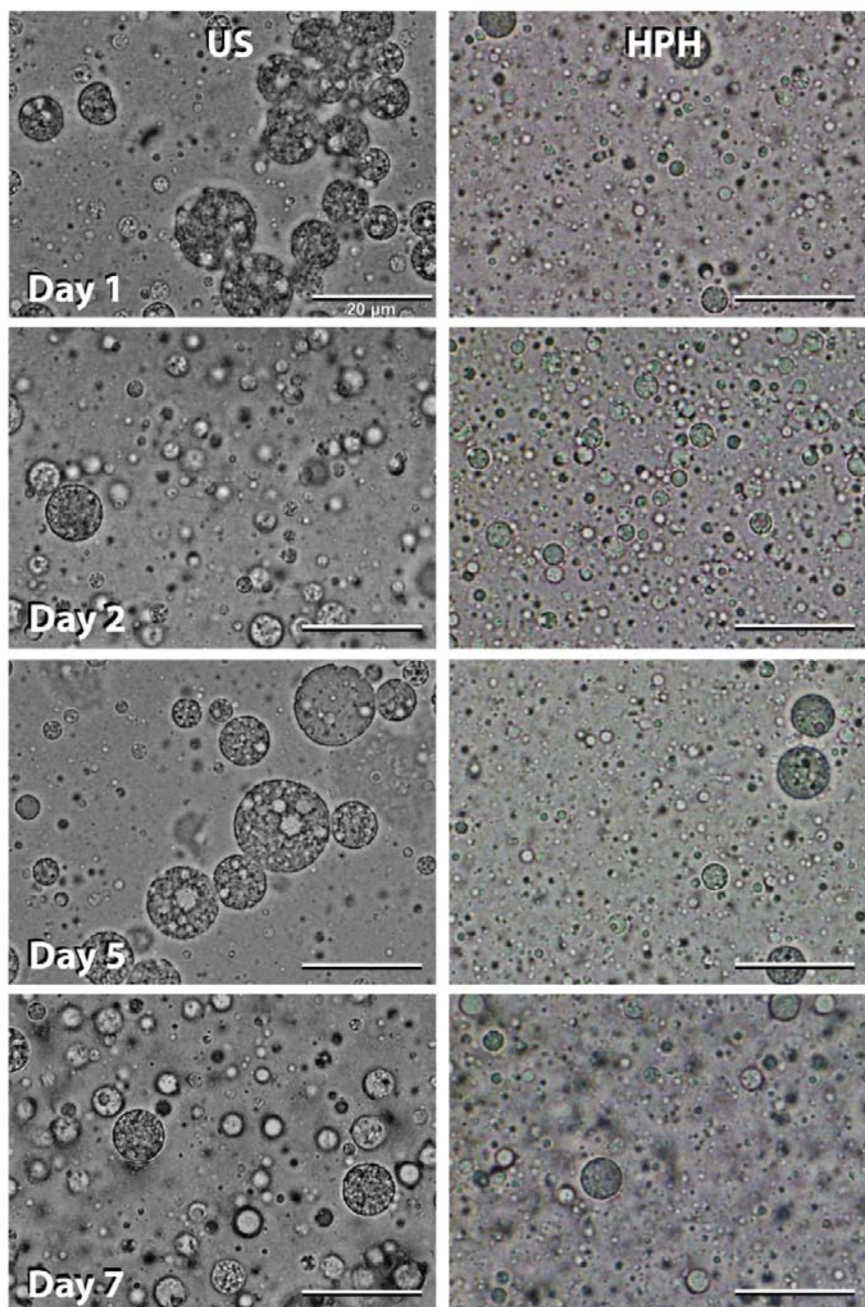
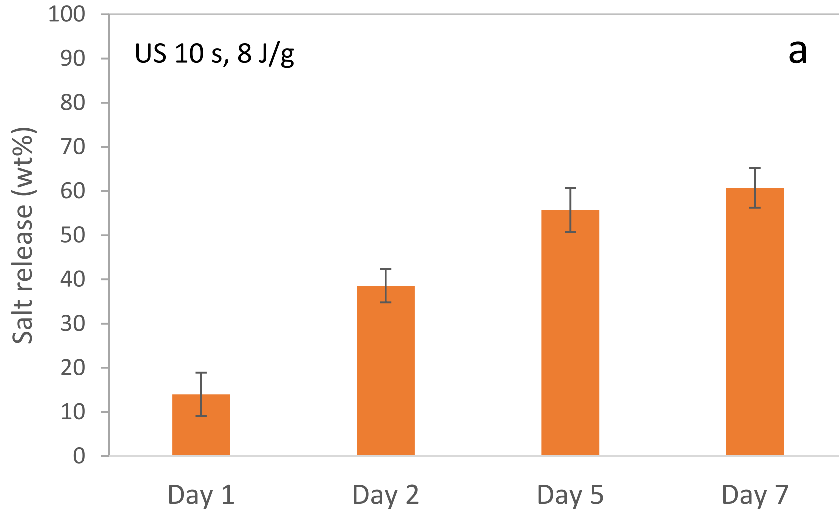



554 Fig. 6. Micrograph images of W1/O/W2 emulsion droplets formed using 10 s sonication
555 compared with HPH formed using 150 bar on days 1, 2, 5 and 7 (scale bar represents 20 μm).
556

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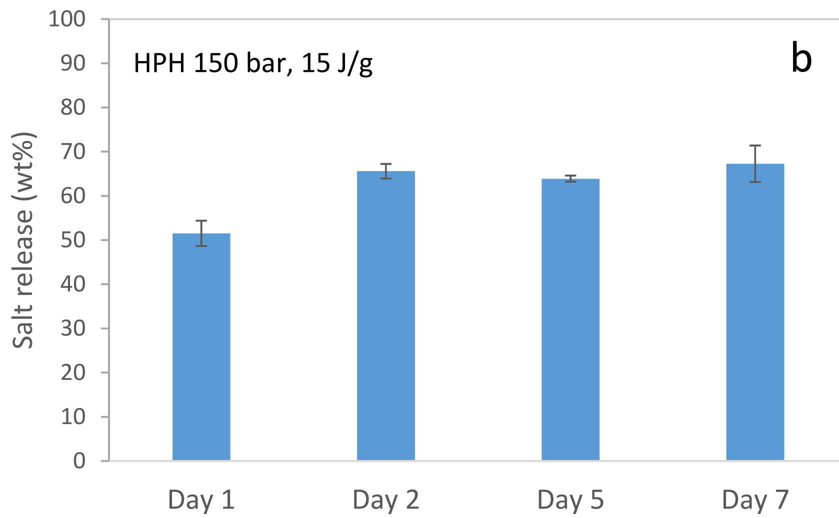


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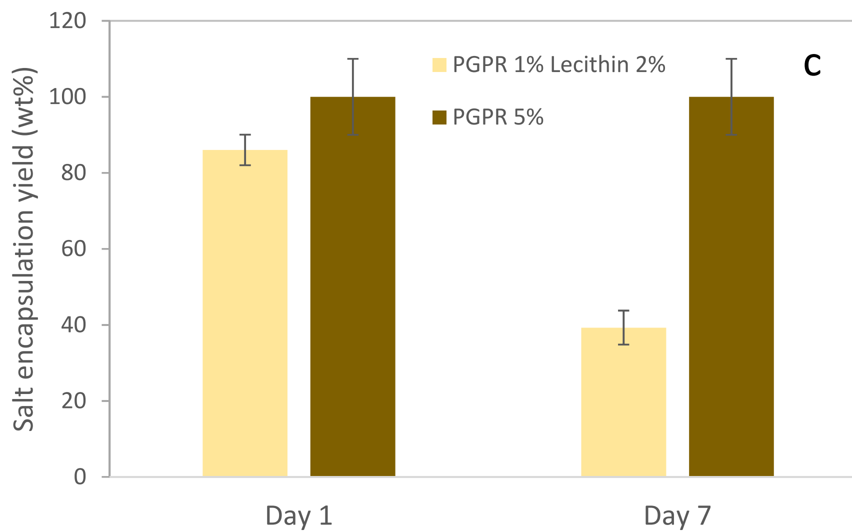
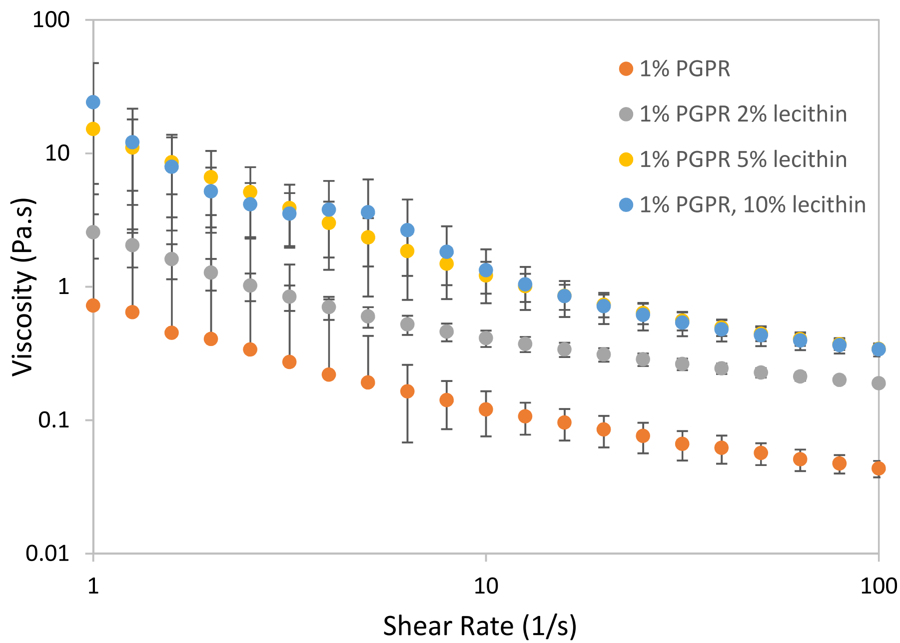


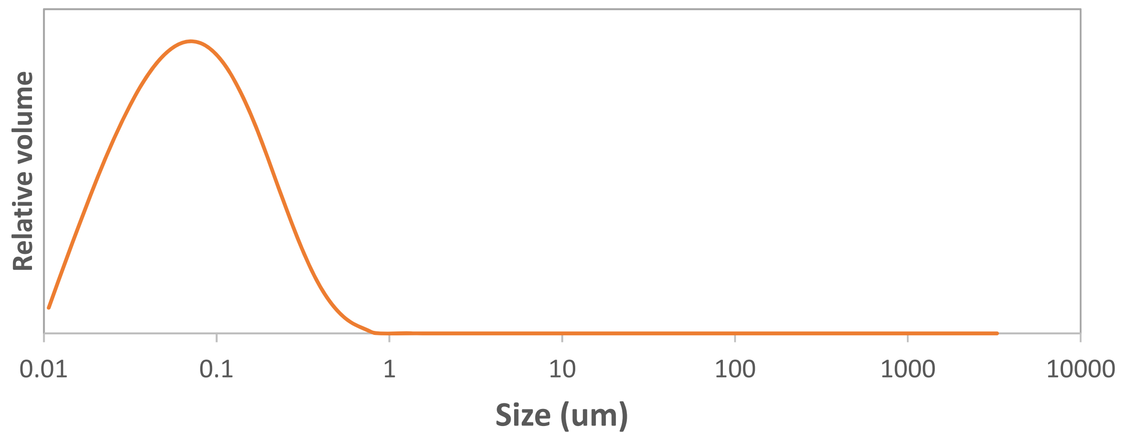

559
560

561 Fig. 7. Salt released expressed as a percentage of the amount loaded into the W1/O emulsion for
562 emulsions formed using a) ultrasonication 10 s and b) HPH 150 bar. c) The salt encapsulation

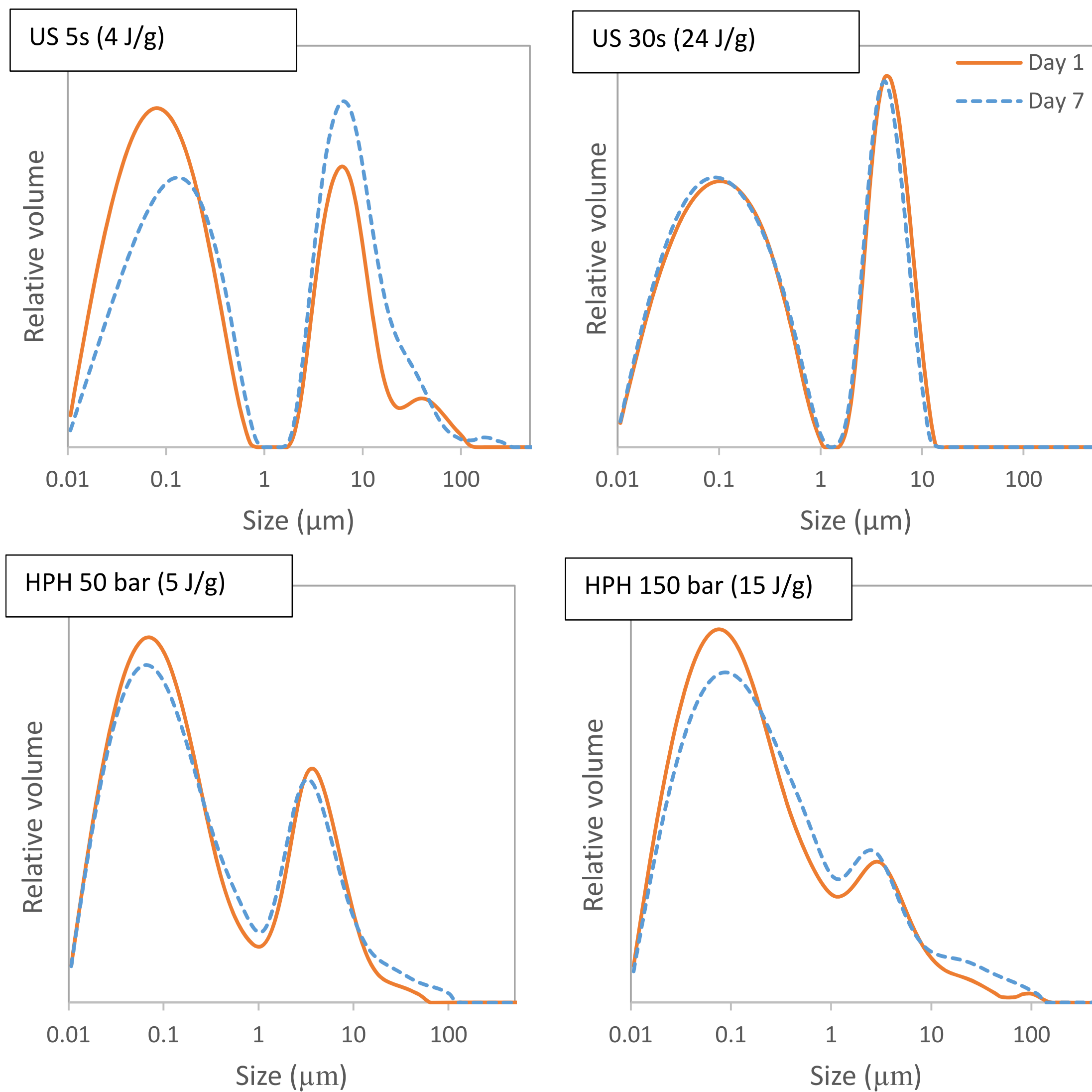
563 yield in emulsions formed using surfactant concentrations of 1% PGPR, 2 % lecithin compared
564 with 5 % PGPR, formed using 10 s sonication. Error bars represent the standard deviation of
565 measurements of triplicate emulsions.
566




567
568 Fig. S1: Shear viscosity as a function of shear rate at a temperature of 30 °C for W1/O emulsions
569 formed with varying concentrations of lecithin. Error bars represent the standard deviation of
570 duplicate measurements.
571




572
573 Fig. S2: Size distribution of casein micelles in skim milk measured using Mastersizer3000
574




575

576 Fig. S3: Particle size distribution change from day 1 to day 7 for emulsions created selected
577 sonication and high pressure homogenisation conditions. The data are the average of triplicate
578 measurements for each emulsion and are representative of the trends from triplicate emulsion
579 samples.
580
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