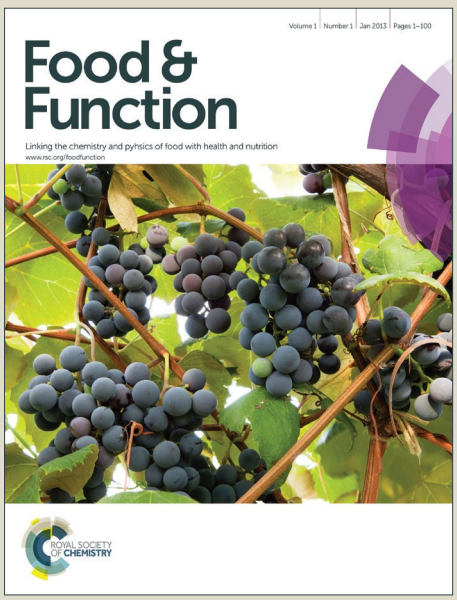
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Influence of droplet size on stability, *in vivo* digestion, and oral bioavailability of vitamin E emulsions

S. Parthasarathi[1,2], S.P. Muthukumar[3] and C. Anandharamakrishnan[1,2*]

[1]Department of Food Engineering

CSIR-Central Food Technological Research Institute

Mysore-570 020, India

[2]Academy of Scientific and Innovative Research (AcSIR), CSIR-CFTRI Campus, India.

[3]Animal House Facility

CSIR-Central Food Technological Research Institute

Mysore-570 020, India

** Correspondence:*

C. Anandharamakrishnan

Ph: +91-821-2513910

Fax: +91-821-2517233

E-mail: **anandhram@cftri.res.in**

Abstract

Vitamin E (α-Tocopherol) is a nutraceutical compound, shown to possess potent antioxidant and anticancer activity; however, its biological activity may be limited by poor bioavailability. Colloidal delivery systems are showing wide applications in the food and pharmaceutical industries to deliver lipophilic bioactive compounds. In this study we developed conventional and nanoemulsions of vitamin E from food grade ingredients (sunflower oil, saponin, and water) and showed nanoemulsion formulation increased the oral bioavailability than the conventional emulsion. The mean droplet diameters in nano and conventional emulsions were 0.277 and 1.285 μm respectively. Stability of emulsion formulation with thermal processing, long-term storage at different temperatures, mechanical stress and in plasma was determined. Results showed that saponin coated nanoemulsion were stable to droplet coalescence during thermal processing (30 – 90 °C), long-term storage and mechanical stress than conventional emulsion. Biological fate of emulsion formulation were studied using male Wistar rats as animal model. Emulsion droplet stability during the passage through gastrointestinal tract was evaluated by introducing them into rat stomachs. Microscopy technique was used to investigate the structural changes during digestion. Both conventional and nanoemulsion formulation showed strong evidence of droplet flocculation and coalescence during *in vivo* digestion. *In vivo* oral bioavailability study revealed that vitamin E in nanoemulsion form enhanced 3 fold increase in AUC than the conventional emulsion. The information reported in this study will facilitate the design of colloidal delivery system using nanoemulsion formulation.

Keywords: Vitamin E, Nanoemulsion, Stability, Invivo bioavailability.

1. Introduction

Emulsion based delivery system is a good candidate for encapsulating and delivering lipophilic bioactive components such as nutraceuticals, micronutrients, catechins, DHA.[1–8] Vitamin E (α-Tocopherol) is one of the nutraceutical compound, effectively used as an antioxidant and it prevents cancer and cardiovascular diseases.[9] Vitamin E is an oily liquid, with poor aqueous solubility and miscibility, which makes poor bioavailability. For any orally administered bioactive compound, bioavailability is depend on the dissolution rate in the intestinal lumen and absorption rate across the intestine. Hence these lipophilic bioactive compounds are usually administered as emulsion-based formulation to enhance their solubilization in the GI tract and to facilitate the biological uptake.[10,11] Further, Vitamin E is a nonpolar antioxidant, more effective in O/W emulsions as they retained in oil droplets. [12] Oil-in-water nanoemulsion have received great attention among the researchers because of their potential advantages like physical stability and enhanced bioavailability.[13,14]

Saponin (Latin *sapo*, means soap), are biosurfactants, received its attention among the food researchers due to increasing evidence of their health benefits like inhibiting cholesterol absorption and decreasing the serum and liver cholesterol level.[15,16] Saponins are widely distributed in more than 500 plant species and used as surface active and foaming agents in the food and cosmetic industries. It is a glycoside - non ionic surfactant, containing hydrophilic part, composed of rhamnose, xylose, arabinose, galactose, fucose, and glucuronic acid; and the lipophilic portion consist of steroidal or triterpene structure.[17,18] Excellent reviews are available to read further about the extraction techniques[19], isolation[20] and clinical significance[21] of saponins. Yang et al.[22] compared the effectiveness of saponin with synthetic

surfactant Tween 80 and suggested that natural surfactant is an effective surfactant and able to replace the synthetic surfactants. Wojciechowski et al.[23] studied the surface activity for saponin/β-casein mixture and reported that saponin can be used as a natural low molecular weight biosurfactant. Yang and McClements[24] and Ozturk et al.[2] successfully employed saponin surfactant for encapsulating vitamin D and E.

Oral administration of bioactive compounds is a most favored route among the consumers since it is easy to administer and requires low level of application skills. However, upon oral administration, a bioactive compound undergoes various physicochemical environment in the GI tract which influences the solubility, stability and bioavailability.[11] Many attempts have been made to understand the fate of emulsion during gastric digestion and their results showed that microstructural changes in emulsion during digestion are closely linked with digestion and release of nutrient in the digestive tract.[25] Hence, understanding the structure/stability of the ingested compound in the digestive environment helps in delivering the nutraceuticals effectively. *In vivo* and *in vitro* approaches are used for studying the fate of emulsified lipid within the GI tract. Various *in vitro* GI models have been developed to unravel the interesting structural and chemical changes of emulsion under simulated gastrointestinal condition.[26,27] But many *in vitro* models were failed to simulate the complex physicochemical and physiological events that happen in the GI tract.[28] However, it is undeniable that *in vivo* animal experiments are more time-consuming, costly and have ethical constraints. But they can provide most realistic and accurate results. Li et al.[29] studied the fate of emulsified lipids in the animal's gastrointestinal tract. Gallier et al.[25,30] unrevealed the effect of milk processing on the digestion of milk fat globules with the animal model.

Food and Drug Administration (FDA) defines bioavailability as "the rate and the extent to

which the therapeutic moiety is absorbed and becomes available to the site of drug action". The overall bioavailability (F) of a lipophilic bioactive component depends on numerous factors and can be elucidated by the following equation[31]:

$$F = F_L \times F_A \times F_D \times F_M \times F_E$$

where F_L is the fraction of bioactive agent liberated into the gastrointestinal tract for absorption, F_A is the fraction that is absorbed by the intestinal epithelial cells, F_D is the fraction of bioactive agents that reaches the site of action after distribution amongst the various tissues of the body, F_M is the fraction that reaches the site of action in metabolically active form and F_E is the fraction of metabolically active bioactive component that remains at the site of action, i.e., without excretion. Song et al.[9] revealed the sustained release of vitamin E encapsulated by Ca-pectinate through *in vivo* animal study. Area under the curve (AUC) for the microencapsulated vitamin E increased about 118 % compared to free vitamin E, which proves the Ca-pectinate encapsulated vitamin E provided sustained-release and improved bioavailability. Food and Drug Administration has approved nanoemulsions for the clinical application of water-insoluble drugs. Gong et al.[32] observed 1.6 fold increase in bioavailability for the nanoemulsion to the marketed vitamin E soft capsules.

To the best of our knowledge, no demonstrated reports were available till date to prove nanaoemulsion could enhance the oral bioavailability of vitamin E than the conventional emulsion. Therefore the current study has the following major aims: (1) to investigate the effect of thermal processing, storage and mechanical stress on the particle size distribution and appearance of conventional and nanoemulsions stabilized by natural surfactant saponin, (2) to study the impact of digestion on the structural changes of emulsion droplets in the gastrointestinal tract, and (3) to assess the influence of particle size on the oral bioavailability of vitamin E. The ultimate aim of this study is to facilitate the rationale usage of nanoemulsion

delivery system for utilization within the pharmaceutical and food industries.

2. Experimental Methods

2.1. Chemicals

Vitamin E (α-Tocopherol) was purchased from HiMedia Laboratories Pvt Ltd, Mumbai, Saponin from Quillaja bark (powder form, active content 20-35 %) was purchased from Sigma-Aldrich, Mumbai. Sunflower oil was purchased from the local market. All other chemicals, reagents and solvents used were of analytical grade or HPLC grade. Milli-Q water was used for the preparation of emulsions and solvents.

2.2. Emulsion Preparation

2.2.1. Nanoemulsion: Vitamin E nanoemulsion was prepared by homogenizing 10% lipid phase (2 wt % vitamin E in sunflower oil) with 90% aqueous phase containing saponin (0.1 wt %) and sodium azide (0.02 wt %). The aim of this study was to understand the effect of emulsion droplet size on the nutrient absorption. For this purpose the saponin concentration was maintained constant in all formulations and this approach would facilitate in studying the change in bioavailability profile of vitamin E based only on droplet size. Then, pre-emulsion was prepared by mixing these two phases using high-speed homogenizer (T18 Ultra Turrax, IKA India pvt. Ltd.) at 15,500 rpm for 5 min at room temperature. Then, nanoemulsion was prepared by passing the pre-emulsion through the microfluidizier (Microfluidics, M110P, Diamond interaction chamber, MA, USA), at a chamber pressure of 12,000 psi for four cycles.

2.2.2. Conventional emulsion: Vitamin E conventional emulsion was prepared by passing the pre-emulsion through the microfluidizier (Microfluidics, M110P, Diamond interaction

chamber, MA, USA), for a single passage at the chamber pressure of 1,000 psi.

2.3. Emulsion stability testing

Stability of the prepared nanoemulsion and conventional emulsion to environmental stresses were studied

2.3.1. Effect of thermal processing: Emulsion samples were incubated in water bath at different temperatures (30 – 90 °C) for 30 min. Then samples were cooled to room temperature and stored at ambient temperature for 24 h prior to analysis.

2.3.2. Long Term storage: Emulsion samples were incubated in temperature controlled environment (5, 25 and 40 °C) for one month prior to analysis.

2.3.3. Effect of mechanical stress: Emulsions were subjected to a mechanical shaking test. Samples were placed randomly on a shaker and agitated at the maximum amplitude of 250 strokes/min for 24 h at room temperature. At the end of each test, samples were analyzed for particle size and visually inspected for signs of phase separation.

2.3.4. Stability in plasma: An aliquot (0.05 mL) of each emulsion was mixed with 0.5 mL of blood plasma. Samples were then incubated at 37 C for 24 h. At the end of the experiment, samples were analyzed for particle size.

2.4. Biological fate of emulsion

The animal experiments were carried out at CSIR-CFTRI, Animal House Facility registered

with Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environments, Forests and Climate Change, Government of India, New Delhi (No: 49/1999/CPCSEA) established under The Prevention of Cruelty to Animals Act, 1960. The experimental protocols were approved by CFTRI - Institutional Animal Ethics Committee (No: IAEC/416/15). Male Wistar rats, weighing 200-220 g, were obtained from CSIR-CFTRI Animal House. Animals were adopted to laboratory conditions by keeping in temperature and humidity controlled animal observation room for 5 days. Prior to experiment, all animals were kept for overnight fasting and allowed free access to water. 1 ml of the sample (conventional and nanoemulsion) was administered into rat's stomach using feeding needle (Orchid Scientifics, Nashik, India). Samples were administered four times for every one hour. After one hour of the final administration, rats were sacrificed with diethyl ether. The gastrointestinal tract was removed and the digesta were collected from stomach and small intestine. Microstructural analysis for the digesta were observed using trinocular microscope (Olympus BX-5, ProgRes C-5 software) fixed with digital camera (ProgRes C-5) to capture images. All the samples were observed under 20X magnification.

2.5. *In vivo* Bioavailability

Male Wistar rats, weighing 200-220 g, were obtained from CSIR-CFTRI Animal House facility. Prior to experiment, all animals were kept for overnight fasting and allowed free access to water. To bring in further clarity to the role of droplet diameter in controlling the absorption of vitamin E, an additional emulsion sample with submicron sized droplet diameters (i.e. 679 nm) was evaluated for its *in vivo* bioavailability. Briefly, rats were randomly divided into three groups (n= 3 per group): nanoemulsion, submicron emulsion and conventional emulsion for oral administration. Rats were administered a single dose of 100 mg/kg of freshly prepared

197 nanoemulsions, submicron emulsion and conventional emulsions. Blood samples (0.5 ml)
198 were collected from retro-orbital vein at 0.5, 1, 2, 3, 4 and 6 h after oral administration.
199 Immediately after blood collection, plasma was separated by centrifugation at 4000 rpm, 10
200 min and stored at -20 °C till HPLC analysis. Mean plasma concentration of vitamin E versus
201 time profiles were plotted and the pharmacokinetic parameters were determined by non-
202 compartmental methods. The maximum plasma concentration (C_{max}) and the time of
203 maximum plasma concentration (T_{max}) were determined directly from the measured data.
204 Pharmacokinetic parameters like area under the curve (AUC), elimination rate constant (K_e),
205 elimination half-life ($t_{1/2}$) were determined using PKSolver 2.0.[33]
206
207 **2.6. Analysis of vitamin E concentration in plasma**
208
209 Vitamin E concentration in the blood samples was determined via isocratic elution in HPLC
210 system (Waters, Milford, USA), fitted with reverse phase Kromasil C18 column (4.6 mm x 250
211 mm) was used. Plasma samples (100 µl) were deproteinized with ethanol (300 µl) and
212 vortexed for 2 min. Then hexane (500 µl) was added and centrifuged the mixture for 10 min at
213 15000 rpm, then supernatant were transfered into amber vials. Hexane extraction procedure
214 was repeated, and the collected organic layer were evaporated to dryness under stream of
215 nitrogen. Prior to analysis, the residues were reconstituted in 100 µl methanol. Elution were
216 carried out at a flow rate of 1 ml/min under isocratic condition in the solvent ratio of methanol
217 to water (98:2 v/v).
218
219 **2.7. Droplet characterization**
220
221 The average droplet size and size distribution of the emulsion formulation were determined by

laser diffraction particle size analyzer (S3500, Microtrac Inc., USA) using triple distilled water as dispersing medium. Emulsion droplet size was reported as the mean diameter of number distribution.

2.8. Statistical Analysis

All experimental works reported here were carried out in duplicates on the freshly prepared samples and the results were reported as averages and standard deviations of the measurements. Statistical analysis of the differences between various treatments was performed using paired Student's *t*-test. A 0.05 level of probability was taken as the level of significance. Area under the curve (AUC) for calculating release of vitamin E in the blood plasma was calculated by trapezoidal approximation.

3. Results and Discussion

3.1. Effect of environmental stress on stability of conventional and nanoemulsion

Nanoemulsions are heterogeneous systems consisting two immiscible liquids, nanometric sized oil droplets dispersed in an aqueous medium and stabilized by food-grade surfactant. However, the definition for nano is still in flux. Nanotechnology deals with the production, processing, and application of materials with sizes less than 1,000 nm.[4] The U.S. Food and Drug Administration (FDA) use a definition of 1-1000 nm for drugs and also European Medicines Agency defines nanoparticle in a size range of less than 1,000 nm. Based on these definitions, we considered the emulsion with average droplet diameter of around 280 nm as nanoemulsion, 500- 1000 as submicron emulsion and more than 1000 nm as conventional emulsion. Emulsion based system should remain stable throughout the anticipated shelf life of

247 the final product. Emulsion become unstable depend on the storage conditions such as pH,
248 ionic strength, thermal processing, freeze-thaw cycle, drying and mechanical agitation.
249 Emulsion undergo following instability mechanisms like flocculation, coalescence, Ostwald
250 ripening and gravitational separation and become unstable.[24] Hence we examined the
251 influence of environmental stresses on the stability of conventional and nanoemulsion.
252
253 **3.1.1. Thermal Processing:** The purpose of this experiment is to examine the influence of
254 thermal processing on the stability of conventional and nanoemulsion (Fig. 2). Both emulsions
255 were held at temperatures ranging from 30 to 90 °C for 30 min, then cooled to room
256 temperature, and stored for 24 h. Creaming was not observed in any of the conventional and
257 nanoemulsion after 24 h at room temperature. Furthermore, no significant change was
258 observed in mean droplet diameter of the nanoemulsion. The stability of nanoemulsion at the
259 elevated temperature might be attributed to the high enough electrostatic and steric repulsion
260 among the droplets which prevented the droplet aggregation.[34] Similarly in the case of
261 conventional emulsion slight change in droplet size were observed. However, we did see
262 some instability in the conventional emulsion when they stored at elevated temperature for a
263 period of 30 days (see next section). Our result suggests that conventional emulsion stable
264 for a short duration thermal exposure and may become unstable to coalescence during the
265 storage at elevated temperatures.
266
267 **3.1.2. Effect of long term storage and temperature:** For commercial applications of
268 emulsion, the long-term stability, i.e., emulsion remain stable throughout their shelf-life is one
269 of the most important factors.[35] Hence we examined the influence of storage time (30 day)
270 and temperature (4, 25, 40 °C) on the stability of conventional and nanoemulsion. The
271 stability of the samples during storage was determined by measuring mean particle diameter

(Table 1). There was insignificant increase in mean particle diameter for nanoemulsion after one month storage: the droplet size ranged from 277 ± 0.02 to 307 ± 0.023 nm (4 ºC), 277 ± 0.02 to 292 ± 0.018 nm (25 ºC) and 277 ± 0.02 to 289 ± 0.003 nm (40 ºC). These findings are agreed with those of Yang et al.[24] in Q-naturale stabilized oil-in-water emulsions stored at 5, 37 and 55 ºC for a period of one month. Increase in droplet size is attributed to the mechanisms like flocculation, coalescence and Ostwald ripening. In the case of conventional emulsions, decrease in the mean particle diameter was observed with increasing storage time for the samples stored at 4, 25 and 40 °C. This decrease in particle diameter might be due to the solubilizaiton mechanism, movement of oil molecules from the emulsion droplets into the surfactant micelles. Furthermore, the rate of solubilization process is decreasing with reducing temperature from 40 to 4 ºC, indicating that it took longer time for the vitamin E with sunflower oil to move from the emulsion droplets into surfactant micelles at lower temperature. These results are in agreement with Ziani et al.[36] who emphasized the reasons behind decrease in mean droplet diameter, (i) movement of oil from the emulsion droplets into swollen micelles, and (ii) growth of some droplets due to Ostwald ripening or coalescence that they became to big to detect.

3.1.3. Stress testing and stability in plasma: Vitamin E emulsion was developed with saponin (biosurfactant), a triterpene backbone structured molecule, with glucuronic acid and saccharides at C-3 and C-28 positions respectively. It contains triterpenoid or steroid backbone as hydrophobic part and saccharide residues, attached to the hydrophobic scaffold via glycoside bonds to act as a hydrophilic part.[37] To access their physical stability, the change in droplet size under mechanical agitation was monitored (Fig. 3). After 24 h of mechanical agitation, no visible free oil or phase separation of the emulsion was observed. Our results suggest that both conventional and nanoemulsion are stable under mechanical

297 stress.

298

299 Vitamin E homologs possess several biological activities in addition to their antioxidant
300 properties. During acute clinical condition, oral administration of vitamin E is less effective for
301 immediate increase in vitamin E concentration.[38] Hence parenteral formulation of vitamin E
302 emulsion is advantageous for high risk patients. Stability of vitamin E emulsion droplets in
303 plasma is an important factor for transdermal/intravenous applications. Flocculation and
304 subsequent coalescence of emulsion droplets in the plasma and serum might cause adverse
305 effects like blocking of lung capillaries.[39] Both conventional and nanoemulsion were stable
306 after 24 h of incubation with plasma (Fig. 3). Nanoemulsion did not aggregate after 24 h of
307 incubation with plasma, however when mixed with conventional emulsion increase in mean
308 droplet size was observed, which may be due to the adsorption of plasma proteins onto the
309 emulsion droplets. The adsorbed proteins can lead to flocculation of oil droplets by bridging
310 and charge neutralization mechanisms.[39]

311

312 **3.2. Fate of emulsion in the digestive tract**

313 Fat digestion is a complex process involving several lipases acting in the stomach and small
314 intestine and the intestinal uptake of fat soluble vitamins are highly dependent on the action of
315 lipolytic enzymes on lipid substrates.[40] In the last decades, serious efforts have been made to
316 understand the physicochemical changes of emulsion during digestion and the reports
317 revealed that food emulsion undergo complicated physical and biochemical stresses.
318 However, current understanding about the structural changes of emulsion is mainly based on
319 the *in vitro* study using simulated gastric fluids. Digestion studies showed the microstructural
320 changes like droplet flocculation, phase separation and bioaccessibility of nutrients.[41] By
321 keeping in mind, the biological fate of conventional and nanoemulsion was studied in the

322 animal's gastrointestinal tract. Rats were fed with emulsion (1 ml) for a period of 4 hour with
323 one hour interval. Then animal was sacrificed one hour after the final administration and the
324 chyme and digesta was collected from stomach and small intestine respectively. Then the
325 collected samples were examined using microscopy techniques without any further treatment
326 to analyze microstructural changes during digestion (Fig. 4). For both conventional and
327 nanoemulsion, strong evidence of droplet flocculation and coalescence were observed for the
328 chyme samples collected from the stomach. Conventional emulsion become susceptible to
329 flocculation in the presence of digestive enzymes which produces the distinct large droplets
330 (see Fig. 4b). On the other hand, nanoemulsion ended with comparatively less flocculation
331 followed by coalescence. Hence we observed lipid droplets in chyme ranges from 0.046-1.61
332 μm for conventional emulsion and 0.042-0.772 μm for nanoemulsion gavaged rats. Similarly
333 in the intestinal digesta, emulsion droplets are in the range of 0.047-1.867 μm for the
334 conventional emulsion and 0.042-0.619 μm for the nanoemulsion.
335
336 In the stomach, the lipid droplets are exposed to highly acidic pH, mixed with digestive
337 enzymes (gastric lipases for initiating lipid digestion and proteases for protein digestion) and
338 experiences complex flow/force profile. After entering the small intestine, digestive enzymes
339 act on the partially hydrolyzed lipids, like pancreatic lipase/co-lipase convert triglycerides and
340 diglycerides into monoglycerides and free fatty acids, protease convert protein to peptides
341 and aminoacids, phospholipases convert phospholipids to free fatty acids.[31] Further, gastric
342 environment promotes the aggregation of emulsion droplets due to flocculation and/or
343 coalescence and consequently the droplets are no longer in their original particle size[42] hence
344 increase in droplet size was observed in this study. Gallier et al.[25] studied the behavior of
345 proteins of raw cream and pasteurized milk cream during digestion, and reported that
346 globules in the digesta are in the range of 1-35 μm for raw cream and 1-40 μm for pasteurized

cream. Similar results were observed for the *in vivo* digestion of β-lactoglobulin stabilized emulsion.[29] For the protein-stabilized emulsion, the proteolytic action of pepsin on the protein would reduce the droplet charge, which removes the steric repulsion barriers. This leads to aggregation and coalescence of emulsion droplets in the gastric environment.[43] In this study, emulsions were stabilized with saponin, which are stable to the acidic pH[24], hence drastic increase in droplet size was not observed during digestion. The obtained results were in agreement with those of Marciani et al.[44] who reported that emulsion stabilized by non-ionic surfactant remain stable during the gastrointestinal digestion because of its high emulsion stability. Our results suggest that nanoemulsions are fairly resistant to droplet coalescence and breakdown in the gastrointestinal tract whereas conventional emulsions are highly susceptible for both droplet coalescence and breakdown, and saponin surfactant can be employed as an alternative for synthetic surfactant.

3.3. *Invivo* bioavailability

The mean plasma concentration versus time profiles of vitamin E in rats following oral administration of conventional, submicron and nanoemulsions were shown in Fig. 5. The pharmacokinetic parameters including half-time (h), maximum plasma concentration (μg/ml), time to reach maximum plasma concentration (h), elimination rate constant (K_e), area under the concentration-time curve (AUC_{0-t} and AUC_{0-inf}) were calculated by non-compartment model (Table 2). Rats administered with conventional emulsion achieved maximum plasma concentration of 2.599 μg/ml at 3 h, submicron emulsion achieved maximum plasma concentration of 7.236 μg/ml at 3 h, whereas nanoemulsion achieved 11.253 μg/ml at 2 h. Maximum plasma concentration (C_{max}) was achieved at/before 3 h for all the administered formulation, hence the study was terminated at 6 h. Similarly half-life ($t_{1/2}$) for conventional,

submicron and nanoemulsion were 1.11 h, 0.98 h and 0.848 h respectively. The $t_{1/2}$ was calculated using the equation $t_{1/2}=0.693/K_e$ (where K_e is the elimination rate constant). The results showed a higher C_{max} and shorter T_{max} for the oral administration of nanoemulsion than the conventional and submicron emulsion. The increase in C_{max} indicates that the nanoemulsion was effectively increasing the vitamin E absorption. The area under the curve ($AUC_{0\text{-}inf}$) of the vitamin E nanoemulsion, submicron and conventional emulsion were 22.294, 17.686 and 7.476 μg/ml/h respectively. Thus our data suggested that the degree of exposure to nano-, submicron emulsion showed 3, 2.4-fold increase in bioavailability respectively, which demonstrates nanoemulsion formulation was able to increase the oral bioavailability of vitamin E.

Absorption of bioactive compound is depend on two factors: (i) solubility in the gastrointestinal lumen, and (ii) capacity to diffuse across the enterocytes.[45] Emulsion-based delivery system was formulated to overcome the solubilization problem. The oral bioavailability of natural vitamin E is not high[32,46]; however the nanoemulsion formulation enhanced the bioavailability of vitamin E by increasing its solubility. Mass flux across the intestine can be be elucidated by the following equation:[10]

$$\frac{\mathrm{dm}}{\mathrm{dt}} = \mathrm{A} \times P_{\mathrm{PT}} \times C_{\mathrm{lumen}} + \frac{V_{\mathrm{max(influx)}} \times C_{\mathrm{lumen}}}{K_{m(\mathrm{influx})} + \mathrm{C}_{\mathrm{lumen}}} - \frac{V_{\mathrm{max(efflux)}} \times C_{\mathrm{ent}}}{K_{m(\mathrm{efflux})} + \mathrm{C}_{\mathrm{ent}}}$$

where A is the surface area of membrane, P_{PT} is the passive permeability, C_{lumen} is the intestinal luminal concentration, C_{ent} is the concentration of bioactive inside the enterocytes, K_m is the Michaelis-Menten constant of the compound with the influx or the efflux and V_{max} is the maximum efflux or influx mediated transport. Abuasal et al.[10,47] reported that total flux of vitamin E is only by the contribution of passive and carrier mediated transport, and the above mass flux equation can be expressed as:

$$\frac{\mathrm{dm}}{\mathrm{dt}} = \mathrm{A} \times P_{\mathrm{PT}} \times C_{\mathrm{lumen}} + \frac{V_{\mathrm{max(influx)}} \times C_{\mathrm{lumen}}}{K_{m(\mathrm{influx})} + \mathrm{C}_{\mathrm{lumen}}}$$

Thus the overall absorption rate of vitamin E is determined by the contribution of saturable (carrier mediated transport) and unsaturable (passive transport) processes. Further, the saturable process is mediated by the Niemann-Pick C1-like 1 (NPC1L1) transporter, a polytopic protein present on the intestinal epithelial cells that facilitates the vitamin E absorption.[48] The uptake of vitamin E is saturated by NPC1L1 transporter, thus the oral bioavailability is decreasing. To overcome this, new formulation approaches like solid lipid nanoparticles (SLN), lipid based formulations like self-emulsifying drug delivery systems (SEDDS), nanoemulsion have been used to enhance its permeability, thus its oral bioavailability. Abuasal et al.[49] reported that solid lipid nanoparticles enhanced the bioavailability of γ-Tocotrienol (member of vitamin E family) by ten-fold compared to the control given as mixed micelles. *In vivo* pharmacokinetic studies also revealed higher plasma concentration of γ-Tocotrienol for SLN formulation (938 ± 63 ng/ml) compared to control (212 ± 111 ng/ml). Gong et al.[32] compared the oral bioavailability for the vitamin E nanoemulsion with marketed soft capsules and reported AUC for the nanoemulsion and marketed soft capsules were 1.48 and 0.907 μg/ml/h respectively. Increased bioavailability for the nanoemulsion might be due to the small-sized droplets offered larger surface area, which enabled large number of lipase molecules to bind at the oil-water interface. Armand et al.[42] reported 3.3 times higher lipolysis for the fine emulsion than the coarse emulsion. Still, the effect of formulation on vitamin E metabolism needs to be addressed by comparing the metabolites between conventional and nanoemulsion.

4. Conclusions

This study mainly focused on understanding the role of emulsion particle size on the stability, digestibility and bioavailability of colloidal dispersion containing vitamin E and natural surfactant (saponin). High-pressure homogenization technique was employed for the formation of conventional and nanoemulsion with droplet diameters 1,285 nm and 277 nm respectively. Both conventional and nanoemulsion were stable at short duration (30 min) of thermal exposure, temperature ranging from 30 to 90 °C. During long term storage (30 days) at different thermal conditions (4, 25, 40 °C), nanoemulsion was physically stable with a slight increase in mean particle diameter. Both conventional and nanoemulsion were stable with no visible free oil or phase separation after 24 h of mechanical agitation. However conventional emulsion aggregated in plasma after 24 h of incubation due to adsorption of plasma proteins onto the emulsion droplets. In the digestive tract, chyme of nanoemulsion had comparatively less flocculation followed by coalescence than the conventional emulsion. Pharmacokinetic study in rats revealed an increase in bioavailability for nanoemulsions; area under the curve ($AUC_{0\text{-}inf}$) of the vitamin E nano-, submicron and conventional emulsions were 22.294, 17.68 and 7.476 µg/ml/h respectively, which proved an increase in bioavailability by 3 fold for nanoemulsion. These results help in designing and producing vitamin E colloidal delivery system based on nanoemulsions for utilization in the pharmaceutical and food industries.

ACKNOWLEDGMENT

Authors wish to thank Prof. Ram Rajasekharan, Director, CSIR-CFTRI, Mysore, India for his support and encouragement. The author (Parthasarathi) thanks the ICMR, India for awarding SRF fellowship.

References

1 G. Davidov-Pardo and D. J. McClements, Food Chem., 2015, 167, 205–212.

2 B. Ozturk, S. Argin, M. Ozilgen and D. J. McClements, Food Chem., 2015, 187, 499–506.

3 P. Karthik, P. N. Ezhilarasi and C. Anandharamakrishnan, Crit. Rev. Food Sci. Nutr., 2015. DOI:10.1080/10408398.2015.1006767

4 P. N. Ezhilarasi, P. Karthik, N. Chhanwal and C. Anandharamakrishnan, Food Bioprocess Technol., 2013, 6, 628–647.

5 P. Karthik and C. Anandharamakrishnan, Food Bioprocess Technol., 2013, 6, 2780–2790.

6 C. Anandharamakrishnan, Techniques for Nanoencapsulation of Food Ingredients, Springer New York, New York, NY, 2014.

7 J. A. Bhushani, P. Karthik and C. Anandharamakrishnan, Food Hydrocoll., 2016, 56, 372–382.

8 P. Karthik and C. Anandharamakrishnan, RSC Adv., 2016, 6, 3501–3513.

9 Y. Bin Song, J. S. Lee and H. G. Lee, Colloids Surfaces B Biointerfaces, 2009, 73, 394–398.

10 B. S. Abuasal, H. Qosa, P. W. Sylvester and A. Kaddoumi, Biopharm. Drug Dispos., 2012, 33, 246–256.

11 Y. Ting, Y. Jiang, C. T. Ho and Q. Huang, J. Funct. Foods, 2014, 7, 112–128.

12 S. S. Kiralan, E. Doğu-Baykut, K. Kittipongpittaya, D. J. McClements and E. A. Decker, J. Agric. Food Chem., 2014, 62, 10561–10566.

13 M. Fanun, Soft Mater., 2009, 7, 258–276.

14 Q. Huang, H. Yu and Q. Ru, J. Food Sci., 2010, 75, R50–R57.

15 L. Vinarova, Z. Vinarov, V. Atanasov, I. Pantcheva, S. Tcholakova, N. Denkov and S.

Stoyanov, Food Funct., 2015, 6, 501–512.

16 L. Vinarova, Z. Vinarov, B. Damyanova, S. Tcholakova, N. Denkov and S. Stoyanov, Food Funct., 2015, 6, 1319–1330.

17 W. Oleszek and A. Hamed, in Surfactants from Renewable Resources, John Wiley & Sons, Ltd, Chichester, UK, pp. 239–249.

18 K. Golemanov, S. Tcholakova, N. Denkov, E. Pelan and S. D. Stoyanov, Soft Matter, 2014, 10, 7034–7044.

19 C. Y. Cheok, H. A. K. Salman and R. Sulaiman, Food Res. Int., 2014, 59, 16–40.

20 R. A. Hill and J. D. Connolly, Nat. Prod. Rep., 2013, 30, 1028–1065.

21 H. Sheng and H. Sun, Nat. Prod. Rep., 2011, 28, 543–593.

22 Y. Yang, M. E. Leser, A. A. Sher and D. J. McClements, Food Hydrocoll., 2013, 30, 589–596.

23 K. Wojciechowski, A. Kezwon, J. Lewandowska and K. Marcinkowski, Food Hydrocoll., 2014, 34, 208–216.

24 Y. Yang and D. J. McClements, Food Chem., 2013, 141, 473–481.

25 S. Gallier, J. Cui, T. D. Olson, S. M. Rutherfurd, A. Ye, P. J. Moughan and H. Singh, Food Chem., 2013, 141, 3273–3281.

26 F. Kong and R. P. Singh, J. Food Sci., 2008, 73.

27 S. J. Hur, B. O. Lim, E. A. Decker and D. J. McClements, Food Chem., 2011, 125, 1–12.

28 L. Chen, X. Wu and X. D. Chen, J. Food Eng., 2013, 117, 183–192.

29 Y. Li, J. Kim, Y. Park and D. J. McClements, Food Funct., 2012, 3, 528.

30 S. Gallier, X. Q. Zhu, S. M. Rutherfurd, A. Ye, P. J. Moughan and H. Singh, Food Chem., 2013, 141, 3215–3223.

31 D. J. McClements and H. Xiao, Food Funct., 2012, 3, 202–220.

32 Y. Gong, Y. Wu, C. Zheng, L. Fan, F. Xiong and J. Zhu, AAPS PharmSciTech, 2012, 13,

961–966.

33 Y. Zhang, M. Huo, J. Zhou and S. Xie, Comput. Methods Programs Biomed., 2010, 99, 306–314.

34 B. Ozturk, S. Argin, M. Ozilgen and D. J. McClements, J. Food Eng., 2014, 142, 57–63.

35 A. H. Saberi, Y. Fang and D. J. McClements, J. Colloid Interface Sci., 2013, 391, 95–102.

36 K. Ziani, Y. Fang and D. J. McClements, Food Res. Int., 2012, 46, 209–216.

37 R. Stanimirova, K. Marinova, S. Tcholakova, N. D. Denkov, S. Stoyanov and E. Pelan, Langmuir, 2011, 27, 12486–98.

38 A. Lassnigg, A. Punz, R. Barker, P. Keznickl, N. Manhart, E. Roth and M. Hiesmayr, Br. J. Anaesth., 2003, 90, 148–154.

39 J. Han, S. S. Davis, C. Papandreou, C. D. Melia and C. Washington, Pharm. Res., 2004, 21, 1573–1580.

40 M. Armand, P. Borel, B. Pasquier, C. Dubois, M. Senft, M. Andre, J. Peyrot, J. Salducci and D. Lairon, Am. J. Physiol., 1996, 271, G172–G183.

41 L. Mao and S. Miao, Food Eng. Rev., 2015, 7, 439–451.

42 M. Armand, B. Pasquier, M. André, P. Borel, M. Senft, J. Peyrot, J. Salducci, H. Portugal, V. Jaussan and D. Lairon, Am. J. Clin. Nutr., 1999, 70, 1096–1106.

43 A. Sarkar, K. K. T. Goh, R. P. Singh and H. Singh, Food Hydrocoll., 2009, 23, 1563–1569.

44 L. Marciani, M. Wickham, G. Singh, D. Bush, B. Pick, E. Cox, A. Fillery-Travis, R. Faulks, C. Marsden, P. A. Gowland and R. C. Spiller, AJP Gastrointest. Liver Physiol., 2007, 292, G1607–G1613.

45 G. Fricker, T. Kromp, A. Wendel, A. Blume, J. Zirkel, H. Rebmann, C. Setzer, R.-O. Quinkert, F. Martin and C. Müller-Goymann, Pharm. Res., 2010, 27, 1469–1486.

46 R. J. Sokol, N. Butler-Simon, J. E. Heubi, S. T. Iannaccone, H. J. McClung, F. Accurso, K. Hammond, M. Heyman, F. Sinatra, C. Riely, J. Perrault, J. Levy and A. Silverman, Ann. N. Y. Acad. Sci., 1989, 570, 156–169.

47 B. Abuasal, P. W. Sylvester and A. Kaddoumi, Drug Metab. Dispos., 2010, 38, 939–945.

48 K. Narushima, T. Takada, Y. Yamanashi and H. Suzuki, Mol. Pharmacol., 2008, 74, 42–49.

49 B. S. Abuasal, C. Lucas, B. Peyton, A. Alayoubi, S. Nazzal, P. W. Sylvester and A. Kaddoumi, Lipids, 2012, 47, 461–469.

542

543

544 **Captions:**

545

546 **Table 1:** Particle size changes after one month storage at different conditions

547 **Table 2:** Pharmacokinetic parameters after oral administration of conventional, submicron
548 and nanoemulsions

549 **Fig. 1** Particle size distribution of the vitamin E conventional emulsions and nanoemulsions

550 **Fig. 2** Influence of thermal processing on the mean particle diameter of conventional and
551 nanoemulsions

552 **Fig. 3** Stability of Vitamin E nanoemulsion and conventional emulsion against plasma and
553 mechanical stress at 24 hrs incubation at 37 °C. Mean (n=6) ± SD.

554 **Fig. 4** Fate of emulsion in the digestive tract. (a) Microscopic image of conventional emulsion;
555 (b) Gastric chyme collected after 60 min after gavaging with conventional emulsion; (c) Small
556 intestinal digesta of rats gavaged with conventional emulsion; (d) Microscopic image of
557 nanoemulsion; (e) Gastric chyme collected after 60 min after gavaging with nanoemulsion; (f)
558 Small intestinal digesta of rats gavaged with nanoemulsion.

559 **Fig. 5** Plasma concentration (μg/ml) versus time (h) profiles after oral administration of
560 vitamin E conventional and nanoemulsion in rats (n=3).

561

562

563

564

565

566

Table 1 Particle size changes after one month storage at different conditions

		0 Day	After one month
Conventional emulsion	4 ºC	1.285 ± 0.003	1.253 ± 0.056
	25 ºC		0.988 ± 0.009
	40 ºC		0.537 ± 0.083
Nanoemulsion	4 ºC	0.277 ± 0.02	0.307 ± 0.023
	25 ºC		0.292 ± 0.018
	40 ºC		0.289 ± 0.003

583

584

585 **Table 2** Pharmacokinetic parameters after oral administration of conventional, submicron and

586 nanoemulsion

587

Pharmacokinetic parameters [(a)]	Conventional emulsion	Submicron emulsion	Nanoemulsion
C_{max} (μg/ml)	2.599	7.236	11.253
T_{max} (h)	3.0	3.0	2.0
K_e	0.622	0.707	0.817
Half life (h)	1.11	0.98	0.848
AUC_{0-t} (μg/ml/h)	6.872	16.534	21.791
AUC_{0-inf} (μg/ml/h)	7.476	17.686	22.294

588

589 [(a)] Data were collected using non-compartmental analysis

590

591

592

593

594

595

596

597

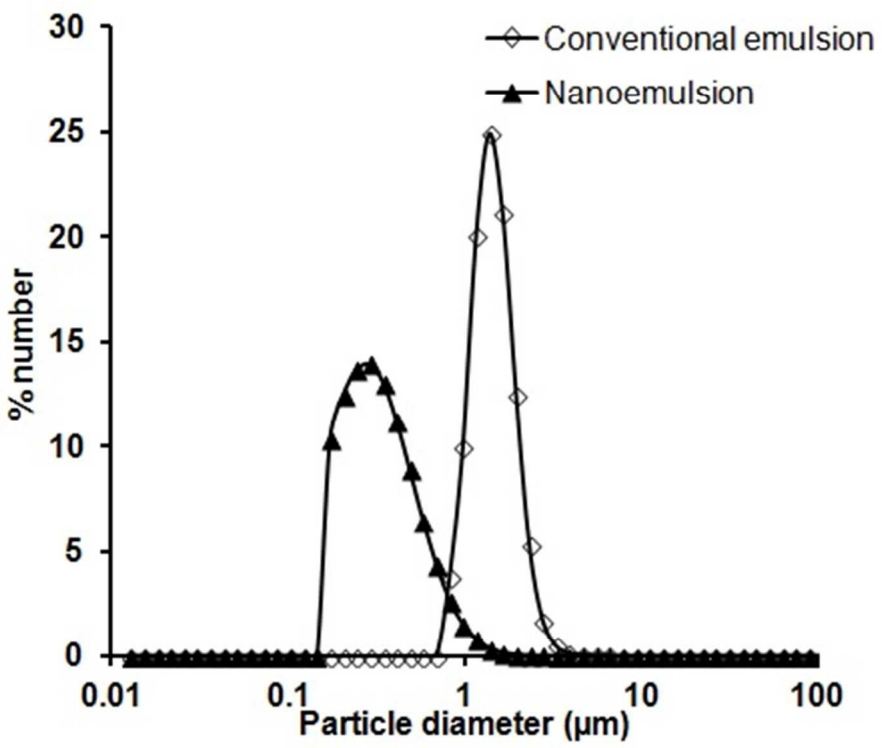
598

599

600 **Fig. 1** Particle size distribution of the vitamin E conventional and nanoemulsion

601

602



603

604

605

606

607

608

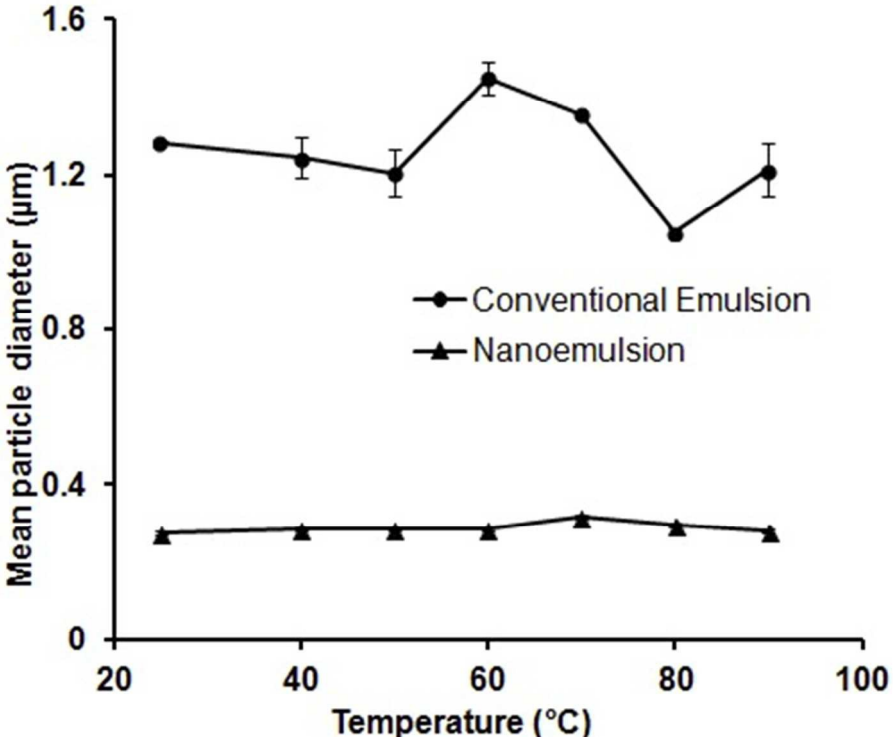
609

610

611

612

Fig. 2 Influence of thermal processing on mean particle diameters of conventional and nanoemulsion.



638

639

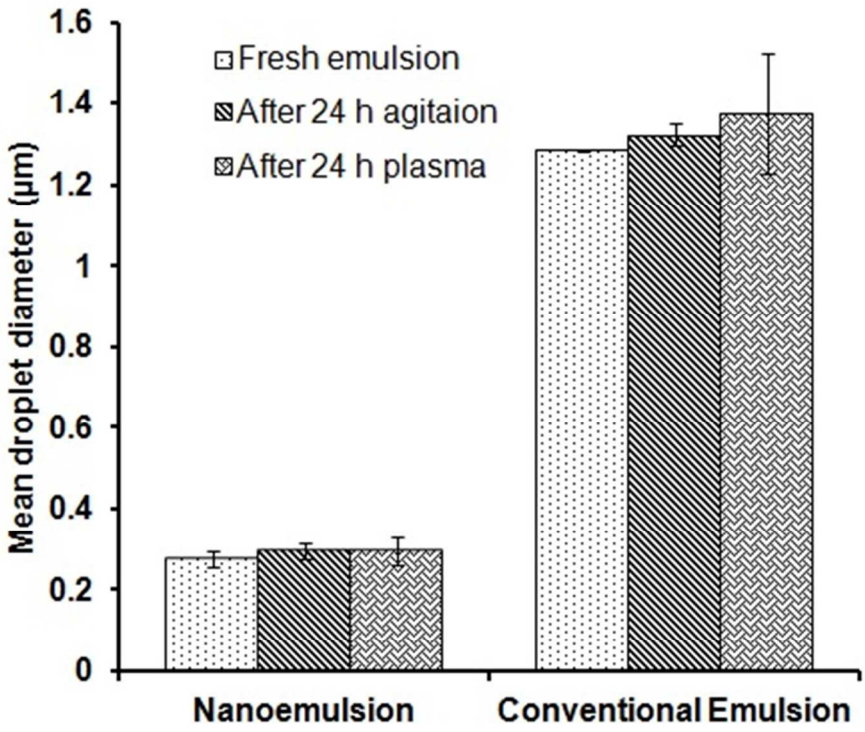
640 **Fig. 3** Stability of Vitamin E nanoemulsion and conventional emulsion against plasma and
641 mechanical stress at 24 hrs incubation at 37 °C. Mean (n=6) ± SD.

642

643

644

645



656

657

658

659

660

661

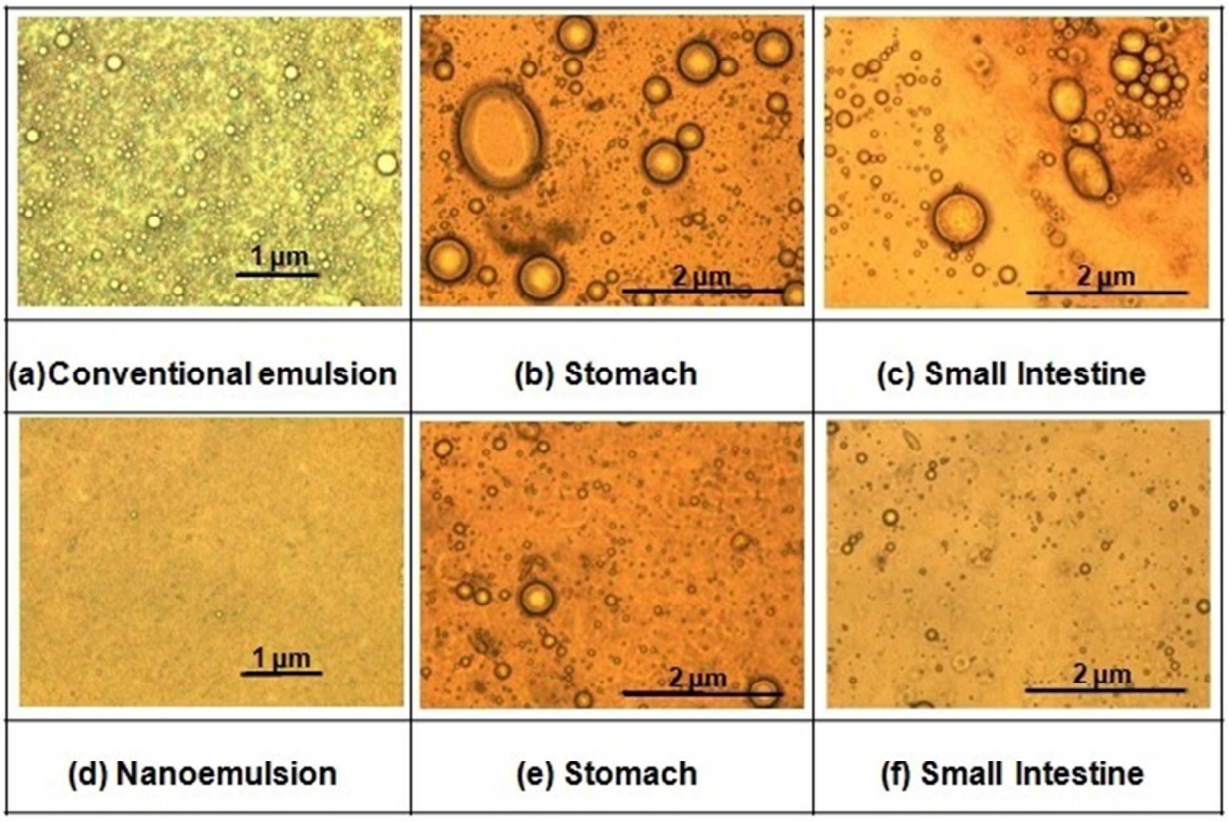
662

663

664

665 **Fig. 4** Fate of emulsion droplets in the digestive tract. (a) Microscopic image of conventional
666 emulsion; (b) Gastric chyme collected after 60 min after gavaging with conventional emulsion;
667 (c) Small intestinal digesta of rats gavaged with conventional emulsion; (d) Microscopic image
668 of nanoemulsion; (e) Gastric chyme collected after 60 min after gavaging with nanoemulsion;
669 (f) Small intestinal digesta of rats gavaged with nanoemulsion.

670



671

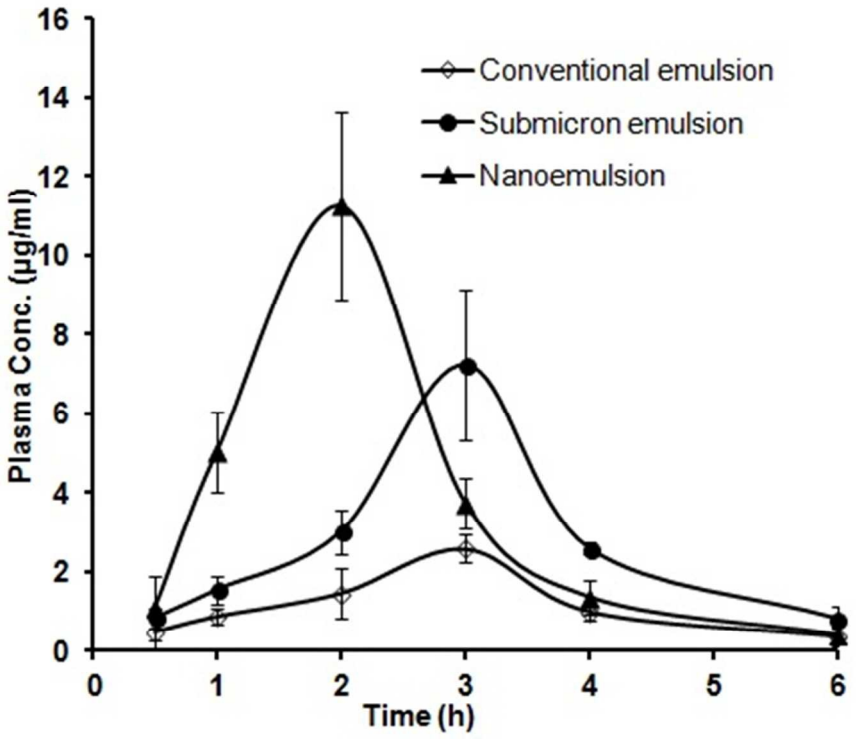
672

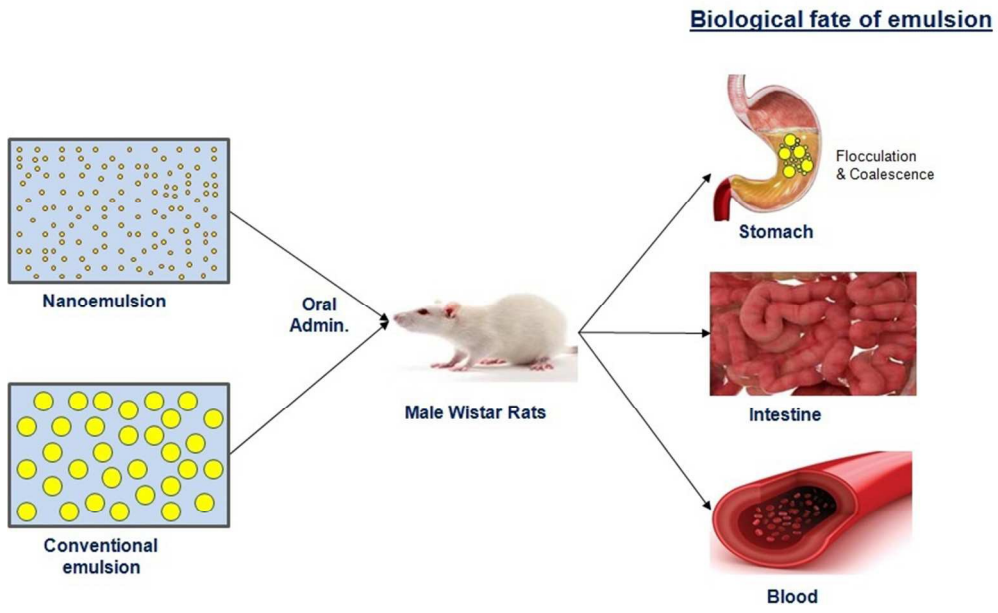
673

674

675

Fig. 5 Plasma concentration (μg/ml) versus time (h) profiles after oral administration of vitamin E conventional, submicron and nanoemulsion in rats (n=3).





237x146mm (96 x 96 DPI)