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Fabrication of nutrient delivery system of docosahexaenoic acid nanoemulsions by high energy techniques

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Abstract

Docosahexaenoic acid (DHA) is the key omega-3 fatty acid for the growth and development of human brain, retina and inevitable for heart health. DHA is highly susceptible to environmental factors due to its increased oxidation and physical instability; hence preventing and extending the shelf life is highly sought. DHA can be made by stable in terms of chemical and kinetic attributes with the help of nanoemulsion system. In this study, DHA nanoemulsion was prepared by oil-in-water emulsion using Tween-40 emulsifier, through high speed homogenization (HSH), high pressure homogenization (HPH) and combination of HSH+HPH techniques. The stability of nanoemulsion was investigated by physiochemical methods under different storage conditions. Fabricated nanoemulsions were less than 88 nm with globular droplets having higher negative charges of -31.1 and -30.2 mV produced by combination and HPH technique respectively. Higher Newtonian flow behavior was observed for combination and HPH technique. Functional groups of DHA were intact without undergoing any changes and functional activity during the nanoemulsification process, which was evident from unchanged fatty acids profile. Similarly, there were no structural changes observed in all the DHA emulsions. Refrigerated (4±1 °C) emulsion exhibited lower lipid oxidation than those stored at 28±1 °C and 40±0.2 °C temperature. Combination of homogenization technique was found to have better physiochemical properties and stable over a period of 100 days during storage than other emulsification techniques for encapsulating DHA.

Keywords: Nanoemulsion, DHA, Emulsification technique, Physiochemical stability studies, Encapsulation.

Introduction

Several studies confirmed the benefits of omega-3 fatty acids (EPA, C20:5n-3 and DHA) supplementation in case of pregnancy, cardiovascular diseases, inflammatory disease, etc. Docosahexaenoic acid (DHA; C22:6n-3) plays a vital role in proper cell membrane function and the development of fetal brain and retina during pregnancy.[1,2] Human body cannot synthesize essential fatty acids; hence it should be supplied from the external sources. However, a major problem associated with DHA is the oxidation due to their high poly unsaturation. Further, it cannot be stable for longer time due to environmental conditions such as temperature, moisture, air, pH, light, etc. To overcome these problems encapsulation through nanoemulsion is the solution to prevent environmental factors and increase the stability of DHA.

Nanoemulsion is a colloidal system, as one phase is dispersed into another phase in tiny droplets which can be either oil or water phase that resides of two immiscible liquids. In case of oil-in-water emulsion system, the oil is dispersed into the water phase. Since, the free energy of formation is greater than zero, the nanoemulsions are kinetically stable systems[3] but their long term physical stability aids them unique characteristics and it sometime resides thermodynamically stable.[4] Moreover, it exhibits great potential to encapsulate a high concentration of oil-soluble nutraceuticals or bioactive food compounds for fortification into food systems.[5] Due to the inherent characteristics of tiny droplet size and higher surface area, the nanoemulsions can improve the bioavailability and solubility of the encapsulated components (carotenoids, polyunsaturated fatty acids, vitamin-E, polyphenols, etc.,) for delivery system.[6,7] Therefore, nanoemulsion can be a primary process for producing

dried nanoencapsulated powders through conventional drying method like spray drying or freeze drying.[8]

In food emulsion, two techniques are used to prepare nanoemulsions such as low-energy emulsification (phase inversion temperature and phase inversion composition) and high-energy emulsification (high-speed homogenization, high-pressure homogenization, microfluidization and ultrasonication) technique. In addition, the low-energy methods are spontaneous formation of very fine oil droplets under the influence of specific system. Whereas, the high-energy methods rely on intense mechanical forces to breakdown the macroscopic phases or oil droplets into the smaller droplets with help of mechanical devices.[9]

For producing tiny droplets in emulsion system, more shear force is applied using high speed homogenizer. At the applied shear force longer time is required to breakdown the oil droplets. In case of high-pressure homogenizer, the combination of intense shear, cavitation and turbulent flow are involved to create tiny oil droplets.[10] Moreover, the HPH yields uniform droplet size distribution and lower mean particle diameter in the nanoemulsions, both which results in higher stability. Whereas, the converse holds true in case of HSH which results in lower emulsion stability. This is the basis of combination technique, which involves two step process such as high speed (primary emulsification) and high pressure (secondary emulsification) technique. During high speed homogenization, oil droplet breakdown can be achieved gradually over a longer period. Subsequently, the high pressure technique reduces the process time and gives monomodal droplets size distribution. However, the emulsification by combination

of high speed and high pressure could be an effective technique for producing nanoemulsion of lower droplet size with higher stability.

The objective of this study is to investigate the different nanoemulsification technique to produce stable DHA nanoemulsion using HSH, HPH and combinations of HSH+HPH techniques. Further, the effectiveness of stability was studied in terms of storage stability, morphology, particle size, particle charge, thermal transition, oxidation, fatty acid composition and structural changes.

Materials and Methods

Preparation of coarse emulsion

Oil in water nanoemulsion was prepared by addition of DHA algae oil (10 %, w/w; Martek Bioscience, Kingstree, SC) into aqueous solution containing Tween-40 emulsifier (2.8 %, w/w; Sigma Aldrich, Bangalore, India). Before using this phase composition, the emulsifiers and oil ratio was varied to formulate stable emulsion. These two phases were mixed using high-speed homogenizer (T18 digital Ultra Turrax, IKA, Bangalore, India) at 1000 rpm for 5 min to prepare the coarse emulsion. After preparation of coarse emulsion, the nanoemulsions were prepared by different emulsification technique.

Preparation of nanoemulsion by HSH

In the preliminary study, different rpm (15,000, 20,000, 24,000) and time intervals (15, 20, 25) were used for producing nanoemulsions. After preparation, the nanoemulsion particle size was measured with respect to different parameters. The obtained results

showed that emulsions prepared at 24,000 rpm and 25 min of homogenization led to lower mean particle when compared to other homogenization speed-time combinations. Hence, based on the optimized results, the mentioned conditions were used for HSH operation. The prepared course emulsion was vigorously homogenized at 24,000 rpm for 25 min to prepare the nanoemulsion using high speed homogenizer. This experiment was performed under the cold condition (4±1 ºC) to prevent heat generation during homogenization.

Preparation of nanoemulsion by HPH technique

The high pressure homogenizer system (GEA Niro Soavi, Panda, NS1001L2K, Italy) was optimized before producing nanoemulsion by varying the pressure (500, 600, 700 and 800 bar) and cycle (5, 6, 7 and 8). However, 800 bar with 8 cycles was chosen based on the lower mean droplet size, poly dispersity index (PDI) and uniform droplet size distribution. With increase in pressure (e.g. 900, 1,000 and 1,100 bar) and no. of cycles (e.g. 9, 10 and 11), there are more chances to affect the stability of DHA during the nanoemulsion preparation. This instability is mainly attributed to the higher disruption and physical stress during the homogenization process. In addition, the high pressure and more number of cycles will increase the oxidation of DHA. After the optimization process, the course emulsion was passed through the high pressure homogenizer at 800 bar for 8 cycles to prepare the nanoemulsion. This experiment was performed under the cold condition (4±1 ºC) to prevent heat generation during homogenization.

Preparation of nanoemulsion by combination (HSH+HPH) technique

The prepared course emulsion was homogenized by combination of high speed homogenizer at 24,000 rpm for 15 min (primary emulsion) and high pressure homogenizer at 800 bar for 8 cycles to form stable nanoemulsion (secondary emulsion). This experiment was performed under the cold condition (4±1 ºC) to prevent heat generation during homogenization. After the preparation, all three types of DHA nanoemulsions were kept in a screw cap glass tube for further analysis.

Determination of zeta potential and particle size distribution

The electrical charge (ζ-potential) of nanoemulsion oil droplets was determined using a Malvern Zetasizer (Nano-ZS90; Malvern Instruments, U.K.). The nanoemulsions particle size distribution was measured by the dynamic light scattering technique using the same instrument. Refractive indices of 1.48 for oil and 1.33 for dispersant medium were used to determine the particle size of emulsions. The analysis of particle size distribution from the emulsion stored at 100^{th} day was carried out using a laser light diffraction particle size analyzer (S3500, Microtrac Inc., USA). This experiment was performed in triplicates.

Morphological studies of nanoemulsions

i. Transmission Electron Microscope (TEM):

The morphology of DHA nanoemulsions were studied using TEM (TECNAI G2, FEI, Germany). Approximately 40 µl of nanoemulsion was negatively stained with 40 µl of 2 % (w/v) phosphotungstic acid and this was placed on a copper grid at room temperature

(28±1 °C). Excess liquid sample was removed using Whatman filter paper and dried at room temperature for 1 hr. Copper grid containing nanoemulsion morphology was acquired at an accelerated voltage of 120 kV.

ii. Confocal laser scanning microscopic (CLSM):

In addition, confocal microscope (Carl Zeiss, LSM 700, Jena, Germany; ZEN 2009 software) was also used to confirm the morphology of nanoemulsions. The nanoemulsion was analyzed using 63X oil immersed objective lens to capture the images. Nile red (fat soluble fluorescent dye) was used to visualize the structural characteristics of nanoemulsion. Fluorescent dye was excited at ʎ=488 nm to observe the images. The fluorescent spectra emitted from nanoemulsion was analyzed by High-sensitive PMT detector with spectral increment 1 nm, with a 70 µm pinhole size, and a scanning time of 983.04 ms. The images were captured at pixels of 512 X 512, with a pixel size of 1.25 µm, and a pixel dwell time of 25.2 µs.

iii.Trinocular microscope:

Morphological behavior changes of nanoemulsions during storage were studied by trinocular microscope (Olympus, model: BX-5, Japan; ProgRes C-5 software). Samples were observed under 100X magnification with oil immersion.

Rheological characteristics

Rheological characterization of DHA algae oil nanoemulsions were performed by rheometer (Rheomatric Scientific, SR5, USA) using a coaxial cylinder (28.8 mm size

184 diameter and 50 mm length) attachment. The shear rate was gradually increased from
185 0.1 up to 100 s^{-1} in a span of 60 s. A pre-shearing process was done for 30 s at 10 s^{-1}
186 and waiting time process was maintained every test for 40 s. The temperature was
187 maintained at 25.0±0.1 °C for all the experiments and the rheological measurements
188 were employed on triplicate basis.
189
190 **Storage stability studies at different temperature**
191 Storage stability studies of DHA nanoemulsions (HSH, HPH and HSH+HPH) were
192 performed at different storage conditions such as refrigeration (4±1 °C), room (28±1 °C)
193 and oven temperature (40±0.2 °C). This study was done to determine the effect of
194 temperature on nanoemulsions stability during storage period. The nanoemulsions
195 changes were observed by morphology and different physiochemical studies.
196
197 **Creaming stability**
198 The nanoemulsions long term creaming stability was studied by visual observation. The
199 prepared nanoemulsions (20 ml) were stored in a transparent measuring cylinder with a
200 stopper. The creaming stability was determined in the samples stored for 1, 3, 5, 8, 10,
201 20, and up to 100 days. It was observed that the emulsions got separated into an
202 opaque layer at the top (cream) and a marginally turbid or transparent layer at the
203 bottom (serum).The emulsion creaming was monitored by measuring the height of the
204 cream layer on top (*HC*) and the height of total emulsion (*HE*) in the tube. Creaming
205 stability in terms of creaming index (%) was obtained using the following equation (1).[11]

$$Creaming\ Index\ (CI\%) = \left(\frac{H_C}{H_E}\right) \times 100 \quad (1)$$

Centrifugation, phase separation and sedimentation stability

Phase separation experiment under centrifugation condition (2 ml of emulsion, 28±1 °C at 3,000 rpm for 2 min; Eppendorf, 5430 R, Germany) was done for all the prepared DHA nanoemulsions.[12] Similarly, phase separation at normal condition without centrifugation and sedimentation was studied for all the emulsions. These measurement study was performed at different storage time intervals such as 1, 2, 3, 5, 10, 40 and 100th day.

Flocculation, coalescence and oiling off stability

Nanoemulsion samples were transferred into 20 ml glass test tube and placed in different storage conditions over the storage period of 100 days. Flocculation and coalescence were studied by observation of emulsion structure using trinocular microscope. All the emulsion samples were observed under 100X magnification with oil immersion. The oiling off of the emulsion sample was determined by measuring height of the oil layer in the emulsion samples (H_0) and total height of the oil layer in a completely destabilized system (H_{total}). Oiling off was obtained using the following equation (2).[13]

$$Oiling\ off\ (\%) = 100 \times \left(H_0 / H_{total}\right) \quad (2)$$

Color analysis

Color (CIE *L* a* b*;* where, *L**-lightness *a**-redness, and *b**-yellowness) of DHA nanoemulsion was evaluated by color measurement analyzer (MINOLTA Spectrophotometer CM-3500d, Spectra Magic software). Nanoemulsions were analyzed at different time intervals of 1, 10, 30 and 60th day of storage.

Thermal transition analysis

Differential scanning calorimetry (DSC; Perkin Elmer instrument, DSC 800, France) was used to determine the thermal transition of DHA nanoemulsions, DHA algae oil and Tween-40. The test sample of about 8.5-9.5 mg was placed in an aluminum pan and hermetically sealed before keeping into the calorimeter thermocouples. The samples were then cooled from 25 °C to -60 °C in 5 °C/min increments and heated up to 60 °C simultaneously in the same process.

Fourier Transform infrared (FTIR) Spectroscopy

The DHA nanoemulsions were analyzed by Fourier transform infrared (FTIR) spectroscopy (Nicolet 5700, M/S. Thermoelectron Corporation, Round Rock, TX). Similarly, DHA algae oil (control) and Tween-40 emulsifier were also analyzed. This study was performed for different time intervals (1st, 10th, 20th and 30th day). FTIR-KBr pure compound was used for sample preparation and the scanning range was fixed at 500-4,000 cm^{-1}. In addition, 32 scans were used for all the samples. Initially, FTIR-KBr was gently grinding the crystals for making fine powder. After, 80 µl of nanoemulsion was added into the KBr fine powder and mixed thoroughly. Further, this mixture was pelletized using pellet making kit and the obtained pellet was transparent in nature.

250

251 **Oxidative stability studies of DHA nanoemulsion**

252 Lipid oxidative stability of DHA nanoemulsions and control DHA algae oil
253 (unencapsulated) was determined over the storage of 30 days. 2 ml of emulsions was
254 taken in capped test tubes. Thiobarbituric acid-reactive substances (TBARS) were used
255 to measure the lipid oxidation reaction products. A solution of TCA (trichloroacetic acid)-
256 TBA-HCl was prepared by mixing 75 g of TCA, 1.68 g of TBA, 8.8 ml of 12 M HCl, and
257 414 g of H_2O. A 100 ml of TCA-TBA-HCl solution was mixed with 3 ml of 2% (w/w)
258 butylated hydroxytoluene in ethanol, and 2 ml of this solution was mixed with the same
259 amount of emulsion sample. This mixture of solution was vortexed and heated in a
260 boiling water bath for 15 min. Later, it was cooled down to room temperature using tap
261 water for 10 min and centrifuged at 1000 g for 10 min. After 10 min, the absorbance was
262 measured at 532 nm using a UV/VIS spectro-photometer (UV-1700 Pharma Spec,
263 SHIMADZU Corporation). TBARS concentrations were measured from the standard
264 curve plotted using 1, 1, 3, 3-tetraethoxypropane.[14]

265

266 **Determination of fatty acid composition**

267 Fatty acid composition was determined by gas chromatography (Shimadzu 2010 plus
268 system, Japan) fitted with a flame ionization detector (FID) and a RTX-2330 capillary
269 column (30 m, 0.25 μm internal diameter and df 0.20 μm). Helium was used as the
270 carrier gas at a constant linear velocity of 20 cm s^{-1}. The temperatures of the injector
271 and FID detector were kept at 250 and 260 ºC, respectively. The oven temperature was
272 initially held at 60 ºC for 10 min before being increased to 150 ºC at 60 ºC min^{-1}. Fatty

acid methyl esters (FAME) were prepared by the procedure of Christie.[15] Briefly, the extracted lipid was added in 0.2 ml of methanolic NaOH (2 N) solution and 1 ml of hexane. The reaction mixtures were vortexed for 15 sec at room temperature and incubate in water bath for 30 min at 50 ºC. Later, 0.2 ml of methanolic HCL (2 N) was added into the above mixture and vortexed for 15 sec. This mixture was incubated in water bath for 15 min at 50 ºC, and then upper layer was removed. Subsequently, the solvent was evaporated by nitrogen gas in the screw capped test tube.

Determination of volatile compounds

Gas chromatography-mass spectrometry (GC-MS) analyses were performed by Perkin Elmer instrument. Helium was used as the carrier gas at a constant linear velocity of 20 cm s^{-1}. The temperature of the injector and the FID detector were kept at 240 ºC. The oven temperature was initially kept at 150 ºC and increased to 5 ºC/min to 220 ºC for 3 min. Electron impact mass spectra were recorded in the range of 40-400 m/z at 0.2 scans/ sec and mode was kept in EI^{+}. Volatiles were determined in both MS library searches and comparison of retention times with control. Moreover, external standard of hexanal was also used to identify the aldehyde.

NMR

The DHA algae oil was extracted from the nanoemulsified DHA using the solvent of chloroform and methanol (1:3 ratio). After, the extracted DHA structural features were studied and it compared with control DHA algae oil and Tween-40 emulsifier. The standard of DHA methyl ester was used as reference to identify the DHA peak in the

algal oil NMR spectra. The samples (liquid form) were dissolved in deuterated chloroform for ^{1}H and ^{13}C NMR spectral studies. The spectral studies were carried out on 500 MHz NMR spectrometer (Avance AQS 500, Bruker, Germany).

Statistical analysis

Results were expressed as the mean value ± SD of three or two independent experiments. Statistical analysis was carried out by analysis of variance (ANOVA) using SPSS statistical software version 16. Comparison of means was performed by Turkey's test. Further, paired-sample for means t-test was performed for creaming and oxidation stability studies using Microsoft office excel 2010 software. The level of significance used was $p<0.05$ for the entire statistical test.

Results and discussion

Particle size distribution and morphology of nanoemulsions

Particle size distribution is one of the essential parameter of food emulsions which interlinks physical properties such as color, viscosity, texture and shelf life.[16] Fig. 1(a) shows the droplet size distribution and polydispersity index (PDI) of DHA nanoemulsions. The HPH and combination (HSH+HPH) technique yielded lower mean particle diameter of 11.17 and 11.31 nm respectively, whereas HSH produced 87 nm particles. Shear forces and turbulence are the high pressure emulsification factors which contribute to the lower particle size in HPH and HSH+HPH nanoemulsions. During high pressure homogenization process (HPH and HSH+HPH), the particle size has reduced and distribution are considerably altered. The smaller mean particle size

offers higher surface area and that improves physical stability due to its unique morphological and functional characteristics. Similarly, polydispersity index of HPH and combination technique also depicted lower value (0.348 and 0.303), which indicates that particle size distributions were monomodal and narrow PDI than HSH (0.516). TEM morphology of DHA nanoemulsions (HSH, HPH and HSH+HPH) are depicted in Fig. 2(a-f). All the emulsions showed spherical shape with droplet diameter in the nano scale. Hence, the morphology of HSH technique exhibited little higher sized droplets than the HPH and HSH+HPH, which highly correlated with the results obtained from particle size distribution. Similarly, the nanoemulsions morphology obtained from confocal microscopy also confirmed the spherical shaped droplets with uniform size distribution in nanometric range (Fig. 2i- iii).

During the storage period of 100 days, there was an increase in mean particle size observed in all the nanoemulsions stored at different temperature (Fig. 3 and Fig. 4a). The mean particle diameter of HPH and HSH+HPH technique showed the range of 238-258 nm for all the storage conditions. Also, it resulted in narrow and uniform particle size distribution (Fig. 4a). On the contrary, HSH emulsion exhibited micrometer sized (2.55-41.54 μm) particle for all the storage conditions. The oven stored emulsion showed higher mean droplet diameter (41.54 μm) than emulsion stored at other temperatures. These destabilizations of emulsions are related to the different forces acting on the emulsion system such as gravitational forces, inter-particle repulsive, attractive forces, flow forces, and molecular forces.[17]

Moreover, uniform nanometer droplet size distribution was found in the samples stored at 4±1 ºC and 28±1 ºC, whereas non-uniform droplet size distribution observed

for the sample stored at 40±0.2 ºC. Therefore, the higher temperature with longer storage period has influence on the instability of emulsions. At higher temperature, there may be an impact on the properties of emulsifier which adsorbed on the surface of oil droplets. Also, it affects the emulsion physiochemical properties and stability.[18] The mean particle diameter increased with increase in storage temperature and this effect was attributed to droplet aggregation. The growth of emulsion droplet size was slower for the emulsion stored at lower temperature than emulsions stored at higher storage temperature. When stored at lower temperature molecular movement is reduced subsequently.[19] Moreover, it is also due to the rate and frequency of inter-droplet collision that reduces the droplet growth effectively.[20] Adjonu *et al*. reported that emulsion stored at higher temperature exhibits larger droplet sizes with multimodal size distributions than the emulsion stored at lower temperature.[21]

Particle charge characteristics of DHA nanoemulsion

Comparison of zeta-potential for the HSH, HPH and combination (HSH+HPH) technique produced DHA nanoemulsions are shown in Fig. 1a. In colloidal dispersion system, the zeta potential may determine the overall physiochemical stability due to its function of electrical charge and their interactions.[22] In this study, zeta potential value of HSH, HPH and HSH+HPH nanoemulsions were found to be of lower negative value. HPH and combination of HSH+HPH resulted in a zeta-potential of -30.2 and -31.1 mV, whereas HSH yielded -25.2 mV which leads to instability in later stage. Earlier researchers have stated that zeta potential value of dispersion system should be around −30 mV to prevent the instability in terms of aggregation and coalescence.[23, 24] Further, it was

reported that increase in surface charge can effectively prevent emulsion instability [25] and this is due to surface charge leading to electrostatic repulsive force between emulsion droplets.[26] HSH nanoemulsion showed higher instability in all the storage conditions. Further, this instability phenomenon was confirmed with storage stability studies of emulsion morphology (Fig. 3). Thus, the combination technique showed higher negative zeta-potential which helps to increase the emulsion stability from the environmental factors.

Rheological Characteristics

Rheological characteristics of DHA nanoemulsions were studied by shear stress as a function of shear rate. Fig. 1b depicts the rheological behavior of nanoemulsion prepared using HSH, HPH and combination techniques. The high pressure treated techniques (HPH, HSH+HPH) exhibit similar rheological behavior when increasing shear rate (s^{-1}) verses shear stress. Presence of a linear relation between shear stress and shear rate in all the emulsification techniques shows that nanoemulsions exhibited a Newtonian flow behavior. By varying the emulsification technique, rheological behavior of nanoemulsions were affected. The emulsion stability and rheological behavior are highly subjected to the interactions between oil droplets and interfacial layer of oil/water in the emulsion system.[27] Thus, the high pressure treated nanoemulsions were stable for longer time against flocculation, creaming and coalescence due to their rheological behavior and it was confirmed by storage stability studies (Fig. 1b).

388 **Stability of DHA nanoemulsions using different emulsification techniques**

389 The storage stabilities of DHA nanoemulsions (HSH, HPH and HSH+HPH) were
390 analyzed by creaming stability, phase separation, centrifugation, flocculation,
391 sedimentation, coalescence, and oiling off with different storage conditions for 100 days
392 and their results are shown in Table 1 and Fig. 3. The color stability was done for 60
393 days at the similar storage conditions.

394

395 **Creaming stability**

396 Creaming is one of the emulsion instability mechanisms which are formed by
397 gravitational separation. The creaming stability of DHA nanoemulsion was analyzed
398 under different storage conditions such as refrigeration (4±0.1 °C), room (28± 1 °C; Fig.
399 4b) and oven (40±0.2 °C) over the storage period of 100 days. The HPH and HSH+HPH
400 emulsion technique showed no cream formation in all the condition during storage of 8
401 days. However, slight change was observed in the emulsion stored at 10^{th} day in all the
402 stored conditions. On the other hand, HSH technique used nanoemulsion was found to
403 be stable only for 4 days and after that creaming had increased rapidly. This is because
404 of the emulsions prepared with HSH yielded uneven droplet size distribution and higher
405 mean particle size. An increasing emulsion droplet collision and aggregation can
406 influence to increase the droplet to cream.[21] Moreover, the HSH emulsion showed 14
407 fold increase for refrigeration (showed in supplementary data) and room temperature
408 (Fig. 4b), whereas 16 fold increase was observed from the emulsion stored at oven
409 temperature (showed in supplementary data). There was large variation (P<0.05) was
410 found between HSH and high pressure treated techniques. However, there was no

411 significant difference observed between HPH and HSH+HPH emulsions stored at all the
412 temperature. During high pressure homogenization process, the decrease in average
413 fat droplets size was achieved (11.17 to 11.31 nm) which helps to reduce the creaming
414 velocity (as per the Stokes' law) and increases the emulsion stability significantly.[28]
415 Whereas, increase in average mean particle size and higher PDI value (0.516) were
416 found in HSH emulsion. In addition, higher droplet-droplet repulsive force acts on the
417 emulsion prepared with high pressure homogenization due to presence of lower
418 negative charge (Fig. 1b). Also, it showed no further increment in creaming at all the
419 stored conditions and it exhibited higher storage stability over the period of 100 days
420 (Fig. 3).
421
422 **Phase separation, centrifugation and sedimentation stability**
423 The initial instability of fine triglyceride oil in water emulsion was due to creaming, that
424 eventually influences the macroscopic phase separation into separate visible regions of
425 cream and serum.[29] In this study, the overall instability was not observed till eight days
426 of storage for all the emulsions at different conditions. However, slight phase separation
427 was observed in HSH nanoemulsion under the storage of oven and room condition. In
428 contrast, HPH and HSH+HPH emulsions showed no changes in all the stored
429 conditions till 20th day. Consequently, there was a change in HPH and HSH+HPH was
430 found on 40th day in all the stored conditions. Another study of phase separation
431 (without centrifugation) also showed no changes till 20th day of HPH and HSH+HPH
432 emulsion for all the conditions. Furthermore, slight changes were observed during the
433 40th day of storage, which is similar to centrifugation study. This may be due to the

change in temperature and increase in droplet size of emulsions. On the contrary, sedimentation study did not show any instability till 40 days (Table 1 and Fig. 3) and more change were found in HSH emulsion over the storage of 100 days for oven temperature. This is because, when increasing in storage temperature the mean particle diameter increased considerably and this is directly influenced to droplet growth (as explained earlier). Due to the increasing droplet diameter, the density of droplets was increased and this was influences to gravitational separation. However, interestingly there was no sedimentation observed in all the emulsion stored at refrigerated conditions. This implies that the DHA nanoemulsions stored at the lower temperature showed an excellent stability towards gravitational separation.

Flocculation, coalescence and oiling off stability

Flocculation was not observed in the emulsion prepared with HPH and HSH+HPH used techniques over the storage period of 100 days at all the stored temperature (Fig. 3). But, more flocculation was observed in HSH technique used nanoemulsion stored at refrigeration and room temperature. The flocculation of emulsion is influenced by van der Waals attraction and it occurs due to their insufficient repulsive energy between the oil droplets.[30] Moreover, during the storage period, oven stored nanoemulsion showed more coalescence. This is because, the small flocculated drops had coalesced into larger drops in presence of elevated heat and prolonged storage.[31] In addition, thinning and disruption of liquid film between the oil droplets caused coalescence which also influenced some oil separation in later stage due to their ultimate joining of droplets in

emulsion system.[32] Therefore, the decrease in storage temperature has influences the increase in nanoemulsion stability towards droplet coalescence.[7]

During coalescence process, large droplets are formed that eventually influences the oiling off. Moreover, in the emulsion system coalescence and oiling-off can happen due to the deformation of oil droplets through external stresses acting on the interfacial layers of oil droplets.[33] In this study, 4% of oiling off was observed in HSH emulsion stored at oven condition on 100th day and further it was confirmed by observing morphology of emulsion (Fig. 3). This instability mechanism is also known as flow-induced coalescence which comprises friction between the emulsion droplets, where droplets merge together by capillary pressure. Beyond the critical pressure, the frictional force between the oil droplets cannot relax by slipping between droplet surfaces. Therefore, this had led to stretching and rupture of the thin film present on oil droplet surface that originated in oiling-off. [33] Also, this oiling off may occur due to the influence of higher storage temperature, emulsification technique and prolonged storage of emulsion. But, there was no oiling off found in HPH and HSH+HPH emulsion. In case of HSH treatment, the droplets in nanoemulsion have coalesced due to the interfacial layer of the systems that was more flexible and liable to disruption.[34] Therefore, the oven stored HSH emulsion showed three different layers that was separation of DHA algae oil with opaque cream at the top, turbid with milky emulsion at middle and more sedimentation at bottom (Fig. 3). Hence, the overall studies were suggested that heating of emulsion has influences the increasing in mean particle diameter, droplet flocculation, coalescence, phase separation, sedimentation, creaming and oiling off.

Color stability

There was no color differences observed in the DHA emulsion at all the storage conditions till 10 days (showed in supplementary data). However, slight changes started to be observed in HSH produced emulsion over the storage of 10th day in all the temperature conditions. This is because of instability produced by high speed homogenization technique and lower stability in elevated storage temperature. Karthik and Anandharamakrishnan have also reported that change in storage temperature influence the color instability of emulsion and it was studied using DHA algae oil emulsion.[12] Hence, refrigerated stored HSH sample showed slight instability, whereas more instability was observed in 28±1 ºC and 40±0.2 ºC temperature at 60th day which confirmed the higher level of b* (yellowness) value. There was no change in color difference observed in high pressure treated emulsion technique during the storage stability studies. These nanoemulsions had higher L* value which showed the strength of white color domination, but not much variation in a* and b* values.

Thermal transition

DSC melting thermograms of DHA algae oil, Tween-40 and nanoemulsified DHA are shown in Fig. 5a and b. DHA algae oil showed broad melting thermopeak (T_m) at -12.05 ºC and followed by one sharp peak at -1 ºC. Tween-40 melting temperature was found at 23.27 ºC. The transition enthalpy (ΔH) values of DHA and Tween-40 found to be 16.04 J/g and 26.01 J/g, respectively (Fig. 5a). The nanoemulsified DHA melting peak was compared with respect to HSH, HPH and combination techniques. The melting peak of HSH, HPH and HSH+HPH nanoemulsified DHA were observed at 3.95 ºC, 4.01

ºC and 3.66 ºC, respectively (Fig. 5b). The transition enthalpy of HPH treated DHA nanoemulsion was found to be higher value 282.03 J/g (HSH+HPH) and 281.39 J/g (HPH). In contrary, lower ΔH value of 275.90 J/g was observed for HSH nanoemulsion. This may be due to the number of oil droplets presence are more per gram of HPH treated nanoemulsion than HSH emulsion. Moreover, the transition temperature and enthalpy change has influenced the effect of emulsifier concentration for the emulsion system.[35] The DHA algae oil showed vast melting temperature differences and melting shift when compared to all the nanoemulsified DHA. Kabri *et al*. reported that lipid nanoemulsion has higher melting temperature when compared to oil melting temperature.[36] Moreover, this obtained DHA nanoemulsions thermogram clearly indicated that there was a protecting layer that acts on the oil droplets to extend the melting temperature of oil. Also, lower oil droplet sizes exhibited lower melting reaction in emulsions.[37] In addition, the result was clear that after nanoemulsification, DHA was effectively protected from environmental conditions and this extended the shelf life.

Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was performed to examine the stability of DHA present in all the nanoemulsified samples. Fig. 6 shows the FTIR spectroscopy of DHA, Tween-40 and nanoemulsified DHA (HSH, HPH and HSH+HPH technique). An intense absorption at 3013.4 cm^{-1} represents –C–H stretching vibration of *cis* double bond of unsaturated fatty acids. However, this observed peak confirms the presence of DHA (control-DHA algae oil) in all the nanoemulsions. Karthik and Anandharamakrishnan had also observed a similar trend in their DHA FTIR spectra.[12] The bands absorbed at 2926.8

and 2854.8 cm^{-1} denotes the asymmetric and symmetric stretching vibration of aliphatic -CH_2 fatty acid hydrocarbon chains.[38] A very strong ester carbonyl (-C=O) functional groups observed at 1743 cm^{-1} indicates the stretching bands of triglyceride. Weak absorption peak was found at 1651.9 cm^{-1} that relates the characteristics of -C=C- stretching vibration of *cis* olefins. Absorption at 1463.7 cm^{-1} indicated medium bending vibrations of -C-H (CH2 and CH3) aliphatic functional group. Peak observed at 1371.3 cm^{-1} belongs to the symmetric bending band of -C-H (CH3) group. Further, very strong stretching and bending vibration of -C-O and -CH_2 group were observed at 1149.3 cm^{-1} [39]. Watanabe et al. have reported the observation of broad band at 719 cm^{-1}, but not in the range of 960-980 cm^{-1} which confirmed the fatty acid was *cis* double bonds but not *trans* ones.[40] These results clearly demonstrated that the fatty acid was *cis*-4, 7, 10, 13, 16, 19-DHA. In addition, concentration of DHA was very low in the nanoemulsions (8 µl of oil in 80 µl of emulsion). Hence, the peaks corresponding to -$(CH2)_n$-and –HC=CH- (*cis*) were not pronounced at 719 cm^{-1}. The Tween-40 emulsifier used in encapsulation of DHA showed almost similar FTIR spectra as compared to the control DHA algae oil. These results clearly indicated that there was no chemical interaction between the oil and emulsifier. Moreover, the 30 days stored emulsion samples at different condition also did not show any major differences when compared to control DHA (data showed in supplementary section).

Oxidative stability of DHA nanoemulsion

The oxidative stability studies of nanoemulsified DHA oil and control was analyzed under different storage conditions. Fig. 4c depicts the oxidative stability of DHA

548 nanoemulsions (stored at 28±1°C) and it was compared with emulsification techniques
549 (HSH, HPH and HSH+HPH) and control during the storage period of 30 days. It was
550 measured by TBARS method which is an identifier of lipid oxidation. The lipid oxidation
551 was monitored at different time intervals and there was no significant difference
552 observed between HPH and HSH+HPH emulsions at initial day. But there was a
553 significant difference (P<0.05) observed when compare to HSH emulsion. Hence, when
554 compared to control DHA, the nanoemulsified DHA showed slightly more oxidation on
555 the first day of storage. This may be due to the emulsification process which involves
556 high pressure processing. However, during the storage period, control DHA showed
557 increase in oxidation at all the temperature conditions. Further, it was found that the
558 control DHA experienced higher oxidation than the nanoemulsified DHA (P<0.05).
559 Oxidation of all the stored emulsions increased slowly, whereas slight differences
560 were observed among the samples kept under refrigeration condition (showed in
561 supplementary data). HSH emulsion showed slightly lower oxidation when compared to
562 the high pressure treated emulsion but later the oxidation was getting close to the HPH
563 and HSH+HPH emulsion. In addition, oxidation of HSH DHA nanoemulsion increased
564 linearly as compared to HPH and HSH+HPH emulsions during storage at room
565 conditions. It clearly indicates the important role of emulsification techniques and
566 storage conditions. On the other hand, the oven stored emulsions showed slightly lower
567 oxidation in HSH+HPH emulsions than HSH emulsions. Also, the oxidation value was
568 found to be comparatively less in HPH DHA emulsion. Among all the emulsification
569 technique, high pressure treated emulsions (HPH and HSH+HPH) resulted in lower
570 oxidative value for both room and oven conditions. In this study, high pressure

processed nanoemulsion showed higher thermal stability and less oxidation than control. Thus, this result strongly reinforces the advantages of high pressure emulsification process.

Determination of fatty acid composition and volatile compounds

The overall fatty acid compositions and the result of DHA algae oil (bulk oil) were compared with the HSH, HPH and HSH+HPH nanoemulsions (showed in supplementary data). DHA algae oil showed 38.11± 0.07 % of DHA from the total fatty acid compositions. In this study, almost similar percentage of DHA fatty acids composition was measured in all the nanoemulsions. Also, there was no change in major fatty acid compositions in all the samples. These results clearly indicated that during emulsification process, there was no loss in DHA and it showed stable against emulsification process. During emulsification process high turbulent force had been applied to make very tiny oil droplets, which might have influenced polyunsaturated oils to be thermodynamically unstable. But at present study, it did not show any changes in DHA fatty acid. The presence of aldehyde compounds was analyzed in HSH, HPH and HSH+HPH DHA nanoemulsion using GC-MS (figure showed in supplementary data). There was no degradation compound observed after the formation of DHA nanoemulsions, which was confirmed with hexanal standards. The retention time (RT) of 3.98 min was observed for hexanal and this RT was compared with nanoemulsified DHA. From this study, it confirmed that there was no peak found at this particular RT. Further, the fatty acids profile spectra were verified by mass spectrometry. These

obtained results were highlighted that after emulsification process, the DHA had showed stable against chemical instability.

NMR

Structural characteristics of DHA algae oil extracted from the nanoemulsions (HSH, HPH and HSH+HPH) were examined using ^{1}H NMR and ^{13}C NMR. In the present study, NMR was used to investigate the effect of emulsification technique to improve the stability of DHA. The NMR spectra of DHA algae oil (control), DHA methyl ester (standard) and Tween-40 emulsifier (data showed in supplementary) were compared with the DHA algae oil extracted from nanoemulsions in terms of chemical shift and integration values (7a-k). In general, algae oil includes other group of fatty acids (e.g. Myristic acid, palmitic acid, stearic acid, docosapentaenoic acid, etc.) along with DHA fatty acid. Hence, a DHA methyl ester (standard, figure 7a-b and 7g) NMR spectrum was used to identify the specific DHA NMR peaks from the group of fatty acid compositions. While integrating terminal methyl group of DHA, it has been calibrated to the value of 3 at 0.99 ppm. Since other fatty acids characteristic peaks are merged with DHA, it was not feasible to separate them in olefinic as well as aliphatic region.

Based on the analysis of NMR data, 12 olefinic protons of DHA were present in the range of 5.32 to 5.45 as multiplets. 10 methylene protons having chemical shift 2.82 to 2.89 were observed as multiplets. 4 methylenic protons next to carboxylic acid having chemical shift in the range of 2.37 to 2.43 integrated to 4 protons. Furthermore, 2 methylenic protons next to terminal methyl and 3 terminal methyl protons were seen at 2.09 (pentet) and 0.99 (triplet) respectively. The proton NMR spectrum of control DHA

was compared with that of DHA extracted from nanoemulsion to identify any structural changes. It was observed that the NMR peaks of the DHA algae oil were comparable to standard DHA. Also, the NMR peaks of the DHA algae oil were integrated at the same δ value as that of standard DHA. And these proton NMR spectra confirm that there was no change in structure of DHA.

The ^{13}C NMR spectrum presented terminal methyl signal at 13.92 ppm and two carbon atoms next to terminal methyl group present at 20.23 and 33.77 ppm. Bunch of methylene was observed at 25.22, 22.25 and 25.319 ppm. Moreover, all the olefins were seen between 125 ppm to 132 ppm and also one carbonyl carbon signal was seen at 173 ppm. From the NMR spectral results it was evident that molecular structure of DHA algae oil has not changed even after the emulsification process and it was confirmed with the spectra of standard DHA. In addition, it was found that DHA has not chemically reacted with the Tween-40 emulsifier during nanoemulsification process. Hence, the NMR study confirms that there was no change in structural behavior after the formation of DHA nanoemulsions. This is because after encapsulating DHA at very tiny molecules, it has been prevented the structure of DHA from several environmental factors.

Conclusions

DHA nanoemulsion was prepared by high energy emulsification techniques (HSH, HPH and HSH+HPH) and physicochemical properties were studied to compare its stability. HPH and HSH+HPH nanoemulsions yielded lower mean particle diameter than HSH. Similarly, HPH and combination of HSH+HPH depicted higher negative charge,

whereas HSH showed lower in negative charge that influences to emulsion instability during the storage period. After studying 100 days at different storage temperature, it clearly indicated that HPH involved emulsification process (HPH and HSH+HPH) produced stable DHA nanoemulsions in terms of lower particle size, morphology and other physical stability studies. There was no change in fatty acid profile and structural changes of DHA in any of the emulsions. Refrigerated HPH and HSH+HPH DHA exhibited lower lipid oxidation than the emulsion stored at other conditions (28±1 °C and 40±0.2 °C). In this study, emulsion prepared by high pressure and combination technique exhibited higher stability in terms of physicochemical studies. In contrary, thermal transition of nanoemulsion results in higher stability towards HSH+HPH than others. However, amongst all the high energy techniques, better stability was achieved in HSH+HPH technique compared to HPH. Thus, the prepared DHA nutrient delivery system can be used in food and pharmaceutical industries to improve the stability and bioavailability of DHA.

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752 **Table 1.** Stability studies of DHA nanoemulsion at different storage temperature
753 conditions.
754

Storage period (days) and samples		Phase separation			Centrifugation			Sedimentation		
		4±1 °C	28±1 °C	40±0.2 °C	4±1 °C	28±1 °C	40±0.2 °C	4±1 °C	28±1 °C	40±0.2 °C
1-8	HSH	–	–	–	–	–	–	–	–	–
	HPH	–	–	–	–	–	–	–	–	–
	HSH+HPH	–	–	–	–	–	–	–	–	–
20	HSH	–	+	+	–	+	+	–	–	–
	HPH	–	–	–	–	–	–	–	–	–
	HSH+HPH	–	–	–	–	–	–	–	–	–
40	HSH	++	++	++	++	++	++	–	–	–
	HPH	+	+	+	+	+	+	–	–	–
	HSH+HPH	+	+	+	+	+	+	–	–	–
100	HSH	++	++	++	++	++	++	–	+	++
	HPH	+	+	+	+	+	+	–	+	+
	HSH+HPH	+	+	+	+	+	+	–	+	+

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756 (-) no change; (+) slight change; (++) more change

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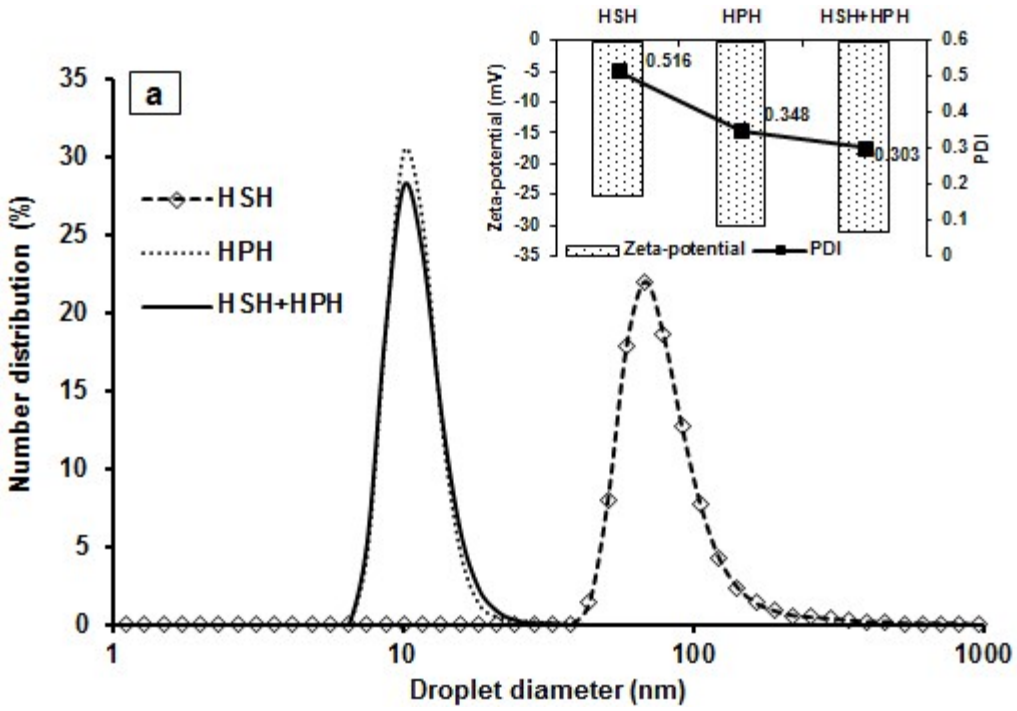


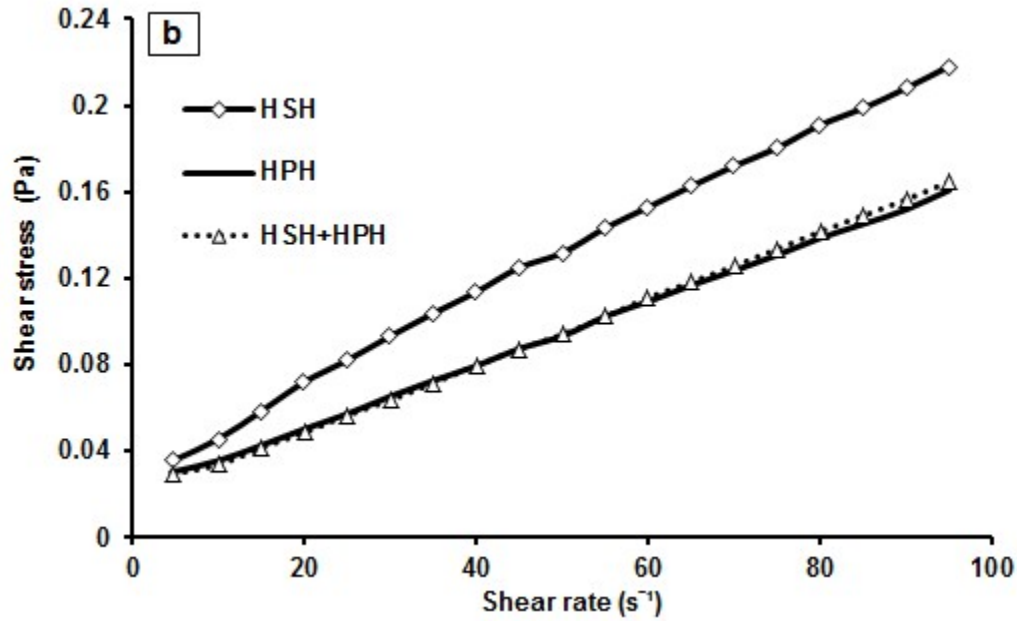


Fig. 1 Effect of different homogenization techniques used DHA nanoemulsions: a) Droplet size distribution, Zeta-potential measurement (mean±SD, *n*=3) and PDI (mean±SD, *n*=3), b) Rheological characteristics.

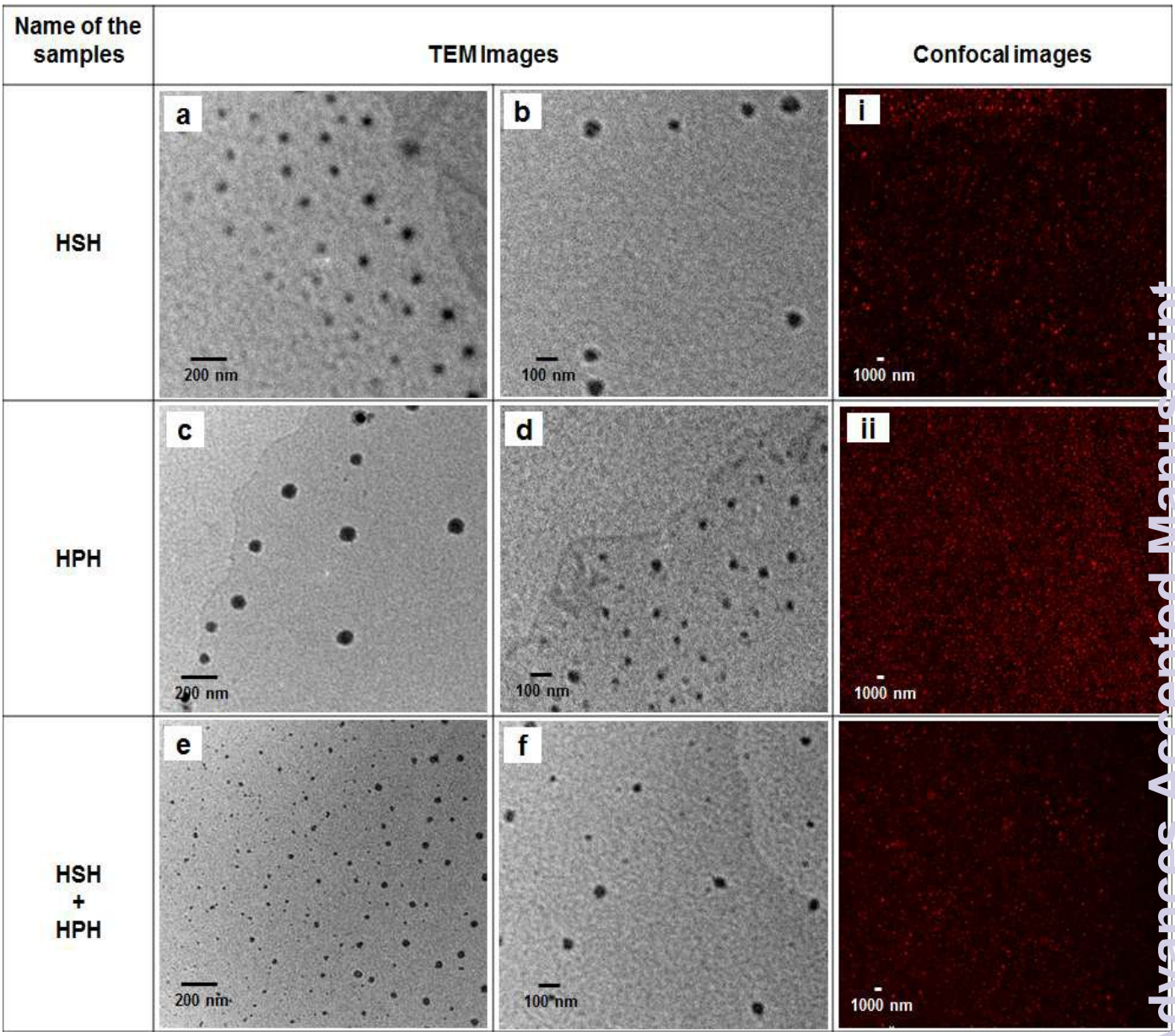


Fig. 2 Transmission electron microscopy (TEM) and confocal laser scanning microscopy images of DHA nanoemulsions.

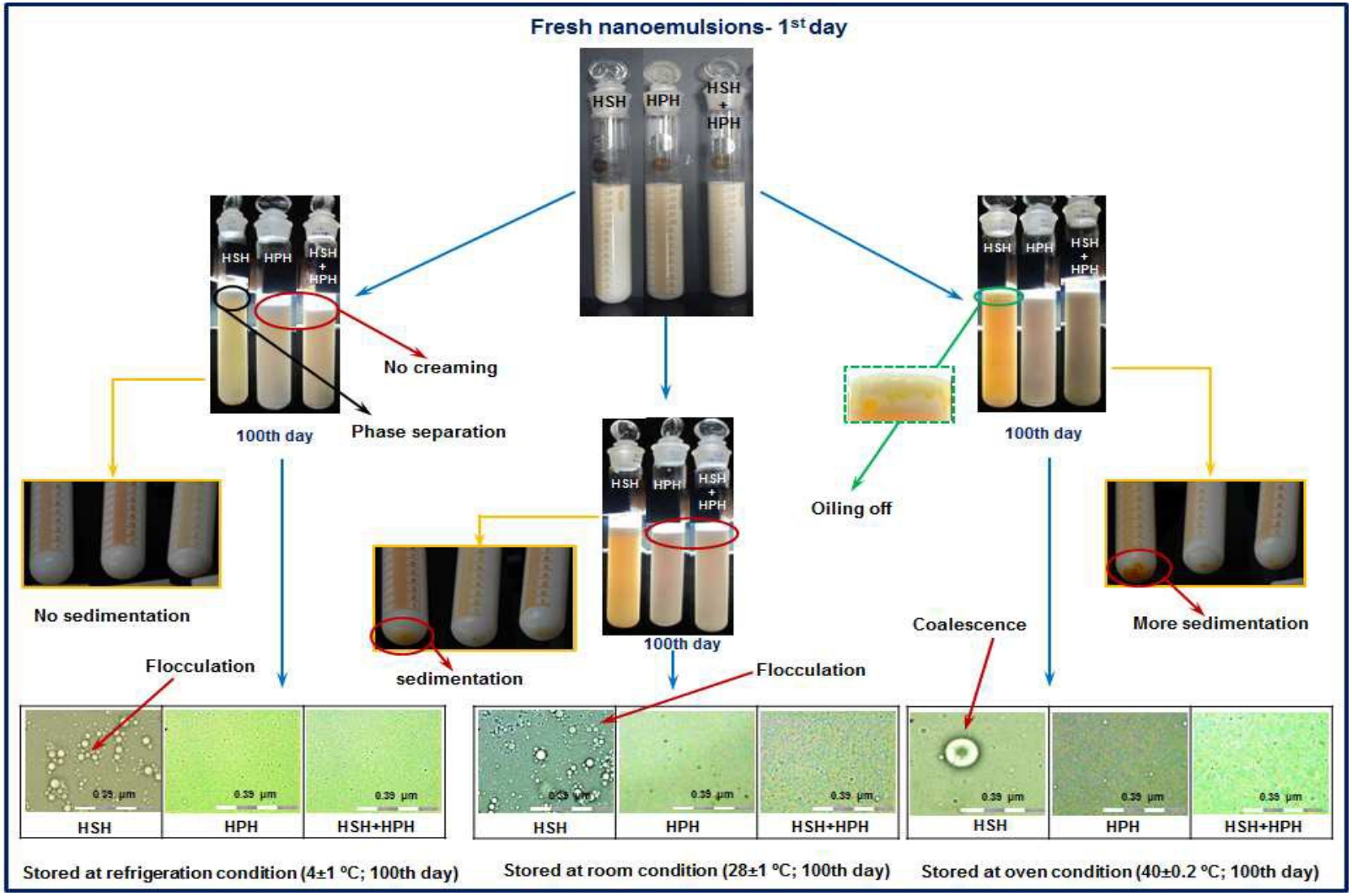
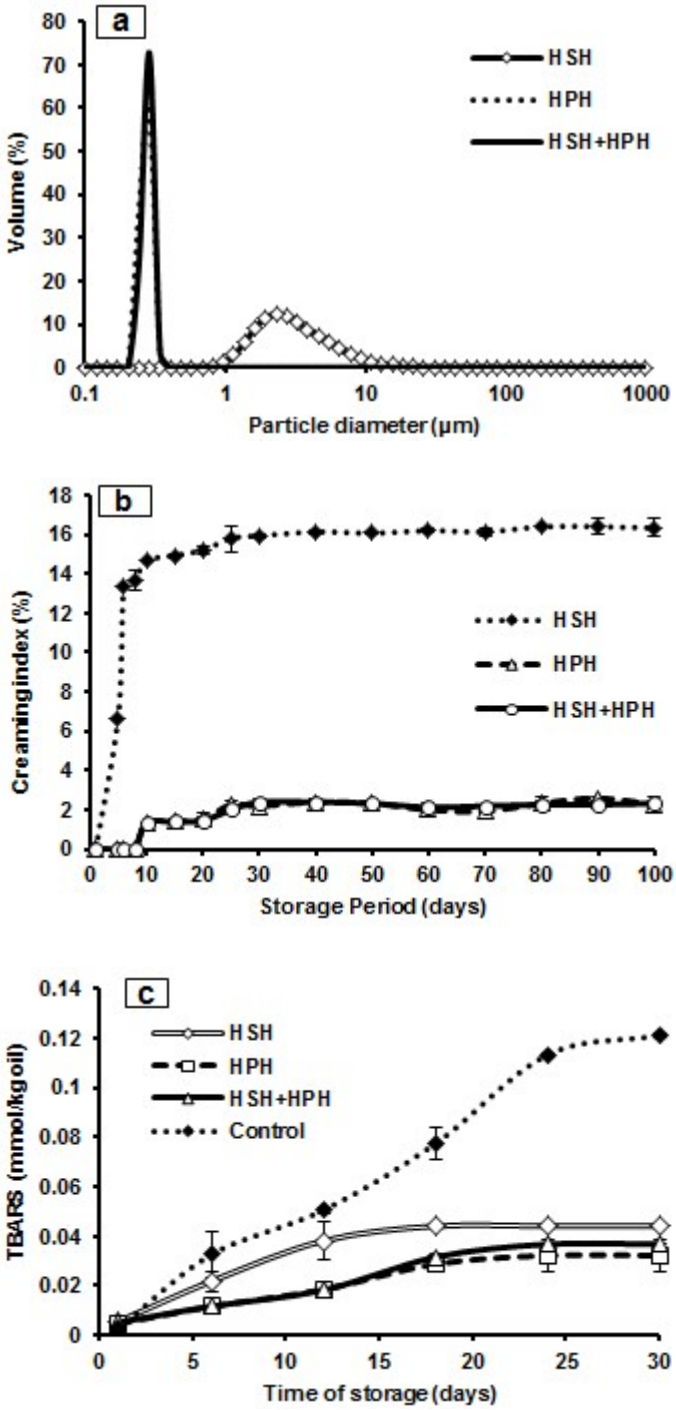


Fig. 3 Physical stability studies of DHA nanoemulsions over the storage period of 100 days at different temperature conditions. Scale bar represents 0.39 µm for all the emulsions.



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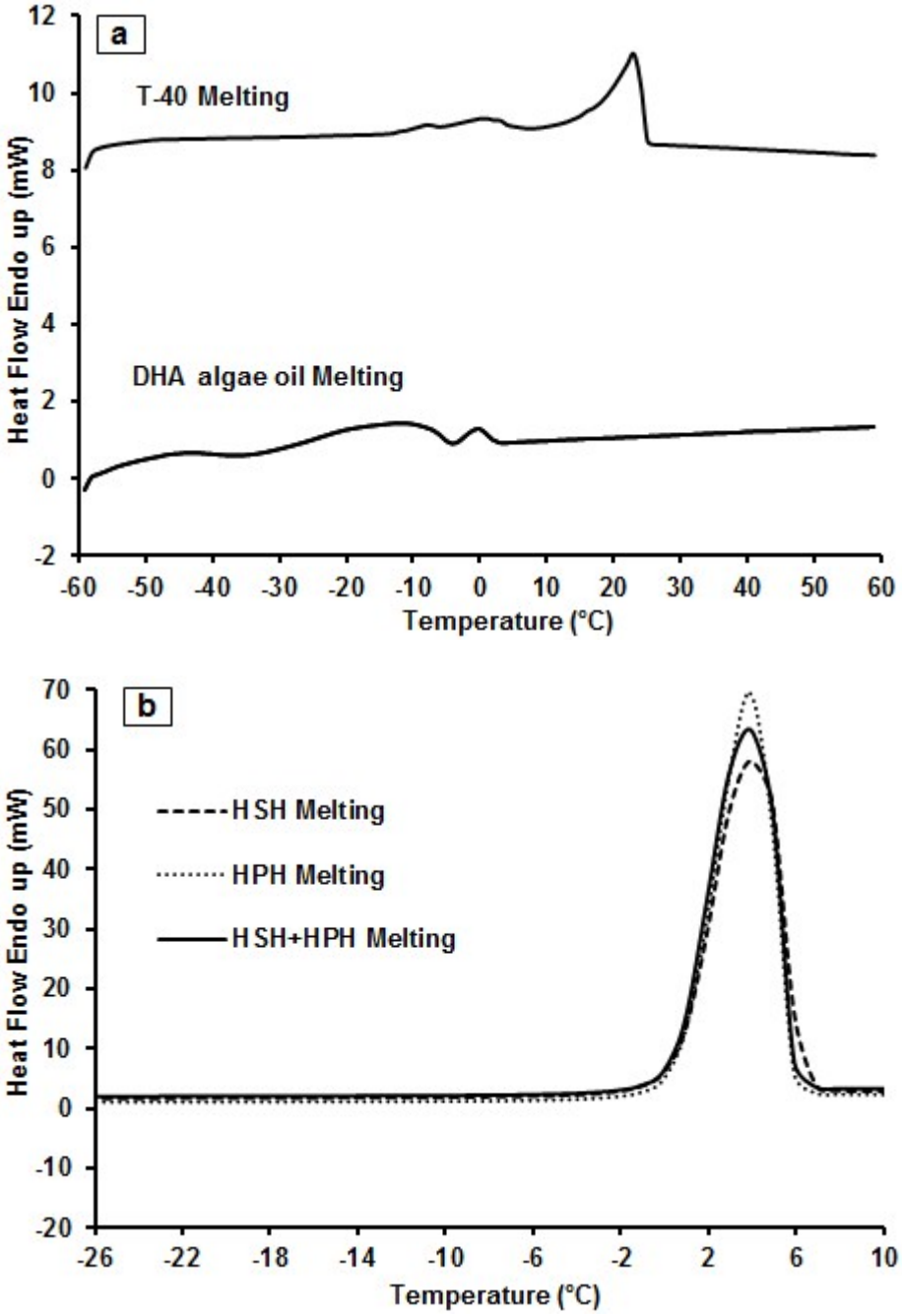
17 **Fig. 4** Storage stability studies of DHA nanoemulsions stored at room condition (28±1

18 ºC): (A) Particle size distribution at 100th day, (B) Creaming and (C) Oxidative study.



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20 **Fig. 5** DSC melting thermograms: (a) DHA and Tween-40; (b) Different high energy

21 emulsification techniques.

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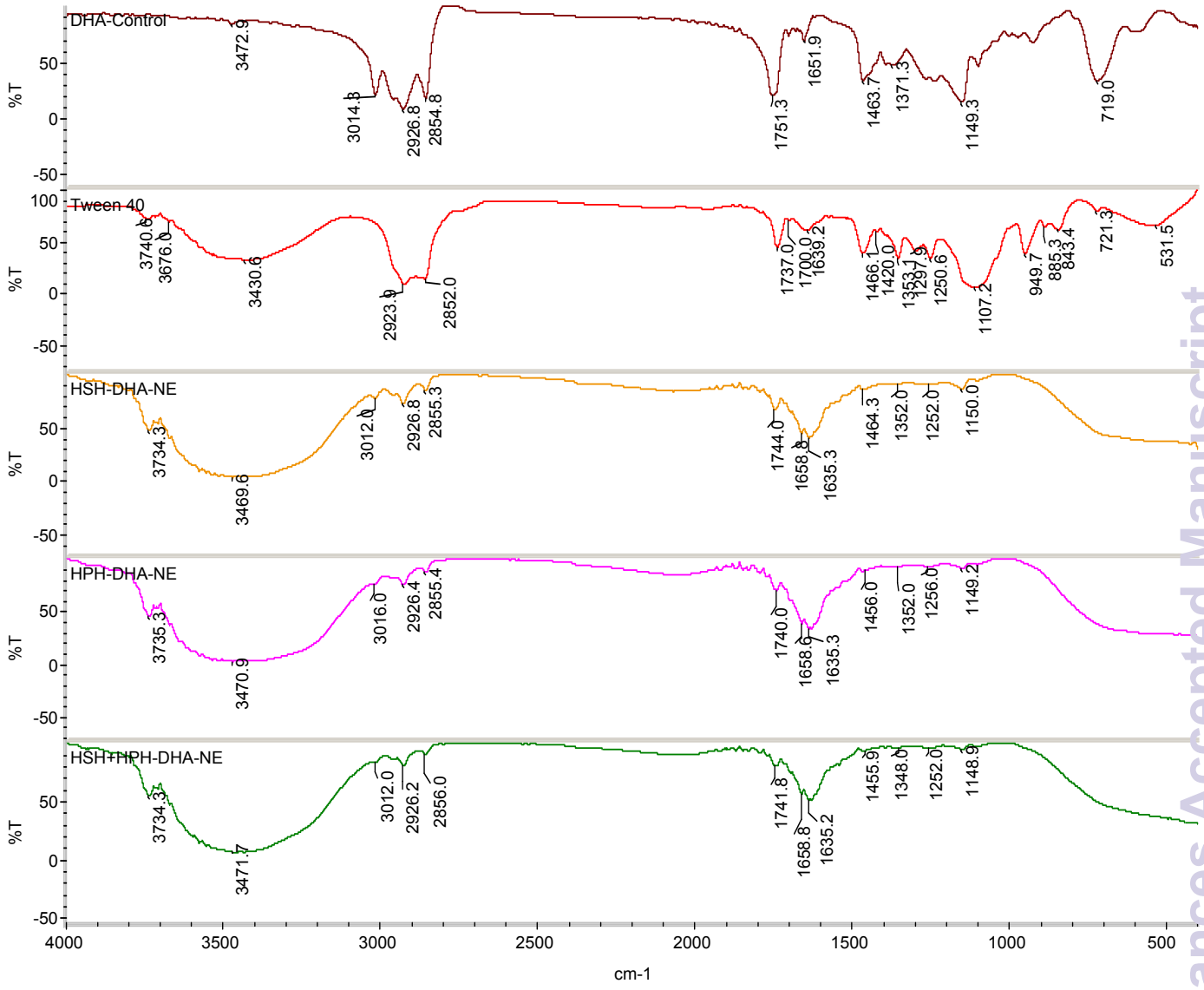
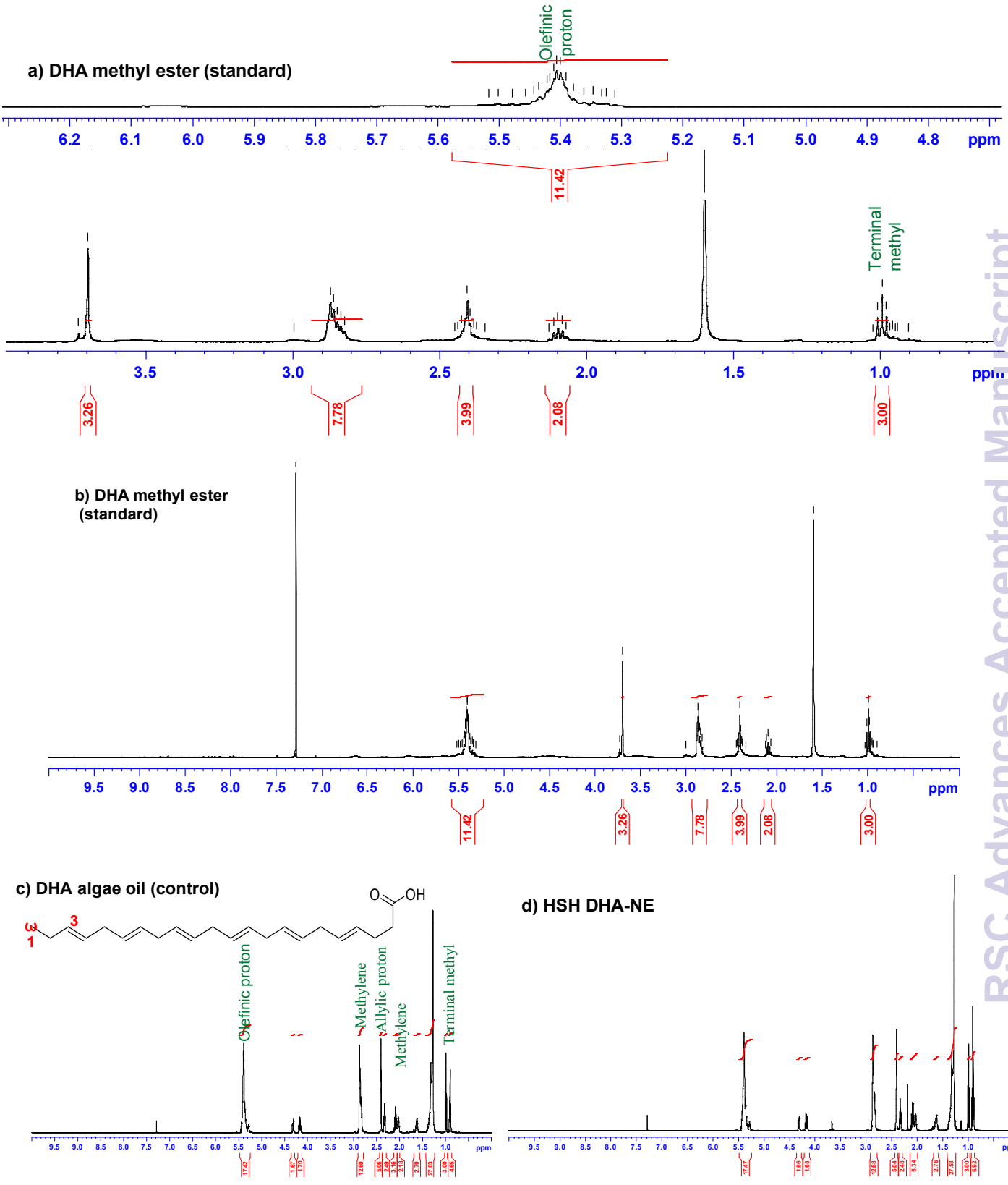
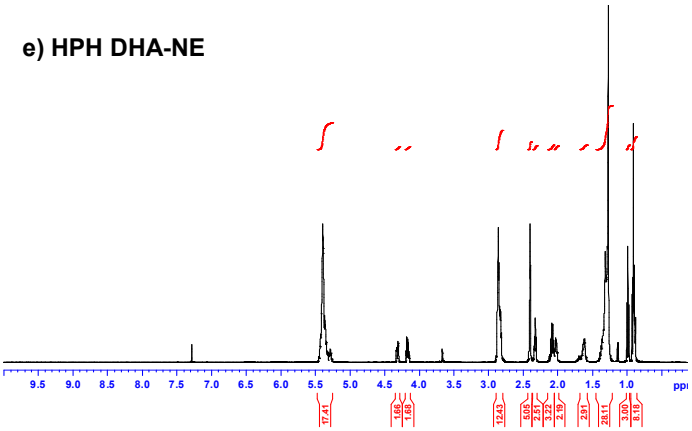


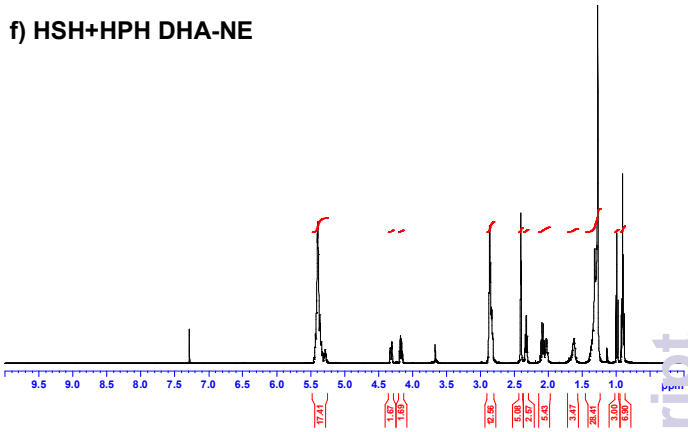
Fig. 6 FTIR spectra of DHA control, Tween-40, HSH-DHA-NE, HPH-DHA-NE and HSH+HPH-NE.



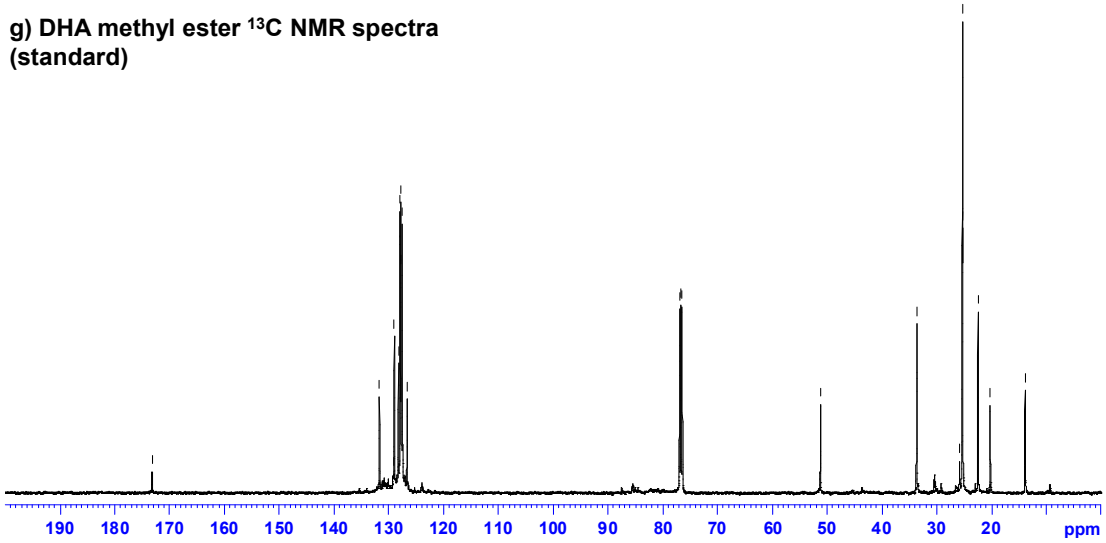
e) HPH DHA-NE



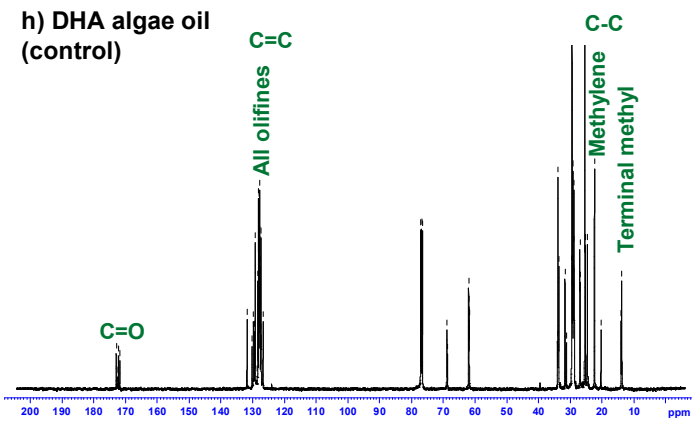
f) HSH+HPH DHA-NE



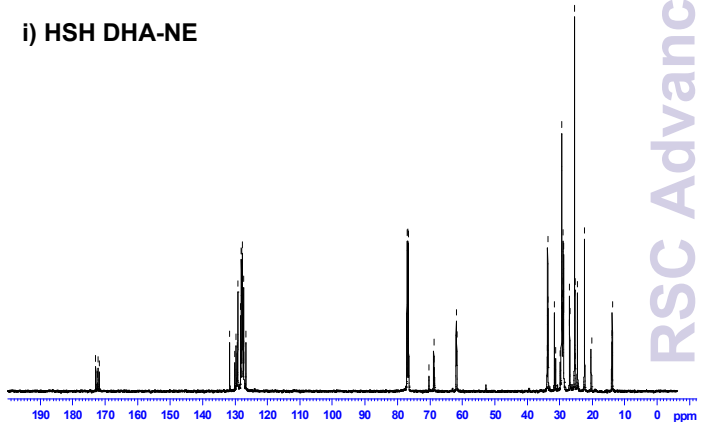
g) DHA methyl ester ^{13}C NMR spectra (standard)



h) DHA algae oil (control)



i) HSH DHA-NE



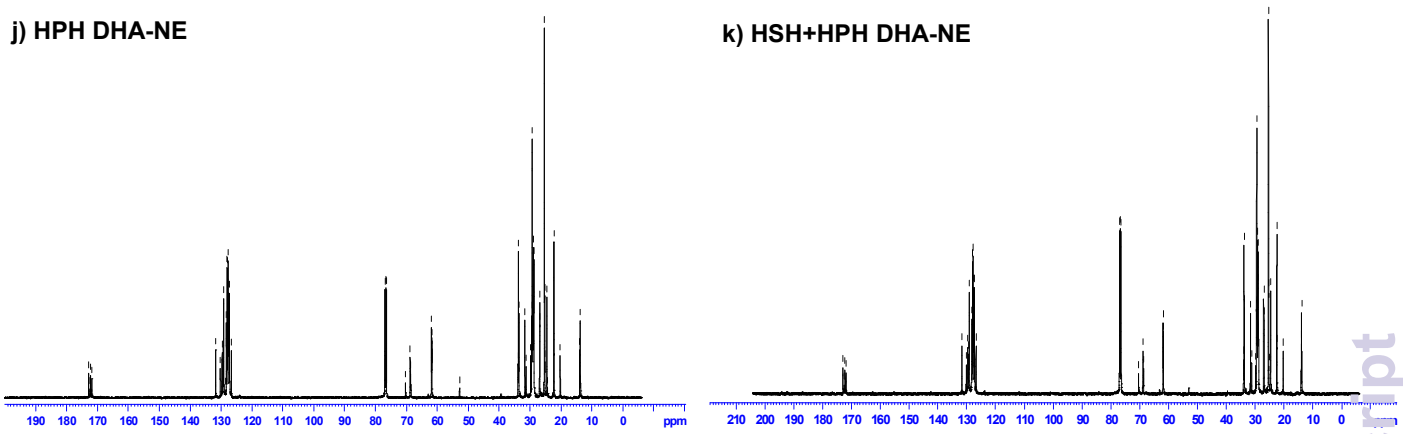


Fig. 7 ^{1}H NMR (a-f) and ^{13}C NMR (g-k) spectra of DHA algae oil, DHA methyl ester (standard) and different high energy techniques used nanoemulsions.

Graphical abstract