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The development of vegetarian omega-3 oil in water nanoemulsions suitable for integration into functional food products

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1 **1.0. Introduction**

2 Long chain omega-3 (*n*-3) polyunsaturated fatty acids (LC ω 3PUFA) in the human diet are
3 mainly obtained from oily fish, fish oil or fish oil based supplements (Bourre, 2007). Recent
4 evidence from Western countries indicates that certain population groups may not be
5 consuming enough LC ω 3PUFA (Elmadfa & Freisling, 2009; Micha et al., 2015). Current UK
6 recommendations state that two portions of fish should be consumed per week, one of which
7 should be oily fish amounting to 140g per week of oily fish (Scientific Advisory Committee on
8 Nutrition, 2004). However, average oily fish consumption in the UK is only around eight grams
9 per day (Bates et al., 2014). Non fish sources of LC ω 3PUFA are particularly important for
10 vegetarians, non-fish eaters and pregnant mothers (Lane, Derbyshire, Li, & Brennan, 2014a).

11 Eicosapentaenoic acid (20:5 *n*-3; EPA) and docosahexaenoic acid (22:6 *n*-3; DHA) comprise of
12 the main LC ω 3PUFA in oily fish and have been linked to healthy aging throughout the life cycle
13 (Swanson, Block, & Mousa, 2012). DHA plays a crucial role in normal human retinal and brain
14 development and is considered by some as an essential fatty acid during early childhood
15 development (Uauy, 2009). Further benefits have also been identified including
16 cardiovascular health, decreased inflammation, improved cognitive function, health
17 promotion and disease reduction (Aberg et al., 2009; Dawczynski, Martin, Wagner, & Jahreis,
18 2010; Mukaro et al., 2008; Murphy et al., 2007; Shahidi, 2015). Vegan diets are completely
19 devoid of DHA and vegetarian diets contain smaller amounts of DHA than that of meat and
20 particularly fish eaters (Ryan & Symington, 2015; Sanders, 2009).

21 The potential health implications of low LC ω 3PUFA intakes coupled with concerns about the
22 sustainability of fish stocks call for innovative approaches to achieve a solution. The use of
23 alternative sources of LC ω 3PUFA to fish oil is likely to be beneficial as based on current
24 production methods, it is estimated that demand for fish oil will far exceed supply by 2025
25 (Jacobsen, Torstensen, & Undeland, 2013). Currently the most significant vegetarian dietary
26 form of LC ω 3PUFA is alpha-linolenic acid (18:3 *n*-3; ALA), which can be found in flaxseeds,
27 walnuts and other seed oils (Edel, Pierce, & Aliani, 2015; Lemahieu et al., 2015; Navas-
28 Carretero et al., 2015). However, previous research has established that in humans,
29 conversion of ALA into the more beneficial longer chain EPA and DHA found in oily fish is
30 limited (Burdge & Calder, 2005; Burdge, Jones, & Wootton, 2002; Deckelbaum & Torrejon,
31 2012; Lane & Derbyshire, 2013b). Microalgal oils are produced in tightly controlled
32 fermentation facilities and may offer a sustainable alternative source of LC ω 3PUFA in the
33 forms of DHA and EPA that are also suitable for vegetarians and vegans (Arterburn et al.,
34 2007; Ryan & Symington, 2015; Salem & Eggersdorfer, 2015; Sanders, 2009).

35 Supplements may provide a substitute, but the National Diet and Nutrition Survey (2014)
36 found that supplements are only used by 11% of the general population (Bates et al., 2014).
37 Supplements are widely available in capsule form, although in some cases their biological
38 effects can be diminished or even lost due to incomplete absorption (Schuchardt & Hahn,
39 2013). Bioavailability is a measurement of the extent an active component reaches the
40 systemic circulation and is available at the site of action (Huang, Yu, & Ru, 2010). Most sources
41 of nutrients function differently when incorporated into food matrixes than in bulk forms,
42 which may affect bioavailability, therefore food based approaches are recommended to
43 optimise the bioavailability of fatty acids (Kris-Etherton & Hill, 2008).

44 A further solution may be offered by nanoemulsions, which have extremely small droplet sizes
45 ranging from 50 to 500nm and can be used to encapsulate sensitive or volatile ingredients
46 (Jafari, He, & Bhandari, 2006; Kentish et al., 2008; Sun et al., 2015).

47 When an emulsion consists of an entire droplet distribution below 80nm there may be
48 advanced properties in comparison to conventional larger sized emulsions including
49 transparency, increased colloidal stability and a large interfacial area in comparison to volume
50 (Kentish et al., 2008). Materials at the nanometre scale equate to 10^{-9} m (Rao & McClements,
51 2011; Silva, Cerqueira, & Vicente, 2011).

52 The incorporation of nutrients into foods using nanotechnology has the potential to improve
53 bioavailability due to small particle sizes and high surface to surface volume ratio (Acosta,
54 2009; Sun et al., 2015). Lipid emulsions behave differently in the digestive tract in accordance
55 with droplet sizes (Armand et al., 1999). Small droplets of nutrients can easily be transported
56 in the body through cell membranes giving increased blood plasma and erythrocyte
57 concentrations (Huang et al., 2010). However, the use of nanoemulsions of omega-3 oils in
58 food matrices may create challenges with consumer acceptability and oxidation stability,
59 which must be considered (Augustin et al., 2015; Jacobsen, 2009; Tippetts & Martini, 2010;
60 Walker, Decker, & McClements, 2015). The objective of this study was to develop stable
61 vegetarian LC ω 3PUFA oil in water nanoemulsion systems suitable for incorporation into
62 functional foods.

63 **2.0. The creation of an oil-in-water nanoemulsion system**

64 **Materials and methods**

65 **2.1. Materials to create emulsion systems**

66 Testing was conducted using vegetarian LC ω 3PUFA source oils rich in DHA or ALA (see Table
67 1).

68 DHA-*Schizochytrium sp* vegetarian algae oil containing 35% of fatty acids as DHA was kindly
69 provided by DSM, London, UK. Flaxseed oil containing 52% of fatty acids as ALA was purchased
70 online from Holland and Barrett, Manchester UK. The fatty acid content of flaxseed and algal
71 oil was verified using lipid extraction and fatty acid analysis using the methods detailed by
72 (Bell et al., 2002). Liquid soy lecithin was purchased from Now Foods, Bloomingdale, IL, USA.
73 Tween 40 was purchased from Sigma-Aldrich Company Limited, Loughborough, UK.

74 **2.2. Preparation methods**

75 All emulsions were of the 'oil-in-water' (o/w) type and were prepared in accordance with
76 methods that are patented by the authors (Lane, Derbyshire, Li, & Smith, 2012). The aqueous
77 continuous phase was deionised water; the lipid dispersed phase was the oil. The emulsifier
78 was either soy lecithin (LE), Tween 40 (TW) or a combination of soy lecithin and Tween 40
79 (TWLE).

80 A solution of 70% (w/w) LC ω 3PUFA oil in combination with 30% (w/w) lecithin was prepared
81 two hours in advance and placed in a water bath at 55°C to dissolve. Tween 40 was introduced
82 directly into deionised water, which had been brought to 55°C in a water bath.

83 Initially, coarse emulsions containing different compositions of oil, emulsifier and deionised
84 water were prepared. Once prepared, samples were placed in a water bath at 55°C for two

85 hours and were hand stirred for 1 min at 30 min intervals. Samples underwent primary
86 homogenisation using a Silverson rotor–stator mixer on a medium setting (4000rpm) for 2
87 min. Development trials took place with 15 and % (w/w) oil content. As stable systems were
88 replicated, further trials were conducted using up to 70% (w/w) oil phase at various intervals.

89 Secondary homogenization was completed by ultrasound using a 24 kHz sonicator (Dr
90 Hielscher series, Model UP 400S, Hielscher Ultrasound Technology, Teltow, Germany). This
91 system consisted of a generator, converter and a sonotrode H22 titanium tip. The horn tip
92 was immersed in the coarse emulsion for the designated time (max depth 45mm) then the
93 ultrasonic processor was turned on at full power (Hielscher Ultrasound Technology, 2007).

94 After initial trials, all experiments were completed using a cold water cooling jacket to control
95 temperature increases and each experiment was duplicated. The cooling jacket facilitated the
96 treatment of a 250mg sample, which was agitated by hand throughout the process to ensure
97 a more even distribution of ultrasound and to avoid hotspots in the sample. Samples were
98 subjected to ultrasound for 30-second intervals then collected after each treatment and
99 examined under a microscope at a 120 magnification using immersion oil and photographed.

100 Once stability and particle size had been established visually, further trials were completed
101 using samples with 20, 25, 30, 40, 50 and 70% (w/w) oil with 2, 3, 4, 5 6, 7 and 8% (w/w)
102 emulsifier. From visual analysis the 20, 50 and 70% (w/w) o/w emulsions consisting of 6%
103 (w/w) lecithin (LE), 6% (w/w) Tween 40 (TW) or a combination of lecithin and Tween 40 in
104 50:50 (w/w) ratio (TWLE) were selected for particle size measurements.

105 **2.3. Analysis of nanoemulsion systems**

106 **2.3.1. Measurement of temperature rises**

107 Temperature increases were measured with a standard laboratory thermometer probe.
108 Temperature increases can significantly reduce the oxidative stability of LC ω 3PUFA rich oils.
109 Research by Alamed *et al.* (2006) indicated that LC ω 3PUFA emulsions can be heated to 90°C
110 for up to 10 min without affecting oxidative stability. Temperature measurements were taken
111 during the ultrasound process to maintain the oxidation stability of oils during product
112 development. To monitor temperature increases during processing, 250ml of coarse
113 emulsion was prepared for each sample using 50% (w/w) oil. Samples were then placed in a
114 cold water cooling jacket and subjected to ultrasound treatment at maximum power output
115 (100 μ m amplitude). Temperature measurements were taken at 30 sec intervals for up to 20
116 min using a thermometer probe, which was immersed directly into the sample.

117 **2.3.2. Methods of measuring emulsion droplet sizes**

118 Particle sizes were determined using a Malvern Mastersizer 2000 (courtesy of Glyndŵr
119 University, Wrexham, UK). Droplet size (DS) distributions were measured for each interval in
120 accordance with the methods used by Akhtar *et al.* (2006) and Akhtar and Dickinson (2003)
121 using a Malvern Mastersizer MS2000 laser light-scattering analyser with a small sample
122 dispersion unit set to 2000rpm. A drop of each emulsion sample amounting to approximately
123 10 μ l were pipetted into the dispersion unit. For the emulsion samples an absorption
124 parameter value of 0.001 and the refractive index ratio of 1.488 for the algae oil and 1.4770
125 for the flaxseed oil were used (Breivik, 2007).

126 Samples were measured in duplicate to ensure accuracy with a 15-sec pause between
127 measurements. For the purposes of this study the d_{32} Sauter mean ($d_{32} = \sum n_i d_i^3 /$
128 $\sum n_i d_i^2$ (Horiba Scientific, 2010)) has been reported as it reflects the surface diameter
129 average value and the droplet size distribution and has been used in a number of previous
130 studies (Abismaïl, Canselier, Wilhelm, Delmas, & Gourdon, 1999; Kentish et al., 2008; Yang,
131 Leser, Sher, & McClements, 2013).

132 **2.3.3. Statistical analysis**

133 Statistical analyses were conducted on the Mastersizer droplet measurement results using
134 SPSS version 19 to assess the effect of oil load, temperature, processing time and type of
135 emulsifier on DS (d_{32} parameter). Prior to statistical analysis, all results were assessed for
136 statistical compatibility using the Kolmogorov-Smirnov and Shapiro-Wilk tests to check for
137 normality (Pallant, 2010). The effect of oil load for the three selected emulsifiers was assessed
138 using the paired t -test function and non-parametric alternative Wilcoxon Signed Rank Test as
139 described by Bell and Rowley (2011). The effect of emulsifier and temperature was measured
140 using a one-way ANOVA test. The effect of the three different emulsifiers on processing time
141 and droplet sizes was assessed using a two-factor repeated measures ANOVA with a
142 Bonferroni adjustment to determine the main effect of the emulsifier and time interaction
143 effects as described by Field (2013).

144 Where a main effect was observed a one-factor and one-factor repeated measures ANOVA
145 test with post-hoc testing was conducted along with the non-parametric alternative Kruskal-
146 Wallis test to identify where the differences were located. For the one-way ANOVA the d_{32}
147 was added as dependant variable and the type of emulsifier was entered as the factor. The

148 Tukey and Scheffe post-hoc tests were selected to identify potential differences. For the
149 Kruskal-Wallis test the d_{32} was entered as the test variable and the type of emulsifier was
150 entered as the grouping variable as described by Pallant (2012).

151 **3.0. Results and discussion**

152 **3.1. The creation of vegetarian omega-3 nanoemulsion systems**

153 Table 2 shows the final oil to water emulsion ratios and experimental design processes used
154 to create the vegetarian LC ω 3PUFA o/w nanoemulsions.

155 **3.2. Temperature rises**

156 The temperature rises of nanoemulsions created using ultrasound were measured to ensure
157 that processing would not promote lipid oxidation. Recent research by Salvia-Trujillo *et al.*
158 (2012) demonstrated that the processing of emulsions under ultrasound caused significant
159 temperature increases, which were particularly prevalent at 100 μ m amplitude maximum
160 ultrasound power, with increases of 27 to 47°C after 180 sec under ultrasound. The use of a
161 cooling jacket in this study ensured that temperatures for samples processed at 100 μ m
162 amplitude over 20 min rose by 20°C maximum and did not exceed 51°C (see Figure 2) which
163 should not impact on oxidative stability (Alamed, McClements, & Decker, 2006).

164 **3.3. Droplet sizes**

165 Droplet measurement confirmed that the 7, 20 and 50% (w/w) oil samples could be classified
166 as nanoemulsion systems in accordance previously defined measurements in the literature
167 (figure 3) (Anton & Vandamme, 2011; Jafari et al., 2006; Kentish et al., 2008).

168 The 70% (w/w) oil load sample was not stable and particle sizes were in the μm range, so it
169 could not be classed as nanoemulsion system. The particle size measurements for the 70%
170 (w/w) flaxseed oil samples indicated that a nanoemulsion had not been successfully created.
171 The oil load for this sample created instability resulting in a system that was prone to
172 separation and appeared to have undergone phase inversion.

173 **3.4. The impact of ultrasound processing energy on droplet** 174 **measurements**

175 To assess the impact of processing energy, samples were prepared using ultrasound at 30, 50,
176 70 and 100- μm amplitude. Initial visual analyses of samples under a standard laboratory
177 microscope (see Figure 1) demonstrated that maximum ultrasound power caused maximum
178 droplet disruption, creating small droplets with minimal processing times. All further samples
179 were prepared at 100- μm amplitude to maximise droplet reduction.

180 Salvia-Trujillo *et al.* (2013) found that ultrasound amplitude and treatment time significantly
181 reduced the droplet sizes of nanoemulsions, with 100 μm amplitude facilitating the largest
182 reductions in 1% (w/w) lemongrass oil–alginate nanoemulsions. Ultrasound treatment of the
183 50 % (w/w) samples at 100 μm amplitude for up to 20 min created nanoscale droplet ranges,
184 with optimum sizes achieved between 10 and 12 min for all emulsifiers (see Table 3). Overall
185 ultrasound processing time had a significant effect on droplet sizes ($P < 0.001$), although this

186 effect was only significant from 0 to 2 min of processing, changes were not statistically
187 significant after 2 min of processing (see Table 4).

188 **3.5. The effect of oil loading on droplet measurements**

189 Statistical analyses assessed the effect of oil loading on droplet sizes and demonstrated that
190 oil loads had a statistically significant effect on d_{32} measurements and that droplet sizes
191 increased with higher oil loads ($P < 0.05$). This was the case for all emulsifiers except lecithin,
192 which was approaching significance for parametric testing ($P = 0.051$) and statistically
193 significant for non-parametric testing ($P = 0.038$) see Table 4.

194 Extensive droplet measurement research using high DHA algae oil nanoemulsions has yet to
195 be published, indicating that this system is novel. However, a number of studies have made
196 similar findings to this research using a variety of other lipid sources including flaxseed oil.
197 Abismaïl *et al.* (1999) found that the d_{32} measurements of kerosene in water emulsions
198 prepared with Tween 60 surfactant and generated using ultrasound, increased with oil load
199 from approximately 250nm at 5% (w/w) to 900nm at 50% (w/w) oil load. Phase inversion
200 was also noted when emulsions contained equal volumes of each phase.

201 Phase inversion did not occur in the present study at equal rates of algae or flaxseed oil and
202 water, but was noted when samples reached a 70% (w/w) oil load. Phase inversion can occur
203 when the droplet interface is only partially covered by surfactant particles and is therefore
204 more likely to occur with increased oil loads (Lee, Niknafs, Hancocks, & Norton, 2012). A study
205 by van Nieuwenhuyzen and Szuhaj (1998) further validates the findings from this study, o/w
206 nanoemulsion droplet sizes were found to increase in lecithin or lecithin and o/w systems

207 from <100 to 300-500nm when the oil/lecithin concentration was increased from 0/1 (lecithin
208 only) to 9/1 in ratio.

209 Previous research identifies the effect of increased oil loads on the droplet sizes of
210 nanoemulsions and validates the findings from the current study. A study by Kentish *et al.*
211 (2009) produced comparable results to this research, although the oil loads were lower.
212 Flaxseed oil 15% (w/w) and 5.6% (w/w) Tween 40 emulsifier were used to produce
213 nanoemulsions using ultrasound. The flaxseed study demonstrated that stable nanoemulsion
214 systems with minimum droplet sizes of 120nm (d_{32}) were created with 15% (w/w) flaxseed oil
215 loads. Further research using 10% (w/w) oil loads demonstrates that systems with a complete
216 distribution below 100nm (d_{32}) can be created using ultrasound in combination with d-
217 limonene oil and 1% surfactant (Li & Chiang, 2012). Oil loads of 1% lemongrass oil were
218 combined with Tween 80 surfactant by Salvia-Trujillo *et al.* (2012) to successfully create
219 translucent systems with extremely small minimum average droplet sizes of 4.31nm (d_{32}) with
220 narrow size distributions. In addition to oil loads, the droplet sizes of nanoemulsions can be
221 affected by the choice of emulsifier.

222 **3.6. The effect of emulsifier on droplet measurements**

223 The emulsifiers in this study were chosen in accordance with their hydrophilic-lipophilic
224 balance (HLB) and favourable attributes identified in the literature (Coultate, 2009; Tadros,
225 2009; Tadros, Izquierdo, Esquena, & Solans, 2004). It was also hypothesised that the
226 combination of the two emulsifiers in equal quantities would create a neutrally balanced HLB
227 giving a system with very small droplet sizes that was less susceptible to lipid oxidation.

228 Temperature monitoring demonstrated that temperature increases were not significantly
229 affected by the choice of emulsifier (see Table 4).

230 Emulsifiers act as surfactants and play an important role in deformation and break-up of
231 droplets. Surfactants allow the existence of interfacial tension gradients, which are crucial
232 for formation of stable oil droplets (Tadros et al., 2004).

233 During emulsification, interfacial tension is lowered causing a reduction in droplet sizes, which
234 will further reduce with increased surfactant quantities until a plateau value is reached.
235 Emulsifiers that adsorb to the interface fastest will stabilise newly formed droplets more
236 quickly than emulsifiers with slower adsorption rates (Lee et al., 2012). Small molecular
237 weight surfactants are usually more efficient in emulsion stabilisation than biopolymers. They
238 are adsorbed to the freshly formed surface of the droplet and stabilise the new interface in
239 milliseconds preventing droplet coalescence (Jafari, He, & Bhandari, 2007). Visual analysis of
240 samples under the microscope demonstrated that a 6% level of surfactant appeared to give
241 optimum droplet reduction (Figure 1). Nanoemulsions have been created with emulsifier
242 quantities around this level in comparable research by Kentish *et al.* (2009).

243 Statistical analysis indicated that there was a statistically significant interaction on droplet
244 measurements between the type of emulsifier and the processing time ($P < 0.001$). It was also
245 established that samples prepared using lecithin had larger droplet sizes than other
246 emulsifiers for flaxseed and algae oils, although this was not statistically significant.
247 Comparable research was conducted by Fomuso *et al.* (2002), who compared the droplet sizes
248 of 10% fish o/w emulsions stabilised with Tween 20, whey protein, mono-diacylglycerols and
249 lecithin. Most emulsions had an average droplet diameter of 0.30-0.37 μm and the droplet

250 size was not found to be significantly influenced by the type of emulsifier; however, lecithin
251 emulsions showed a population of particles with a larger diameter of 4.7 μm . During the
252 emulsification process, lecithin forms water vesicles within the continuous phase, which must
253 disperse before the emulsifier can adsorb to the surface of droplets.

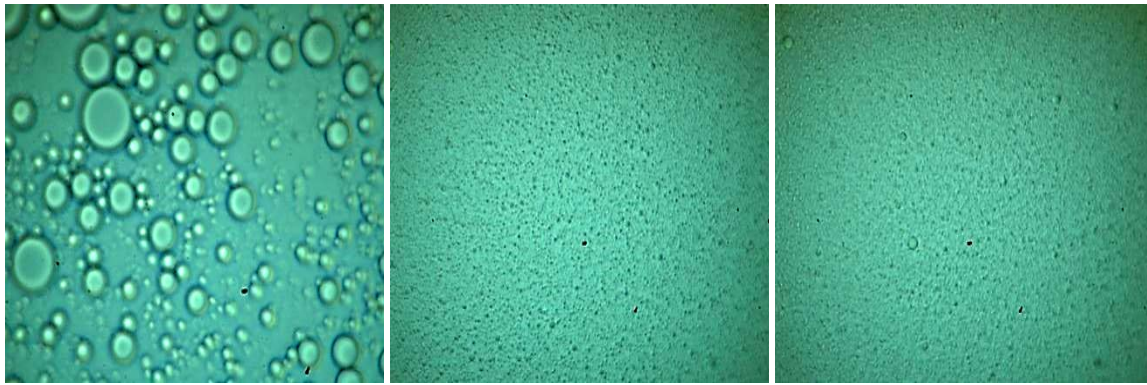
254 Interfacial tension is therefore reduced more slowly so larger droplets are formed by lecithin,
255 which may explain why there were larger droplets for the lecithin samples in this study (Lee
256 et al., 2012).

257 A review of current literature and patent applications demonstrates that the creation of o/w
258 nanoemulsions using ultrasound and high DHA algae oil, which can be considered as an
259 alternative to fish oil, has yet to be undertaken. Furthermore, papers and patent applications
260 relating to the creation of a 50% nanoemulsion system using ultrasound have not been
261 previously published, indicating that this system and the method of creating it is novel. This
262 technique can be applied to create vegetarian LC ω 3PUFA nanoemulsions suitable for
263 integration into enriched functional food products to provide a suitable alternative to fish oil
264 with the potential to increase DHA bioavailability (Lane & Derbyshire, 2013a; Lane et al.,
265 2014a; Lane, Li, Smith, & Derbyshire, 2014b, 2014c). The addition of a 50% (w/w) system is
266 also less likely to have a detrimental effect on food matrices than systems with lower oil loads
267 as lower volumes of nanoemulsion can be added to achieve optimum enrichment levels.

268 **4.0 Conclusion**

269 Stable oil in water emulsion systems were successfully created using flaxseed and high DHA
270 algae oil in combination with lecithin and Tween 40 emulsifiers. Particle size measurements

271 established that nanoemulsion systems had been created with up to 50% (w/w) oil loads. The
272 ratio of oil to water was found to affect droplet sizes, which rose significantly with higher oil
273 loads. It was not possible to create nanoemulsions with a 70% (w/w) oil load as phase
274 inversion occurred at this level. Statistical analysis of the d_{32} means for the 50% flaxseed
275 system showed that time under ultrasound significantly affected droplet sizes and that the
276 optimum processing time to create the smallest droplets was between 10 and 12 minutes.
277 Further research is now warranted to further develop appropriate food matrixes for
278 fortification and to analyse the physical and oxidation stability of the 50% (w/w) o/w
279 nanoemulsion systems.



280

281 Coarse emulsion

1 minute under ultrasound

2 minutes under ultrasound

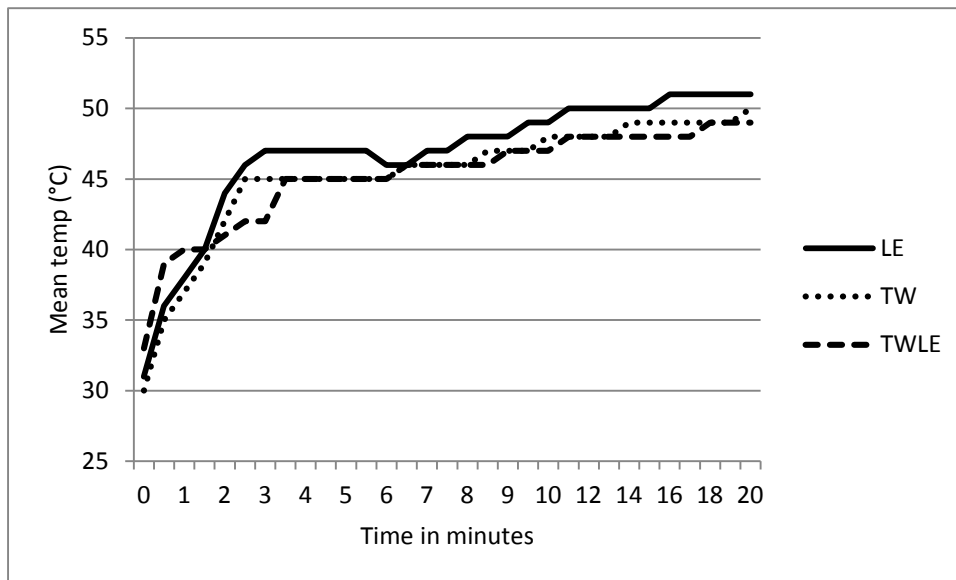
282

283

284 Figure 1 - Visual microscopic slide pictures of 50% (w/w) TW emulsion at 120 magnification
285 using immersion oil

286

287

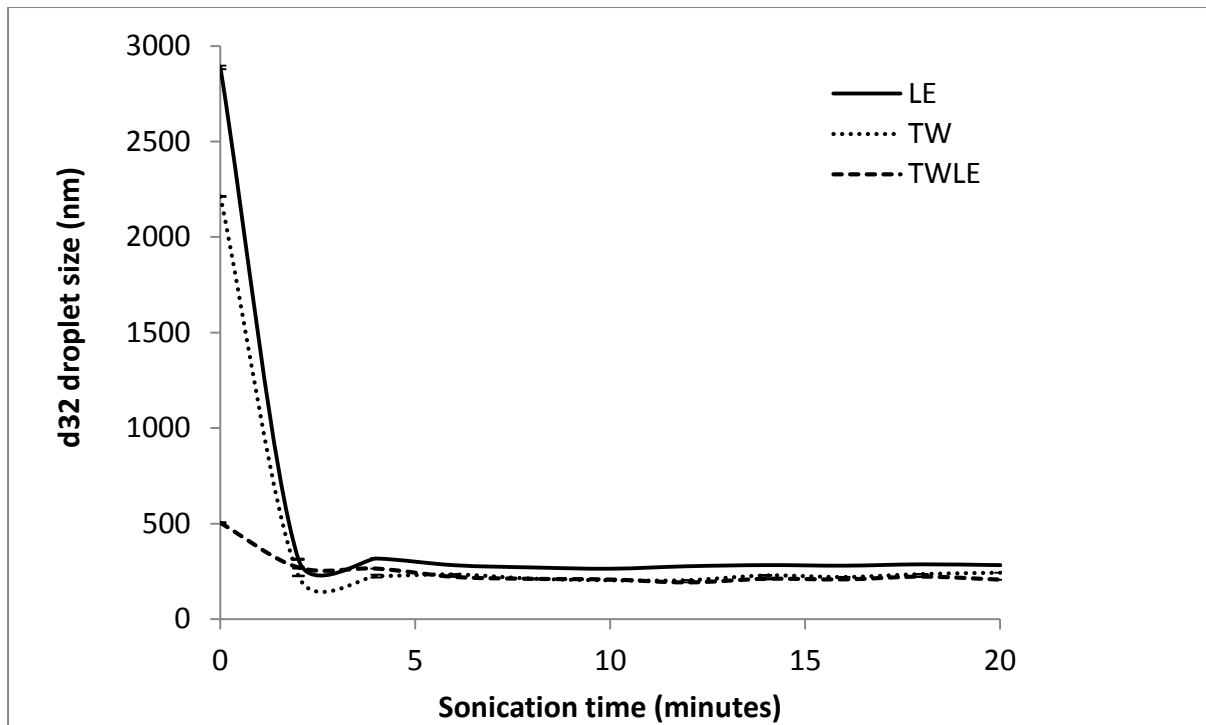


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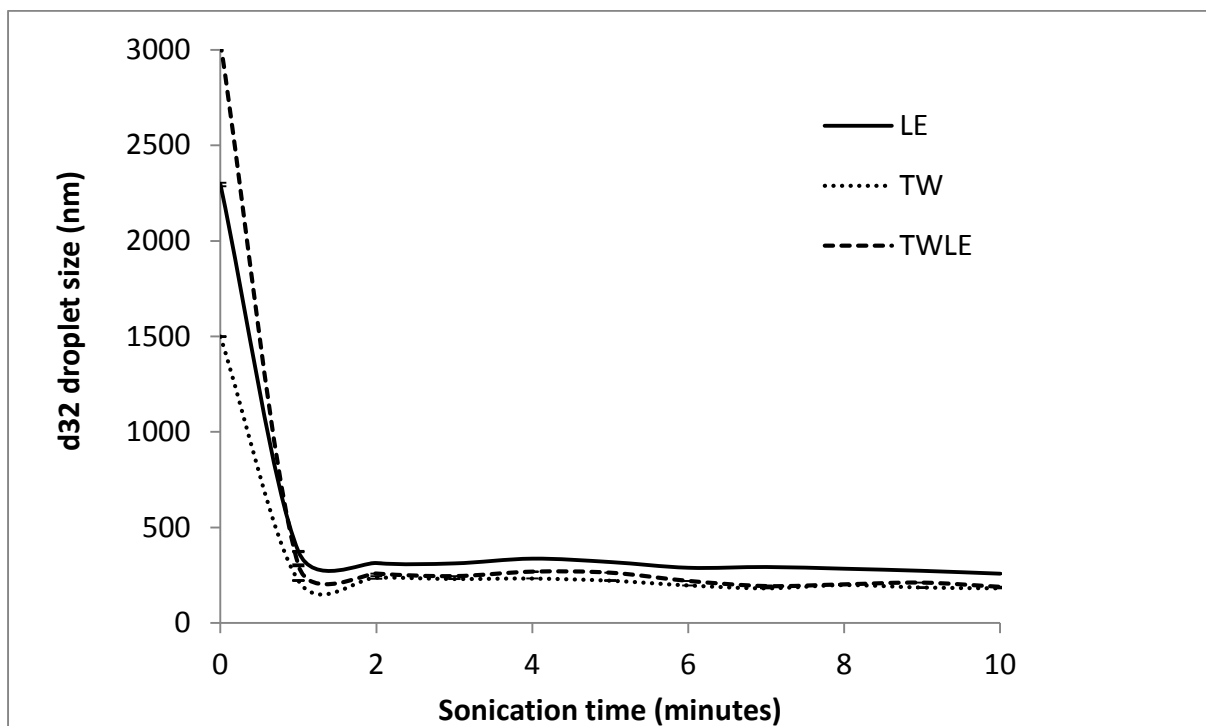
289 Figure 2 - Ultrasound temperature rises for 50% (w/w) flaxseed oil system processed at
290 100µm amplitude with a cold water cooling jacket

291

292



293



294

295

296 Figure 3 – The effect of sonication time for a 50% (w/w) 250ml flaxseed oil in water emulsion
 297 sample on the particle size (d_{32}). Error bars represent the standard error of measurements
 298 (top) and the effect of sonication time for a 50% (w/w) 250ml algae oil in water emulsion
 299 sample on the particle size (d_{32}). Error bars represent the standard error of measurements
 300 (bottom)

301

302

303 Table 1. - Fatty acid composition of flaxseed oil and DHA-S™ algal oil

Fatty acid	Flaxseed oil (g/100g)	DHA-S™ algal oil (g/100g)
16:0	6.00	0.00
18:0	3.00	0.00
18:1<i>n</i>-9	16.00	0.00
18:2<i>n</i>-6 (Linoleic acid)	17.00	1.27
18:3<i>n</i>-6		0.28
20:2<i>n</i>-6		0.00
20:3<i>n</i>-6		0.41
20:4<i>n</i>-6		1.06
22:4<i>n</i>-6		0.11
22:5<i>n</i>-6		15.63
18:3<i>n</i>-3 (ALA)	52.00	0.11
18:4<i>n</i>-3		0.36
20:3<i>n</i>-3		0.00
20:4<i>n</i>-3		0.82
20:5<i>n</i>-3 (EPA)		1.19
22:5<i>n</i>-3		0.47
22:6<i>n</i>-3 (DHA)		35.22
Total LCω3PUFA	52.00	38.17

304

305

306 Table 2. - Final emulsion ingredient ratios

Sample	Flaxseed/algae oil (%)	Tween 40 (%)	Lecithin (%)	Lecithin: oil premix (g) (30:70)	Deionised water (%)
Lecithin (LE)	20	0	6	20	74
Tween 40 (TW)	20	6	0	0	74
Combined (TWLE)	7	3	3	10	74
Lecithin (LE)	50	0	6	20	44
Tween 40 (TW)	50	6	0	0	44
Combined (TWLE)	50	3	3	10	44
Lecithin (LE)	70	0	6	20	24
Tween 40 (TW)	70	6	0	0	24
Combined (TWLE)	70	3	3	10	24

307 Key: The lecithin premix is shown for completeness and was included as part of the lecithin
308 and oil. All ratios are displayed as percentage measures.

309

310 Table 3. - The effect of sonication time on droplet measurements d_{32} for 50% w/w samples

Min	Algae TW	Flax TW	Algae LE	Flax LE	Algae TWLE	Flax TWLE
0	1499	2213	2296	2888	3039	490
30 secs	261	283	1968	1569	1606	285
1	224	253	402	361	321	283
	219	228	347	331	283	268
2	238	227	319	313	267	270
	238	233	309	314	250	259
3	238	233	305	298	246	241
	224	235	319	306	245	244
4	233	225	316	318	271	265
	233	230	358	317	267	264
5	222	209	319	313	263	282
6	197	234	289	282	220	221
7	182	193	293	269	193	204
8	199	212	284	271	202	211
9	186	203	273	286	211	211
10	<u>182</u>	<u>203</u>	<u>258</u>	<u>264</u>	<u>189</u>	207
12		204		277		<u>192</u>
14		229		283		211
16		220		280		208
18		236		287		222
20		243		283		207

311 Key: Optimum mean d_{32} droplet measurements are underlined and emboldened. Statistical
312 analysis indicated no significant differences for type of emulsifier.

313

314 Table 4. - Temperature, emulsifier type, processing time and droplet measurement statistical
 315 analysis

Effect	Parameters	Parametric test	P value	Non-parametric test	P value
Type of emulsifier on temperature	50 % oil load LE, TW and TWLE emulsifiers	One-way ANOVA	>0.05 (NS)	Kruskal-Wallis	>0.05 (NS)
Type of emulsifier and processing time on d ₃₂	50 % oil load LE, TW and TWLE emulsifiers	Two-factor repeated measures ANOVA	<0.001***	N/A	
Type of emulsifier on d ₃₂	50 % oil load LE, TW and TWLE emulsifiers	One-way repeated measures ANOVA	=0.918	Friedman	<0.01**
Processing time on d ₃₂	50 % oil load LE, TW and TWLE emulsifiers	One-way repeated measures ANOVA	<0.001***	Friedman	<0.001***
0 min			<0.001***		
2 min			>0.05 (NS)		
4 min			>0.05 (NS)		
6 min			>0.05 (NS)		
8 min		One-way ANOVA	>0.05 (NS)		
10 min			>0.05 (NS)		
12 min			>0.05 (NS)		
14 min			>0.05 (NS)		
16 min			>0.05 (NS)		
18 min			>0.05 (NS)		
20 min			>0.05 (NS)		
Oil loading on d ₃₂	LE 20 and 50% oil	Paired t-test	=0.051(NS)	Wilcoxon	0.038*
	TW 20 and 50% oil	Paired t-test	<0.001***	Wilcoxon	<0.001***
	TWLE, 7 and 50% oil	Paired t-test	<0.05*	Wilcoxon	<0.001***

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