The composition and oxidative stability of vegetarian omega-3 algal oil nanoemulsions suitable for functional food enrichment

(Running title: Composition and oxidative stability of omega-3 algal oil nanoemulsions)

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Abstract

Background: Long chain omega-3 polyunsaturated fatty acid (LCn3PUFA) nanoemulsion enriched foods offer potential to address habitually low oily fish intakes. Nanoemulsions increase LCn3PUFA bioavailability, but may cause lipid oxidation. This study examined oxidative stability of LCn3PUFA algal oil-in-water nanoemulsions created by ultrasound using natural and synthetic emulsifiers during 5-weeks of storage at 4, 20 and 40°C. Fatty acid composition, droplet size ranges and volatile compounds were analysed.

Results: No significant differences were found for fatty acid composition at various temperatures and storage times.

Lecithin nanoemulsions had significantly larger droplet size ranges at baseline and during storage regardless of temperatures. While combined Tween 40 and lecithin nanoemulsions had low initial droplet size ranges, there were significant increases at 40°C after 5-weeks storage. Gas chromatograms identified hexanal and propanal as predominant volatile compounds, along with 2-ethylfuran; propan-3-ol; valeraldehyde. The Tween 40 only nanoemulsion sample showed formation of lower concentrations of volatiles compared to lecithin samples. Formation of hexanal and propanal remained stable at lower temperatures although higher concentrations were found in nanoemulsions than bulk oil. The lecithin only sample had formation of higher concentrations of volatiles at increased temperatures despite having significantly larger droplet size ranges than the other samples.

Conclusions: Propanal and hexanal were the most prevalent of five volatile compounds detected in bulk oil and lecithin and/or Tween 40 nanoemulsions. Oxidation compounds remained more stable at lower temperatures indicating suitability for enrichment of refrigerated foods. Further research to evaluate the oxidation stability of these systems within food matrices is warranted.

Keywords: Omega-3 fatty acids; Algal oil; Nanoemulsion; Oxidative stability; Lecithin; Tween 40.

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1 Introduction

Oily fish consumption continues to fall short of recommended levels in Western populations, causing potential implications for overall health and increased risk of other non-communicable diseases [1-3]. The use of long chain omega-3 polyunsaturated fatty acid (LCn3PUFA) rich oil-in-water nanoemulsion enriched foods offers a potential solution to this problem, particularly for population groups that consume little or no oily fish such as vegetarians and vegans.

New approaches, including food-based strategies have the potential to improve intakes and LCn3PUFA nanoemulsion enriched functional foods may offer an alternative solution for groups with low habitual oily fish consumption [4, 5]. Eicosapentaenoic acid (20:5 n3; EPA) and docosahexaenoic acid (22:6 n3; DHA) are thought to be the most beneficial forms of LCn3PUFA [6, 7]. The European Food Safety Agency [8] have approved health claims in relation to foods naturally rich or fortified with EPA and DHA. Microalgal oils have recently emerged to offer a sustainable alternative source of EPA and DHA that is also suitable for vegetarians and vegans [9-11].

Nanoemulsions, similar to conventional oil-in-water emulsion systems but with considerably smaller droplet sizes can be used to incorporate lipid based components into aqueous foods [12]. The use of nanoemulsions offers potential benefits including increases in water dispersibility of oils, good physical and chemical stability and improved bioavailability of various hydrophobic lipid components [12-14]. However, utilisation of LCn3PUFA rich oils in nanoemulsions may further promote lipid oxidation in these already vulnerable oils.

EPA and DHA are characterised by long carbon chain lengths and a high degree of lipid unsaturation, which increases their susceptibility to oxidation when exposed to air, light and heat, all of which are inevitable during emulsion processing. The mechanism of lipid oxidation is proposed to include three stages initiation, propagation and termination [15]. A high variety of volatile compounds of different polarity, stability and small molecular weight are formed which can be detected by dynamic headspace analysis [16]. Once the initiation phase has begun the rate of oxidation increases exponentially and foods are quickly spoiled. Lipid oxidation in enriched/functional foods impacts the shelf-life, safety, nutritional value, functionality and flavour of the subsequent food products [17]. The creation of LCn3PUFA nanoemulsions may further increase the oxidation susceptibility of these oils due to high droplet surface areas and the need for greater processing.

In previous work, novel oil-in-water nanoemulsion systems suitable for functional food enrichment were successfully created using ultrasound processing with commercially available high DHA vegetarian algal oil loads up 50% (w/w), which has not been previously achieved [18]. The addition of a 50% (w/w) system is less likely to have a detrimental effect on food matrices than systems with lower oil loads as lower volumes of nanoemulsion can be added to achieve optimum enrichment levels [19]. The systems were also demonstrated to significantly increase the bioavailability of total LCn3PUFA and DHA in a pilot randomised crossover trial [14], although additional larger, longer studies are needed to further ratify these findings. Preliminary evaluation to analyse total oxidation values (totox) indicated these systems remained within safe ranges when stored at 4°C for 37 days [18]. However, there were significant detrimental changes in the sensory properties of yogurt when the nanoemulsion systems were used as a fortification vehicle, which may indicate the presence of volatile

oxidation compounds [20]. A further 16-day shelf life sensory evaluation of 50% (w/w) algal oil and lecithin nanoemulsion enriched strawberry yogurt indicated that detrimental sensory changes were detected after 2 days of storage but significant improvements to several sensory attributes were found after 16 days of refrigerated storage, which warrants further investigation [21].

Lecithin, a zwitterionic natural emulsifier has good emulsifying properties due to its molecular structure, which has hydrophilic and lipophilic groups and a hydrophilic-lipophilic balance (HLB) of 8 making it well suited to the successful creation of LCω3PUFA algal oil and marine based oil nanoemulsions [22]. Lecithin has been used successfully to create physically stable LCn3PUFA nanoemulsion systems in various previous studies [16, 19, 23-25] and has been found to increase oxidative stability of emulsions whilst maintaining physical stability [26]. The use of Tween 40 in the creation of LCn3PUFA nanoemulsions has also previously been demonstrated to have a protective effect on lipid oxidation [27-29]. However, a study to evaluate the oxidation stability of high load DHA algal oil lecithin and Tween 40 nanoemulsions using more comprehensive methods has yet to be undertaken. Therefore, the aim of the current study was to evaluate the oxidative stability of algal oil nanoemulsions created with ultrasound using lecithin and Tween 40 solely and in combination to maximise their physical and chemical stabilising properties, which coupled with the gas chromatography headspace method is a novel and useful study. The oxidative stability of algal oil was compared in bulk form and nanoemulsion under the same conditions with no other added components other than deionised water and the two types of emulsifier.

2 Materials and Methods

2.1 Materials

Algal oil (Life DHATM S35-O300) was purchased from DSM Ltd., (Columbia, USA). The algal oil used in this study is a commercially available product that contains added antioxidants (tocopherols (0.025%) and ascorbyl palmitate (0.025%) [30]). L-α-Phosphatidylcholine (P3644-100G) of soybean and Type IV-S. ≥30% (enzymatic), Polyoxyethylenesorbitan monopalmitate (Tween 40, P1504) were purchased from Sigma-Aldrich, UK. Sodium chloride (99.5%) was purchased from ACROS, Spain. Hexane (HPLC Grade) was purchased from Fisher Scientific, (UK). Methanol (HPLC Grade), Sulphuric acid 95%. Sodium sulphate anhydrous were purchased from VER BDH PROLABO chemicals, EC (UK). Distilled and deionized water was added to all nanoemulsions.

2.1.1 Preparation of nanoemulsion samples

The 50% (w/w) oil-in-water nanoemulsions of LCn3PUFA algal oil were prepared by following the method developed by Lane et al, [19], in which 6% (w/w) of the selected emulsifiers i.e. lecithin, Tween 40, and equal ratio Tween 40 and lecithin (3% w/w of each) were used. A solution of lecithin premix containing algal oil (70g) combined with lecithin (30g) was placed in a water bath at 55°C for 2 hours to ensure the lecithin was completely dissolved. Tween 40 premixes containing deionised water (44g) mixed with Tween 40 (6g) were prepared and placed in a water bath at 55°C.

Appropriate measures of algal oil (36g) and water (44g) were combined with the lecithin premix (20g) to create the lecithin samples. Measures of algal oil (50g) were combined with 50g of Tween 40 premix to create the Tween 40 samples.

Both emulsion premixes (10g lecithin premix, 22g Tween 40 premix) were combined with algal oil (43g) and deionised water (25g) to give a 50% algal oil sample with a combination of lecithin and Tween 40 containing 3% w/w of each emulsifier.

After premixing, the coarse emulsions were replaced in the water bath for a further 2 hours and hand stirred for 1 min at 30 min intervals. The temperature was controlled at 55°C. Samples underwent primary homogenisation using a L5 series Silverson rotor–stator mixer (Silverson Machines Ltd, England), on a medium setting (668.12 x g) for 2 min, then processed under an ultrasonic processor (BSP-1200 Ultrasonic processor, New York, USA) using Amplitude 100% with power 850w, operated at 19650Hz for 10 minutes to create nanoemulsions. A cold-water cooling jacket was used to ensure sample temperatures remained below 55°C.

2.1.2 Fatty acid composition analysis by Gas Chromatography (GC)

The fatty acid composition analysis of algal oil and nanoemulsion samples was performed using fatty acid methyl ester (FAME) analysis whereby 0.5000 g algal oil /1.000 g of nanoemulsion sample and 10 ml Reagent A (2.5% w/v KOH solution in Methanol) were added into a MARSXpress vessel microwave digestion tube, then closed. The tube was placed into the Kevlar sleeves of a Mars 6 microwave (CEM Ltd., UK). The temperature was increased to 90°C in 5 min and held for 10 min. After cooling to room temperature, 15ml reagent B (2% sulphuric acid v/v in methanol) was added to the tube.

158 After closing, the tube was placed back into the Kevlar sleeves of the Mars 6 microwave and
159 the temperature was increased to 120 °C for 6 min. After cooling to room temperature, 10 ml
160 Hexane was added to the tube and inverted once.

161

162 The sufficient saturated salt solution was added to bring the hexane to the top layer. The
163 upper hexane layer containing fatty acid methyl esters was obtained for GC analysis using a
164 GC Clarus 480. 200µl of the upper hexane layer containing fatty acid methyl esters and 800µl
165 hexane was added into the GC vial with a small amount of added anhydrous sodium sulphate.
166 The samples were analysed by GC Clarus 480 system (PerkinElmer Inc, USA) equipped with an
167 auto sampler, Flame Ionization Detector, 30 m, 0.25 mm id 0.25 µm film thickness GC capillary
168 column (SGE Analytical Science Pty Ltd, Australia) and Total Chrom Navigator software system
169 (Version 6.3.2 PerkinElmer Inc, USA). The injector and detector temperature were 220°C and
170 250°C respectively, 1.5 µl of sample was injected in each time and hydrogen flow rate was set
171 at 8.4 psi. The temperature program for the column was increased from 60 to 170°C at a rate
172 of 20°C/min and to 200°C at a rate 1 °C/min, holding 1 min; the total run time was 36.5 min.
173 Fatty acids were identified by reference to the retention time of standards. Analysis was
174 performed in triplicate on individual vials for each time point.

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176 **2.1.3 Measurement of emulsion droplet size**

177 Nanoemulsions are classed as systems with droplet sizes ranging from 50 to 500 nm [31, 32]. The
178 droplet size of emulsion samples prepared was determined by Mastersizer 3000 laser light-
179 scattering analyzer (Malvern Instruments Ltd, Malvern, UK) with a small sample dispersion

unit set 2400 rpm. For the emulsion samples, an absorption parameter value of 0.001 was selected and a refractive index ratio 1.488 for algal oil [19]. For the purposes of this study, Sauter mean ($d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ [33]) has been reported as it reflects the surface diameter average value and the droplet size distribution and has been used in a number of previous studies [19, 34].

2.1.4 Lipid oxidation compound analysis: Gas Chromatography Headspace Analysis (GCHS)

Gas chromatography (GC) was performed using 2 g nanoemulsion samples prepared with 1 ml 1% NaCl solution, added to HS vial and vortexed for 30 secs. The samples were heated in a Headspace (HS) sampler (TuborMatrix 40 PerkinElmer Inc, USA) at 100°C for 60 min and injected under the following conditions: vial pressure 30psi; pressurise time 0.2 min; needle temp 100 °C; injection time 4.8 sec; withdrawal time 6 sec.

To account for possible production of volatile compounds during heating, peak areas for heating times of 20, 40 and 60 min at 100°C were compared and 60 min chosen as this gave the most consistent peak composition results. The samples were analysed by Gas Chromatography Headspace Analysis (GCHS) Clarus 580, (PerkinElmer Inc, USA) equipped with a Flame Ionization Detector and 60 m 0.32 diameter column, 1.8 µm film thickness (Agilent Technologist, USA) under the conditions: Hydrogen flow rate 17 psi; injector temperature 230°C; detector temperature 230°C; oven temperature: from 40°C, ramp to 230°C at 20°C /min and hold at 230°C for 1 min, total time was 10.5 min. The volatile compounds were identified by reference to the retention time of standards. Analysis was performed in triplicate on individual vials for each time point.

Values for volatile compounds were determined by measuring the GC peak area to give quantitative measurements compatible for statistical analysis as demonstrated in previous studies [35, 36].

2.1.5 The storage trial for algal oil and algal oil emulsion

The algal oil and algal oil nanoemulsions were stored in the dark in single chamber incubators set at 4 °C, 20 °C and 40 °C for 5-weeks. The droplet sizes and volatile compounds were determined at baseline, week 1, week, 2 and week 5. Fatty acid composition was analysed for the bulk oil at baseline and bulk oil and nanoemulsions during week 1 and week 5 of the storage trial.

2.1.6 Experimental design and data analysis

All measurements were performed in duplicate or triplicate. Results are reported as the mean ± the standard deviation where applicable. Statistical analysis was completed using IBM SPSS® 24.0, (SPSS Inc. Chicago, USA). Significant differences were identified ($p < 0.05$) by two-way analysis of variance (ANOVA) with a Tukey post hoc test at confidence intervals of 95%.

3. Results and discussion

3.1 Fatty acid composition

Changes in fatty acid profiles were monitored at baseline and during the storage trial. Fatty acid composition levels for bulk oil were of a similar level to the manufacturer's specification in Table 1. The linoleic acid (18:2 n6; LA) composition of samples containing lecithin was

223 increased in comparison to the bulk oil and Tween 40 only samples due to the fatty acid
224 composition of lecithin which is abundant in LA and contains small amounts of the other main
225 fatty acids that were measured [37].

226 Two-way ANOVA testing revealed no significant differences for overall fatty acid composition
227 when the results from the bulk DHA algal oil (baseline) were compared with the lecithin (LN),
228 lecithin and Tween 40 (LTN) and Tween 40 (TN) nanoemulsions during storage. No significant
229 differences in the percentage of DHA for sample, temperature and storage period, indicates
230 that there was no sign of DHA degradation in samples throughout the storage trial. However,
231 minor DHA degradation in samples may not have been detected within the storage timescales
232 using this method.

233

234 All of the nanoemulsion samples showed small non-significant reductions for percentage DHA
235 composition in comparison to the unprocessed oil with some more noticeable decreases at
236 the higher temperatures after 1 week of storage. In addition, small non-significant decreases
237 were found for LA in samples containing lecithin at higher temperatures. This indicates that
238 small amounts of oxidation products could have been created during ultrasound processing
239 and that accelerated temperatures may have further promoted the production of volatile
240 compounds although this was not significant [38]. Relatively small amounts of oxidation
241 products can have a detrimental effect on the flavour of LCn3PUFA enriched foods [17]. The
242 small though non-significant LA and DHA losses for the nanoemulsion samples could explain
243 previous detrimental changes to the sensory profiles of algal oil nanoemulsion enriched
244 yogurt [20]. The results of this study demonstrate that following the initial non-significant fatty
245 acid composition reductions for nanoemulsions created using ultrasound, DHA composition

remained relatively stable during storage at lower temperatures, which is comparable to other work in the field [28].

3.2 Emulsion Droplet Sizes

The droplet size ranges of the nanoemulsion samples were measured by laser light scattering particle sizer at baseline and intervals during storage at the different temperatures. The mean droplet sizes and droplet ranges of the nanoemulsions prepared with lecithin, Tween 40 and in combination are shown in Figures 1 and 2. At baseline the nanoemulsions prepared with Tween 40 (TN) and lecithin and Tween 40 combined (LTN) showed significantly smaller ($p < 0.05$) droplet sizes (242 ± 0.002 nm and 172 ± 0.002 nm respectively) than those prepared with lecithin alone (LN) (340 ± 0.001 nm). Statistical analysis using two-way ANOVA testing revealed the lecithin samples had significantly larger droplet ranges throughout the 5-week storage period at all temperatures ($p < 0.05$).

The combined LTN sample had the smallest droplet size ranges at baseline; however, there were significant increases in droplet sizes from baseline and 5-weeks at 20 and 40°C ($p < 0.05$). Larger droplet ranges for lecithin samples in this study may be explained by the molecular weight of lecithin, which is greater than that of Tween 40 [39, 40]. The sole use or use of high ratios of lecithin is more likely to produce larger droplets with thicker interfacial surface areas [41]. The inclusion of Tween 40 may have reduced the droplet size ranges, because the lower molecular weight and higher hydrophilic-lipophilic balance (HLB) value of 15.6 [22]. Tween 40 combined with lecithin which has a lower HLB of 8 [22] creates a balance giving a system with smaller droplet sizes.

3.3. Oxidation and volatiles produced in nanoemulsion preparation and storage

GCHS is used widely to measure volatile compounds produced in the terminal stages of lipid oxidation and is beneficial as no oil extraction methods are required [42]. The oxidised volatile compounds produced by hydroperoxides (ROOH) breakdown in the last stage of oxidation. Products with lower molecular weights than those of ROOH can therefore be used as a measure of lipid oxidation [43].

Hexanal and propanal were the predominant volatile compounds detected in this study (Figure 3) and these have also been previously identified as common indicators of secondary oxidation for LCn3PUFA nanoemulsions [44]. Further gas chromatograms identified oxidised compounds 2-ethylfuran, propan-3-ol and valeraldehyde were produced by the algal oil and its nanoemulsions (see Figure 2 and Tables 3 to 6) all of which have been associated with rancid off flavours in oxidised LCn3PUFA oils and emulsions [42, 45]. Significant differences were found for all volatiles except valeraldehyde in both lecithin nanoemulsion samples stored at 40°C after 2 weeks ($p < 0.05$). The Tween 40 samples showed formation of higher concentrations of hexanal in comparison to the lecithin only sample and this was further accelerated by increases in temperature.

The combined lecithin and Tween 40 sample showed formation of lower concentrations of volatiles at week 1 than the lecithin only sample; however, the Tween 40 only sample had the lowest concentrations of the five volatile compounds when analysing storage time and temperature. These differences may due to the nature of the emulsifiers, since lecithin consists of phospholipids that can also be susceptible to lipid oxidation [13, 27].

It may be that LA, which is abundant in lecithin [46] positioned in the interfacial (outer) region of emulsion droplets and closer to the aqueous phase of the system was subjected to increased exposure to oxidation accelerants [47], which could explain higher levels of volatile compounds in these samples. Following ultrasound processing, the DHA in these samples may have retained some stability as it was located within the interior of nanoemulsion oil droplets that were surrounded by lecithin molecules at the interfacial region [12, 37].

All samples remained the most stable at 4°C with the least amount of significant differences in volatile compounds found at this temperature. Increases in sample storage temperature led to significant increases in the development of volatile compounds for all samples, so the degradation of fatty acids and production of oxidised compounds was temperature dependant particularly for the changes in the range of 20 °C to 40 °C.

Both lecithin samples demonstrated formation of higher concentrations of volatiles for storage times and temperature than the Tween only samples at 40°C, again indicating that Tween 40 may offer an overall protective effect whilst lecithin is less stable to oxidation particularly at increased temperatures. This is comparable to research by Uluata, McClements, & Decker [27] and Arancibia et al [13] who found that lecithin nanoemulsions had higher propanal development at increased temperatures.

311 The fatty acid composition of samples in this study showed no significant differences for
312 temperature and storage times although small non-significant changes were noted for LA and
313 DHA in some samples at higher temperatures as discussed earlier. The droplet size range
314 measurements showed the lecithin only samples had significantly larger droplet size ranges
315 throughout the trial, which suggests that larger droplet size ranges do not offer a protective
316 effect for the development of volatile compounds. Karthik & Anandharamakrishnan [24] also
317 found lecithin samples had larger droplet ranges and that Tween 40 could be used to create
318 systems with smaller droplet ranges and offered a protective effect for oxidative stability. In
319 the current study, samples created using the synthetic emulsifier Tween 40 had the smallest,
320 most stable droplet ranges and developed fewer volatile compounds than the lecithin
321 containing samples.

322

323 The present work evaluated volatile compounds found in the headspace of the bulk algal oil
324 and its various nanoemulsions. Systems remained the most stable 4°C which coupled with our
325 previous totox work [29] indicates the potential use of these systems as a refrigerated food
326 enrichment vehicle. Further work is now needed to analyse the effect 50% (w/w) algal oil
327 nanoemulsion enrichment may have on various appropriate food matrixes. In terms of
328 application and further development for a larger bioavailability trial there may be concerns
329 over the commercial acceptability of synthetic emulsifier Tween 40 in functional food
330 products [12], which would also need to be addressed.

331 **4. Conclusion**

This study was the first to use GCHS to analyse the oxidative stability of 50% (w/w) algal oil-in-water nanoemulsions created using ultrasound. The algal oil nanoemulsion prepared with lecithin was determined to have significantly larger droplet size ranges, which remained significantly larger throughout the 5-week storage trial regardless of storage temperatures. The nanoemulsion prepared with Tween 40 and lecithin combined had a low initial droplet size range distribution; however, there were significant increases in droplet ranges at 40°C after 5-weeks of storage. There were no significant differences in the DHA percentage composition for all samples throughout the storage trial at all temperatures.

Five oxidised compounds were detected in bulk oil algal and its nanoemulsions prepared with lecithin and/or Tween 40 over 5-weeks at the tested temperatures. Propanal was the main component of oxidised compounds followed by hexanal. It was noted that propanal production was temperature dependent and it was relatively stable at lower temperatures (4°C and 20°C) compared to 40°C. However, the nanoemulsions prepared in this study were less stable than bulk oil at low temperatures due to formation of higher concentrations of volatiles overall and particularly propanal and hexanal.

In terms of application, this work indicates good potential for further development in collaboration with the food industry to create innovative functional foods that offer alternative vegetarian sources of LCn3PUFA with improved bioavailability to alleviate habitually low oily fish consumption. The use of an algal oil source of LCn3PUFA offers a solution for vegetarians, vegans and non-fish eaters. Further research to evaluate the oxidation stability and potential improvements to LCn3PUFA bioavailability using algal oil-in-water nanoemulsions within food matrixes on a larger scale is warranted.

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