1 **Encapsulation of ω -3 fatty acids in nanoemulsion-based delivery**
2 **systems fabricated from natural emulsifiers: Sunflower**
3 **phospholipids**

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5 Jennifer Komaiko^a, Ashtri Sastrosubroto^a, David Julian McClements^{a,b*}

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8
9 ^a *Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA*

10 ^b *Department of Biochemistry, Faculty of Science, King Abdulaziz University, P. O. Box 80203*
11 *Jeddah 21589 Saudi Arabia*

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19
20 *To whom correspondence should be addressed. Tel: (413) 545-1019. Fax: (413) 545-1262.
21 E-mail: mcclements@foodsci.umass.edu

ABSTRACT

Nanoemulsions have considerable potential for encapsulating and delivering ω -3 fatty acids, but they are typically fabricated from synthetic surfactants. This study shows that fish oil-in-water nanoemulsions can be formed from sunflower phospholipids, which have advantages for food applications because they have low allergenicity and do not come from genetically modified organisms. Nanoemulsions containing small droplets ($d < 150$ nm) could be produced using microfluidization, by optimizing phospholipid type and concentration, with the smallest droplets being formed at high phosphatidylcholine levels and at surfactant-to-oil ratios exceeding unity. The physical stability of the nanoemulsions was mainly attributed to electrostatic repulsion, with droplet aggregation occurring at low pH values (low charge magnitude) and at high ionic strengths (electrostatic screening). These results suggest that sunflower phospholipids may be a viable natural emulsifier to deliver ω -3 fatty acids into food and beverage products.

Keywords: ω -3; fish oil; Nanoemulsion; Natural surfactants; Sunflower; Phospholipids

1. Introduction

Omega-3 fatty acids (ω -3s) have numerous potential health benefits, when consumed at sufficient levels, including those related to neurodevelopment and vascular health (Adkins & Kelley, 2010; Lopez-Huertas, 2010; Uauy & Valenzuela, 2000). However, many American adults do not currently consume sufficiently high levels of ω -3s (Papanikolaou, Brooks, Reider, & Fulgoni, 2014). Therefore, there is an interest in developing functional foods and beverages enriched with ω -3s (Gogus & Smith, 2010; Walker, Decker, & McClements, 2015). Fish oil is of particular interest, as an ω -3 source, because it contains high levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are the most important ω -3 fatty acids for human health (Ward & Singh, 2005).

However, ω -3s cannot simply be incorporated into many functional food products, due to their poor water-solubility, chemical instability and low bioavailability (Augustin, Patten, De Luca, Abeywardena, Lockett, Head, et al., 2011; Augustin & Sanguansri, 2015; Shahidi & Zhong, 2010; Taneja & Singh, 2012). Therefore, there is a pressing need to develop effective delivery systems, to overcome these challenges, so that these bioactive lipids can be incorporated into a wider range of functional food products (McClements, 2010). Oil-in-water nanoemulsions,

which consist of small ($d < 200$ nm) oil droplets dispersed in an aqueous medium, are particularly suitable templates for the development of delivery systems because of their good water-dispersibility, high optical clarity, enhanced physical stability, and improved bioavailability (McClements & Rao, 2011). Nanoemulsions can be prepared using either low- or high-energy methods, which each have their own advantages and disadvantages. Low-energy methods rely on the spontaneous formation of small oil droplets in certain surfactant-oil-water systems when their composition or environmental conditions are altered in a specific manner (Solans & Sole, 2012). The main advantages of these methods are that they are simple to implement, and do not require any expensive specialized equipment (McClements & Rao, 2011). However, they typically require high surfactant-to-oil ratios, and the only surfactants that have so far proved effective at producing small droplets are synthetic ones, such as Tweens and Spans (Hessien, Singh, Kim, & Prouzet, 2011; Saberi, Fang, & McClements, 2013). This approach is therefore unsuitable for many types of functional food products, due to the desire to have “clean labels” that are free of synthetic ingredients (Downham & Collins, 2000).

High-energy methods of producing nanoemulsions rely on specialized equipment (“homogenizers”) to effectively disrupt the oil and water phases so as to generate fine oil droplets (McClements, 2011). Within the food industry, high-energy methods are currently the most commonly used because they are capable of large-scale production, and can produce nanoemulsions from a range of different components (McClements & Rao, 2011). In particular, they can form nanoemulsions from both synthetic and natural emulsifiers, such as surfactants, phospholipids, proteins, and polysaccharides. High-pressure valve homogenizers (HPVHs) are currently the most widely utilized mechanical devices for producing emulsions in the food industry. However, there is increasing interest in the utilization of microfluidizers in the food industry, because they are actually more efficient at producing nanoemulsions than HPVHs (Galooyak & Dabir, 2015; Qian & McClements, 2011). Microfluidizers produce small droplets using specially designed channels to split an inputted coarse emulsion into two streams that are then directed towards each other at a high velocity (Kim & Baianu, 1991). The intense disruptive forces generated within the interaction chamber are highly efficient at promoting droplet fragmentation and nanoemulsion formation (McClements & Rao, 2011). Due to its advantages over other high-energy approaches, a microfluidizer was used to form the ω -3-enriched nanoemulsions prepared in this study.

There are a variety of natural emulsifiers that can be utilized to form food emulsions and nanoemulsions including phospholipids, proteins and polysaccharides (Kralova & Sjoblom, 2009; van Nieuwenhuyzen & Szuhaj, 1998). In the current study, the focus was on the utilization of phospholipids to form ω -3-enriched nanoemulsions. Phospholipids are amphiphilic molecules that have a hydrophilic head group and two hydrophobic tails, and so they can adsorb to oil-water interfaces and stabilize oil droplets (Klang & Valenta, 2011; Mezdoor, Desplanques, & Relkin, 2011). However, there are a number of concerns associated with the application of phospholipids as food ingredients, such as the fact that they may come from sources that contain allergens, *e.g.*, eggs (Herman, 2003), or from sources that are genetically modified, *e.g.*, soybeans (Mezdoor, Desplanques, & Relkin, 2011). For these reasons, there has been considerable interest in investigating the utilization of sunflower phospholipids, which are not a common source of allergens (Herman, 2003; Kruger, Burmann, & Morlock, 2015) and which are not obtained from a genetically modified organism (GMO) (Cabezas, Madoery, Diehl, & Tomas, 2012; Guiotto, Cabezas, Diehl, & Tomas, 2013). In a recent study, we showed that oil-in-water emulsions could be fabricated using a low-energy method (spontaneous emulsification) using sunflower phospholipids (Komaiko, Sastrosubroto, & McClements, 2015). The main advantage of using this low-energy method is that it is very simple and inexpensive to implement, since it simply involves titrating a mixture of oil and phospholipids into water. However, this method also has some major disadvantages for commercial applications, since the phospholipid-to-oil ratio required is relatively high, and the droplet size produced is relatively large ($> 10 \mu\text{m}$).

The main objective of this research was, therefore, to determine whether stable ω -3 nanoemulsions containing small oil droplets could be fabricated using microfluidization and sunflower phospholipids. In addition, the role of electrostatic interactions on the stability of the phospholipid-coated oil droplets was established by measuring the influence of pH and ionic strength on their electrical charge and aggregation. The knowledge gained from this study could help identify functional food products where ω -3-enriched nanoemulsions made with sunflower phospholipids could be successfully applied.

2. Materials and methods

2.1. Materials

Four different natural phospholipid surfactants derived from sunflower oil were kindly donated by Perimondo (New York, NY) (Table 1). Additional information, about the fatty acid composition, can be found in previous manuscripts (Komaiko, Sastrosubroto, & McClements, 2015). Fish oil was kindly donated by DSM Nutritional Products Ltd (Ropufa 30 n-3 food oil, Basel, Switzerland). The aqueous phase for all emulsions was prepared using a sodium phosphate buffer solution (5 mM; pH 2-7). Distilled and deionized water (Milli-Q®) was used to prepare all solutions and emulsions.

2.2. Methods

2.2.1. Emulsion preparation

Initially, an aqueous emulsifier solution was prepared by dispersing the phospholipids in the aqueous phase and stirring overnight at room temperature to ensure complete dissolution. Coarse emulsions were then prepared by blending organic (fish oil) and aqueous phases (buffer and phospholipids) together using a high-speed blender (Bamix, Switzerland) for 2 min at room temperature. The coarse emulsions were then passed through a microfluidizer (Microfluidics M110L, Newton, MA, USA) for 3 cycles at 12,000 psi to produce fine emulsions or nanoemulsions.

2.2.2. Variables tested

Five main variables were tested.

2.2.2.1. Phospholipid composition

Four different sunflower phospholipids were tested (Sunlipon™ 50, 65, 75, and 90), which mainly differ in percentage of phosphatidylcholine (PC) they contained (Table 1).

2.2.2.2. Surfactant-to-oil ratio

The influence of surfactant concentration was investigated by varying the surfactant-to-oil ratio (SOR). The total oil content in the final systems was held constant at 5%, while altering the amounts of surfactant and buffer in the final system varied the SOR:

$$\text{SOR} = \frac{m_s}{m_o} \quad (1)$$

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$$m_b = 100 - m_o - m_s \quad (2)$$

139 Here, m_s , m_o and m_b are the mass percentages of surfactant, oil and buffer in the overall system,
140 respectively.

141 *2.2.2.3. Oil presence*

142 To better understand the difference between liposome and emulsion formation, systems
143 were made up with, and without, fish oil at a phospholipid concentration of 1.25%. In systems
144 without oil, the aqueous phases (buffer and SunliponTM) were still stirred overnight and then
145 mixed using a high-speed blender (Bamix, Switzerland) for 2 min at room temperature prior to
146 microfluidization. Emulsions containing 5% fish oil were prepared at a similar phospholipid
147 level as described in Section 2.2.1.

148 *2.2.2.4. Effect of pH on emulsion stability*

149 Emulsions were prepared at pH 7 and then diluted 10-fold using buffer solutions with
150 different pH values (pH 2 to 7). Samples were adjusted to ensure that they were at the specified
151 pH values and stored for 24 h prior to measurements.

152 *2.2.2.5. Effect of salt on emulsion stability*

153 Emulsions were prepared at pH 7 and then diluted 10-fold using solutions containing a range
154 of salt levels to achieve final values of 0 to 500 mM NaCl. Samples were stored for 24 h prior to
155 measurements.

156 *2.2.3. Emulsion characterization*

157 Samples were analyzed using a variety of techniques including particle size analysis, zeta
158 potential analysis, visual observation, and optical microscopy.

159 *2.2.3.1. Particle size analysis*

160 The particle size distribution and mean particle diameter ($D[3,2]$ or $D[4,3]$) were measured
161 using a commercial static light scattering (SLS) instrument (Mastersizer 2000, Malvern
162 Instruments Ltd., Malvern, Worcestershire, UK). Samples were diluted in double distilled water
163 prior to analysis to avoid multiple scattering effects. The diameter is displayed, primarily, as the
164 surface-weight diameter ($D[3,2]$) except for the effect of salt on physiochemical stability where
165 the mean volume-weighted diameter ($D[4,3]$) is also shown because the mean-volume weighted
166 diameter is more sensitive to particle aggregation (Guiotto, Cabezas, Diehl, & Tomas, 2013).

2.2.3.2. ζ -potential analysis

The electrical charge (ζ -potential) on the droplets was determined using particle electrophoresis (Zetasizer Nano ZS-90, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were diluted in buffer solutions (same pH as sample) prior to measurement, to avoid multiple scattering effects.

2.2.3.3. Visual observation

Samples were observed visually and recorded using a digital camera (Lumix ZS8, Panasonic, Osaka, Japan). All pictures were taken against a black background in a photo box using ambient light.

2.2.3.4. Microscopy

An optical microscope (C1 80i Digital Eclipse, Nikon, Tokyo, Japan) with a 20 $\times$ objective lens and 10 $\times$ eyepiece was used to investigate the microstructure of the samples.

2.2.4. Experimental design

All measurements were performed on two freshly prepared samples in triplicate. The mean and standard deviations were calculated from this data. Statistical analysis was performed through subjection of the data to analysis of variance (ANOVA) using commercial statistics software (Minitab 16.2.4, Minitab Inc., State College, PA). Means were subject to Tukey's test and a P -value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Influence of phospholipid composition and surfactant-to-oil ratio

The influence of phospholipid type and concentration on the properties of emulsions and nanoemulsions formed by microfluidization was investigated (Figure 1). For Sunlipon 50, 65 and 90, a similar trend of decreasing mean particle size with increasing surfactant concentration was observed (Figure 1a). This trend can mainly be attributed to the fact that there were more surfactant molecules available to cover the oil-water interfaces created during homogenization, which enabled smaller droplets (larger surface area) to be formed (Tcholakova, Denkov, & Danner, 2004). In addition, a higher surfactant concentration typically leads to faster coverage of the oil droplet surfaces within a homogenizer, thereby leading to a more rapid reduction in

interfacial tension (thereby facilitating droplet disruption) and leading to a more rapid formation of a protective coating (thereby inhibiting coalescence) (Jafari, He, & Bhandari, 2007). For Sunlipon 75, it was found that the mean particle diameter initially decreased with increasing surfactant concentration (up to SOR 0.1), but then it increased steeply and the droplets formed were always large at high surfactant levels (Figure 1a). Visual observation of these samples indicated that they were highly viscous at high SOR values, *i.e.*, the Sunlipon 75 emulsions would not flow to the bottom of the test tubes used to contain them (Figure 1c). It is, therefore, possible that the high viscosity of these samples prevented effective droplet disruption within the microfluidizer leading to the generation of large droplets. It is not clear why this system had such a higher viscosity than the other types of phospholipids studied, but it may be due to differences in its phase behaviour. Previous studies using sunflower oil (30 wt%) and sunflower lecithin (0.1-2%) have also reported a decrease in particle diameter with an increase in lecithin concentration using homogenization methods (Cabezas, Madoery, Diehl, & Tomas, 2012). However, the surfactant-to-oil ratio used in that study was much lower ($\text{SOR} \approx 0.07$) and so the minimum droplet diameter formed ($d[3,2] \approx 20 \mu\text{m}$) was much larger than that obtained in this study. For many applications of ω -3 fatty acids in functional foods and beverages it is important that the particle size is relatively low to protect against gravitational separation and ensure a high bioavailability (Walker, Decker, & McClements, 2015). Thus, it may be necessary to use relatively high levels of phospholipids to achieve these small particle sizes.

The major components in sunflower phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA) (Mezdour, Desplanques, & Relkin, 2011). Sunlipon 50, 65, 75 and 90 mainly differed in the amount of PC they contained, with the manufacturers designation number roughly corresponding to the weight percentage of PC present (Table 1). Emulsifier performance has previously been linked to phospholipid composition, with studies showing that PC is capable of forming well-ordered mono-layers or bi-layers in a lamellar structure around fat droplets, which is proposed to facilitate emulsion formation and enhance emulsion stability (van Nieuwenhuyzen & Szuhaj, 1998). For sunflower oil-in-water emulsions, it was reported that the highest emulsion stability occurred when there was a high ratio of PC to PE (Guiotto, Cabezas, Diehl, & Tomas, 2013). Other studies have also shown that lecithin ingredients enriched with PC have the best emulsifying properties and are the most effective at stabilizing lipid droplets (Cabezas, Madoery,

Diehl, & Tomas, 2012). Therefore, based on our starting materials, we would have predicted that Sunlipon 90 would have formed the smallest droplets, followed by Sunlipon 75, 65 and 50. However, we found no significant difference in the minimum droplet diameter obtained using Sunlipon 50, 65, and 90, and a significantly larger droplet size with Sunlipon 75 (at SOR = 2). Thus, these results do not support the hypothesis that higher PC levels lead to better emulsion formation or stability. It is therefore clear that other factors are also important in determining the performance of the different sunflower phospholipid emulsifiers. For example, the electrical characteristics of the phospholipid head groups would impact the formation of the interfacial layers around the fat droplets, as well as their aggregation stability. In addition, the rheology of the emulsions formed by the phospholipids will influence the efficiency of droplet disruption within a microfluidizer. Finally, phospholipid ingredients may contain different minor components or impurities that impact their performance. The darker colour of Sunlipon 50, 65, and 90 suggests that they may contain Maillard reaction products generated during their manufacture (e.g. due to PE and sugar interactions), or coloured impurities, such as carotenoids, melanoids and porphyrins (van Nieuwenhuyzen & Tomas, 2008), which could influence their emulsifying ability. It should also be noted that the Sunlipon 50, 65, and 90 ingredients were pastes, whereas the Sunlipon 75 ingredient was a white powder (Table 1), which suggests that the different phospholipids had different structural organizations in the dehydrated state. Nevertheless, this difference is likely to be less important after the phospholipid ingredients had been hydrated.

When comparing the ability of emulsifiers, to form emulsions, it is useful to calculate their surface loads, *i.e.*, the amount of emulsifier required to cover a given surface area (McClements, 2007). The surface load (Γ) of an emulsifier is related to the mean droplet diameter (d_{32}), disperse phase volume fraction (ϕ), and concentration of the adsorbed emulsifier (C_s): $\Gamma = d_{32}C_s/6\phi$ (Tcholakova, Denkov, & Danner, 2004). Consequently, the surface load can be calculated from the slope of a plot of d_{32} versus $1/C_s$: $\Gamma = \text{slope}/6\phi$. This approach assumes that the droplet size is limited by the amount of emulsifier present, rather than by the disruptive forces generated by the homogenizer, and therefore only the data at relatively low emulsifier concentrations is used in the calculations. The surface loads of the Sunlipon 50, 65, 75, and 90 calculated using this approach were 2.04, 2.27, 3.67 and 10.0 mg m⁻² (Table 1). These calculations indicate that smaller amounts of Sunlipon 50 and 65 are required to cover a given

droplet surface area than for Sunlipon 75 or 90. A possible explanation for this effect is that Sunlipon 75 and 90 formed multiple layers of phospholipids at the droplet surfaces, whereas the other two phospholipids only formed monolayers. Indeed, the calculated surface loads for Sunlipon 50 and 65 are close to the values reported for small molecule surfactants that typically form monolayers (McClements, 2007). An alternative explanation is that an appreciable fraction of the phospholipids were not adsorbed to the droplet surfaces for Sunlipon 75 and 90, and instead formed liposomes or other structures in the aqueous phase surrounding the fat droplets. In this case, not all of the phospholipid molecules would be adsorbed to the lipid droplet surfaces, and therefore the surface load would be over-estimated. In future studies, it would be useful to examine the microstructure of the emulsions using electron microscopy or other high-resolution methods to establish the precise nature of the structures formed, *e.g.*, the number, size, and organization of any liposomes present.

The ζ -potential measurements indicated that phospholipid type had a pronounced influence on the electrical characteristics of the droplets (Figure 1b). For example, the droplets in emulsions made with Sunlipon 50 had a high negative charge, those made with Sunlipon 65 had a modest negative charge, and those made with Sunlipon 75 and 90 were slightly positive. For each phospholipid type, the ζ -potential remained relatively unchanged for all SOR levels tested. The relatively low cationic charge on the droplets coated by Sunlipon 75 and 90 can be attributed to the fact that they contained relatively high levels of PC, which has a zwitterionic head group that does not carry much net charge at a neutral pH. Conversely, the anionic nature of the droplets coated by Sunlipon 50 and 90 may be due to the presence of other phospholipids (Table 1) that do have a net negative charge at this pH (García-Moreno, Horn, & Jacobsen, 2014).

Overall, our results indicate that there was not a direct relationship between the size of the droplets formed by homogenization and the electrical characteristics of the phospholipids. This conclusion is based on the fact that droplets formed by Sunlipon 50, 65, and 90 had similar minimum droplet diameters but different particle charges, and that Sunlipon 75 and 90 had similar electrical charges but very different mean particle diameters (Table 1).

Visually, the emulsions containing droplets coated by Sunlipon 50 and 65 appeared stable to phase separation across the entire surfactant range studied (Figure 1c). For Sunlipon 50 a slight brown colour could be observed at the highest surfactant concentrations, while Sunlipon 65 always appeared milky white. Sunlipon 75 was unstable to phase separation at the lowest SORs

and was highly viscous at the highest SORs studied. For Sunlipon 90, a layer of free oil (“oiling off”) was observed at the top of the emulsions after storage. This suggests that some droplet coalescence may have occurred due to the relatively low charge on the droplets in this system allowing them to come close together.

3.2. Influence of oil presence

In the absence of oils, phospholipids can form colloidal structures when they are dispersed in aqueous solutions, such as vesicles and liquid crystals (Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008). These colloidal structures may influence the formation, stability, and properties of the emulsions prepared from phospholipids in the presence of oils. Therefore the properties of phospholipid dispersions in the absence of oils were examined (presumably liposomes), and compared them to the properties measured in the presence of oils (presumably emulsions). Interestingly, the general trends in particle size and charge were fairly similar for the dispersions prepared in the absence and presence of oil Sunlipon 50, 65 and 90 formed relatively small particles, whereas Sunlipon 75 formed relatively large ones (Figure 2a). Sunlipon 50 and 65 formed negatively charged particles, whereas Sunlipon 75 and 90 formed positively charged ones (Figure 2b). The similar electrical characteristics of the two systems can be attributed to the fact that both liposomes and fat droplets are coated by a layer of phospholipids. Visually, there were distinct differences between the overall appearance of the dispersions with and without oil, which depended on phospholipid type (Figure 2c). The emulsions (with oil) were all optically opaque and milky white in appearance, whereas the liposomes (without oil) varied from transparent (Sunlipon 50 and 65) to cloudy (Sunlipon 75). These differences in optical properties can be attributed to differences in the light scattering characteristics of the colloidal dispersions. In general, the light scattering behaviour of a colloidal dispersion depends on particle size, concentration, refractive index, and spatial distribution (McClements, 2002). The milky white appearance of the emulsions can therefore be attributed to the fact that the fat droplets had an appreciably different refractive index than the surrounding water phase, and that they contained particles with dimensions similar to the wavelength of light. In contrast, the smaller degree of light scattering by the liposomes can be attributed to the fact that the phospholipid bilayers had dimensions that were much smaller than the wavelength of light. In this case, it was thickness of the bilayers, rather than the overall particle size, that mainly governed their light scattering behaviour. The fact that the liposome

suspensions formed using Sunlipon 75 had a higher turbidity than for the other phospholipids may be because the phospholipid layers were packed closer together, either due to aggregation or multilayer formation. The ζ -potential for all systems without oil was significantly less negative (Sunlipon 50 and 65) or more positive (Sunlipon 75 and 90) than those with oil (Figure 2b), which implies that the fish oil contributed some negative charge to the droplets. This could have been due to hydrolysis of triacylglycerols into free fatty acids with anionic carboxylic acid head-groups that accumulated at the fat droplet surfaces. Indeed, previous studies have shown that free fatty acids may partition into phospholipid bilayers (Langner, Isac, & Hui, 1995).

3.3. Effect of pH on physicochemical stability

The aim of this research was to create nanoemulsion-based delivery systems for ω -3 fatty acids that could be incorporated into a variety of different functional food products ranging from acidic soft drinks ($\approx$ pH 2) to neutral nutritional beverages ($\approx$ pH 7). Therefore, the influence of pH on the stability of nanoemulsions made using sunflower phospholipids was investigated. In this series of experiments, the performance of phospholipids containing around 50% PC (Sunlipon 50) and 90% PC (Sunlipon 90) was compared to provide some insight into the role of phospholipid composition. For Sunlipon 50, the mean particle diameter remained relatively low from pH 7 to 3, but increased at pH 2 (Figure 3a). Correspondingly, these emulsions remained visibly stable to gravitational separation from pH 7 to 3, but creamed at pH 2 as demonstrated by some clearing in the bottom of the test tubes (Figure 3b). The creaming observed at pH 2 can be attributed to the increase in particle size, since the creaming velocity is known to increase with particle size (McClements, 2011). The physicochemical origin of pH-stability of this system can be attributed to changes in the electrical characteristics of the droplets with pH (Figure 3b). The droplets coated by Sunlipon 50 had a relatively high negative charge from pH 7 to 3 ($\approx$ -43 to -19 mV), which would generate a strong electrostatic repulsion between them, thereby inhibiting aggregation. Conversely, the droplets had a relatively low positive charge at pH 3 ($\approx$ +3 mV), which would not be sufficient to inhibit droplet aggregation.

For Sunlipon 90, the mean droplet diameter remained relatively low across the entire pH range studied (Figure 3a), which suggested that the individual emulsion droplets were relatively small. However, these emulsions were highly unstable to creaming across the whole pH range,

with an opaque creamed layer being observed at the top of the samples (Figure 3c). Interestingly, there appeared to be less creaming in the emulsions stored at pH 2, since the lower serum layer was more opaque than for the other pH values. The fact that creaming was observed in these emulsions suggests that there was some aggregation of the oil droplets, which was confirmed by optical microscopy at neutral pH (Figure 3d). The emulsions formed from Sunlipon 90 were seen to contain large aggregates, whereas those containing Sunlipon 50 were not. The fact that the particle size measured by light scattering was relatively small, even though droplet aggregation occurred, suggests that these emulsions were flocculated. The origin of droplet flocculation can again be attributed to the influence of electrostatic interactions. The ζ -potential of the droplets in the emulsions stabilized by Sunlipon 90 remained relatively low across the entire pH range, with the exception of pH 2 where there was a modest positive charge (+15 mV) (Figure 3b). The relatively low droplet charge from pH 3 to 7 would account for the fact that the droplets were highly susceptible to flocculation in this pH range, because there would only be a very weak electrostatic repulsion between them. The modest positive charge at pH 2 may have been responsible for the fact that these emulsions were more stable to creaming than at other pH values.

3.4. Effect of salt on physiochemical stability

Commercial foods and beverages differ in the ionic compositions of their aqueous phases, with beverages like mineral water having a low ionic strength and foods like soups and sauces having high ionic strengths. The influence of ionic strength on the stability of the ω -3 enriched nanoemulsions was, therefore, examined for the Sunlipon 50 emulsions. The Sunlipon 90 emulsions were not included in this series of experiments since they were already unstable to flocculation in the absence of salt (Section 3.3).

The influence of salt addition (0 to 500 mM NaCl) on the mean particle diameter, ζ -potential, physical appearance, and microstructure of the nanoemulsions was measured (Figure 4). In this case, the mean particle diameter was represented by both $d[3,2]$ and $d[4,3]$ to highlight the presence of a population of large aggregates observed in the particle size distribution data (not shown). There was clear evidence of an increase in particle size with increasing salt concentration (Figure 4a), which suggested that some droplet aggregation occurred when the ionic strength was increased. This effect was confirmed by optical

microscopy images of the samples, which showed that they were highly susceptible to droplet coalescence at higher salt concentrations, *i.e.*, there was an increase in the size of the individual droplets (Figure 4d). The origin of droplet aggregation upon addition of salt can be attributed to a reduction in the electrostatic repulsion operating between oil droplets with increasing ionic strength (McClements, 2011). Indeed, the addition of salt to the emulsions caused an appreciable decrease in the magnitude of the ζ -potential (Figure 4b), which can be attributed to electrostatic screening effects. Surprisingly, there was little change in the visual appearance of the emulsions containing different salt levels (Figure 4c). One would have expected larger oil droplets to move more rapidly to the top of the emulsions. The fact that creaming was not observed may have been because the aggregated droplets formed a three-dimensional network that inhibited their movement, or because the presence of liposomes in the aqueous phase inhibited their movement. These results are in agreement with earlier studies, which have also shown that oil-in-water emulsions stabilized by phospholipids are unstable in the presence of electrolytes (De Vleeschauwer & Van der Meeren, 1999).

4. Conclusions

We have shown that ω -3-enriched nanoemulsions can be produced by microfluidization using certain kinds of natural sunflower phospholipids. The initial size of the droplets produced depended on phospholipid type and surfactant-to-oil ratio. There appeared to be no simple correlation between the phosphatidylcholine content of the phospholipid ingredients, and their ability to form small droplets. Emulsions made with the highest overall phospholipid concentration produced the smallest droplet sizes. The emulsions formed were primarily stabilized by electrostatic repulsion, and were therefore susceptible to aggregation under conditions where the droplets had low net charges or where the aqueous phase had a high ionic strength. These results suggest that sunflower phospholipids can be used as natural emulsifiers to form ω -3 nanoemulsion-based delivery systems suitable for application in certain types of foods and beverages. However, it is important to ensure that the pH and ionic strength of a particular product do not promote droplet aggregation by reducing electrostatic repulsion. In future studies, it would be interesting to investigate the chemical stability of these systems, as oils rich in polyunsaturated lipids are known to be highly unstable to oxidation.

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References

- Adkins, Y., & Kelley, D. S. (2010). Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. *Journal of Nutritional Biochemistry*, 21(9), 781-792.
- Augustin, M. A., Patten, G., De Luca, A., Abeywardena, M., Lockett, T., Head, R., & Sanguansri, L. (2011). Intestinal passage of microencapsulated fish oil in rats following oral administration. *Food & Function*, 2(11), 684-696.
- Augustin, M. A., & Sanguansri, L. (2015). Challenges and Solutions to Incorporation of Nutraceuticals in Foods. In M. P. Doyle & T. R. Klaenhammer (Eds.), *Annual Review of Food Science and Technology*, Vol 6, vol. 6 (pp. 463-477).
- Cabezas, D. M., Madoery, R., Diehl, B. W. K., & Tomas, M. C. (2012). Emulsifying Properties of Different Modified Sunflower Lecithins. *Journal of the American Oil Chemists Society*, 89(2), 355-361.
- De Vleeschauwer, D., & Van der Meeren, P. (1999). Colloid chemical stability and interfacial properties of mixed phospholipid–non-ionic surfactant stabilised oil-in-water emulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 152(1–2), 59-66.
- Downham, A., & Collins, P. (2000). Colouring our foods in the last and next millennium. *International Journal of Food Science and Technology*, 35(1), 5-22.
- Galooyak, S. S., & Dabir, B. (2015). Three-factor response surface optimization of nano-emulsion formation using a microfluidizer. *Journal of Food Science and Technology-Mysore*, 52(5), 2558-2571.
- García-Moreno, P. J., Horn, A. F., & Jacobsen, C. (2014). Influence of Casein–Phospholipid Combinations as Emulsifier on the Physical and Oxidative Stability of Fish Oil-in-Water Emulsions. *Journal of Agricultural and Food Chemistry*, 62(5), 1142-1152.

443 Gogus, U., & Smith, C. (2010). n-3 Omega fatty acids: a review of current knowledge.
444 *International Journal of Food Science and Technology*, 45(3), 417-436.

445 Guiotto, E. N., Cabezas, D. M., Diehl, B. W. K., & Tomas, M. C. (2013). Characterization and
446 emulsifying properties of different sunflower phosphatidylcholine enriched
447 fractions. *European Journal of Lipid Science and Technology*, 115(8), 865-873.

448 Herman, E. M. (2003). Genetically modified soybeans and food allergies. *Journal of*
449 *Experimental Botany*, 54(386), 1317-1319.

450 Hessien, M., Singh, N., Kim, C., & Prouzet, E. (2011). Stability and Tunability of O/W
451 Nanoemulsions Prepared by Phase Inversion Composition. *Langmuir*, 27(6),
452 2299-2307.

453 Jafari, S. M., He, Y. H., & Bhandari, B. (2007). Production of sub-micron emulsions by
454 ultrasound and microfluidization techniques. *Journal of Food Engineering*, 82(4),
455 478-488.

456 Kim, H.-H. Y., & Baianu, I. C. (1991). Novel liposome microencapsulation techniques for food
457 applications. *Trends in Food Science & Technology*, 2, 55-61.

458 Klang, V., & Valenta, C. (2011). Lecithin-based nanoemulsions. *Journal of Drug Delivery*
459 *Science and Technology*, 21(1), 55-76.

460 Komaiko, J., Sastrosubroto, A., & McClements, D. J. (2015). Formation of oil-in-water
461 emulsions from natural emulsifiers using spontaneous emulsification: Sunflower
462 phospholipids. *Journal of Agricultural and Food Chemistry*.

463 Kralova, I., & Sjoblom, J. (2009). Surfactants Used in Food Industry: A Review. *Journal of*
464 *Dispersion Science and Technology*, 30(9), 1363-1383.

465 Kruger, S., Burmann, L., & Morlock, G. E. (2015). Comparison and Characterization of Soybean
466 and Sunflower Lecithins Used for Chocolate Production by High-Performance
467 Thin-Layer Chromatography with Fluorescence Detection and Electrospray Mass
468 Spectrometry. *Journal of Agricultural and Food Chemistry*, 63(11), 2893-2901.

469 Langner, M., Isac, T., & Hui, S. W. (1995). INTERACTION OF FREE FATTY-ACIDS WITH
470 PHOSPHOLIPID-BILAYERS. *Biochimica Et Biophysica Acta-Biomembranes*,
471 1236(1), 73-80.

472 Lopez-Huertas, E. (2010). Health effects of oleic acid and long chain omega-3 fatty acids (EPA
473 and DHA) enriched milks. A review of intervention studies. *Pharmacological*
474 *Research*, 61(3), 200-207.

475 McClements, D. J. (2002). Colloidal basis of emulsion color. *Current Opinion in Colloid &*
476 *Interface Science*, 7(5-6), 451-455.

- 477 McClements, D. J. (2007). Critical review of techniques and methodologies for characterization
478 of emulsion stability. *Critical Reviews in Food Science and Nutrition*, 47(7), 611-
479 649.
- 480 McClements, D. J. (2010). Emulsion Design to Improve the Delivery of Functional Lipophilic
481 Components. In M. P. Doyle & T. R. Klaenhammer (Eds.), *Annual Review of*
482 *Food Science and Technology*, Vol 1, vol. 1 (pp. 241-269).
- 483 McClements, D. J. (2011). Edible nanoemulsions: fabrication, properties, and functional
484 performance. *Soft Matter*, 7(6), 2297-2316.
- 485 McClements, D. J., & Rao, J. (2011). Food-Grade Nanoemulsions: Formulation, Fabrication,
486 Properties, Performance, Biological Fate, and Potential Toxicity. *Critical Reviews*
487 *in Food Science and Nutrition*, 51(4), 285-330.
- 488 Mezdour, S., Desplanques, S., & Relkin, P. (2011). Effects of residual phospholipids on surface
489 properties of a soft-refined sunflower oil: Application to stabilization of sauce-
490 types' emulsions. *Food Hydrocolloids*, 25(4), 613-619.
- 491 Mozafari, M. R., Johnson, C., Hatziantoniou, S., & Demetzos, C. (2008). Nanoliposomes and
492 Their Applications in Food Nanotechnology. *Journal of Liposome Research*,
493 18(4), 309-327.
- 494 Papanikolaou, Y., Brooks, J., Reider, C., & Fulgoni, V. L. (2014). U.S. adults are not meeting
495 recommended levels for fish and omega-3 fatty acid intake: results of an analysis
496 using observational data from NHANES 2003–2008. *Nutrition Journal*, 13, 31-
497 31.
- 498 Qian, C., & McClements, D. J. (2011). Formation of nanoemulsions stabilized by model food-
499 grade emulsifiers using high-pressure homogenization: Factors affecting particle
500 size. *Food Hydrocolloids*, 25(5), 1000-1008.
- 501 Saberi, A. H., Fang, Y., & McClements, D. J. (2013). Fabrication of vitamin E-enriched
502 nanoemulsions: Factors affecting particle size using spontaneous emulsification.
503 *Journal of Colloid and Interface Science*, 391, 95-102.
- 504 Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical*
505 *Society Reviews*, 39(11), 4067-4079.
- 506 Solans, C., & Sole, I. (2012). Nano-emulsions: Formation by low-energy methods. *Current*
507 *Opinion in Colloid & Interface Science*, 17(5), 246-254.
- 508 Taneja, A., & Singh, H. (2012). Challenges for the Delivery of Long-Chain n-3 Fatty Acids in
509 Functional Foods. In M. P. Doyle & T. R. Klaenhammer (Eds.), *Annual Review of*
510 *Food Science and Technology*, Vol 3, vol. 3 (pp. 105-123).

511 Tcholakova, S., Denkov, N. D., & Danner, T. (2004). Role of surfactant type and concentration
512 for the mean drop size during emulsification in turbulent flow. *Langmuir*, 20(18),
513 7444-7458.

514 Uauy, R., & Valenzuela, A. (2000). Marine oils: The health benefits of n-3 fatty acids. *Nutrition*,
515 16(7-8), 680-684.

516 van Nieuwenhuyzen, W., & Szuhaj, B. F. (1998). Effects of lecithins and proteins on the stability
517 of emulsions. *Fett-Lipid*, 100(7), 282-291.

518 van Nieuwenhuyzen, W., & Tomas, M. C. (2008). Update on vegetable lecithin and phospholipid
519 technologies. *European Journal of Lipid Science and Technology*, 110(5), 472-
520 486.

521 Walker, R., Decker, E. A., & McClements, D. J. (2015). Development of food-grade
522 nanoemulsions and emulsions for delivery of omega-3 fatty acids: opportunities
523 and obstacles in the food industry. *Food & Function*, 6(1), 42-55.

524 Ward, O. P., & Singh, A. (2005). Omega-3/6 fatty acids: Alternative sources of production.
525 *Process Biochemistry*, 40(12), 3627-3652.
526

Table 1: Properties of the sunflower phospholipids used in this study. Phospholipid composition was provided by the manufacturer.

		Sunlipon™ 50	Sunlipon™ 65	Sunlipon™ 75	Sunlipon™ 90
		Weight %			
Phospholipid Composition	Phosphatidylcholine	58	65	74	90
	1-Lysophosphatidylcholine	1	1	1	0.5
	2-Lysophosphatidylcholine	3	5	4	2
	Phosphatidylinositol	1	<1	-	-
	Lysophosphatidylinositol	-	-	-	-
	Phosphatidylserine-Na	-	-	-	-
	Lysophosphatidylserine	-	-	-	-
	Sphingomyelin	-	-	-	-
	Phosphatidylethanolamine	5	4	0.5	-
	Lysophosphatidylethanolamine	1	<1	-	-
	acyl-Phosphatidylethanolamine	3	2	2	-
	Phosphatidylglycerol	1	1	-	-
	Phosphatidic acid	-	<1	-	-
	Lysophosphatidic acid	0.2	-	-	-
	Other	2	1	1	0.1
Physical Appearance		Brown Paste	Brown Paste	White Powder	Amber Paste
<i>I</i> _{sat} (mg/m ²)		2.04	2.27	3.67	10.0
D _{min} (μm)		0.124	0.138	0.356	0.137

Figure 1: Effect of surfactant-to-oil ratio and phospholipid type on (a) mean particle size and microstructure, (b) z-potential, and (c) physical appearance of emulsions. The microstructures of emulsions formed with Sunlipon 50 and 75 are shown as insets in figure 1a (SOR = 2).

a) Particle size

b) Zeta potential

c) Physical appearance

	Surfactant-to-Oil Ratio							
	0.025	0.05	0.1	0.25	0.5	1	2	
Sunlipon 50
Sunlipon 65
Sunlipon 75
Sunlipon 90

Figure 2: Exploration of liposome versus emulsion formation by looking at the effect of the presence of oil on the a) particle size, b) zeta potential, and c) physical appearance of the systems. Different lower case letters (a, b) mean statistical differences in the particle diameter or zeta potential of a given surfactant type (i.e., the effect of oil versus no oil)

a) Particle size

b) Zeta potential

c) Physical appearance

Figure 3: Effect of pH on the stability of emulsion systems formed using Sunlipon 50 and Sunlipon 90 at an SOR of 1 after 24 hours. The microstructure was investigated at pH 7

a) Particle size

b) Zeta potential

c) Physical appearance

Sunlipon 50						
pH	2	3	4	5	6	7

Sunlipon 90						
pH	2	3	4	5	6	7

d) Microstructure at pH 7

Figure 4: Effect of salt on the stability of emulsion systems formed using Sunlipon 50 at an SOR of 1 and pH of 7 after 24 hours. Particle size is represented as D[3,2] and D[4,3] since the D[4,3] better represented the large particles that were formed.

a) Particle size

b) Zeta potential

c) Physical appearance

[NaCl] (mM)					
50	100	200	300	400	500

d) Microstructure