ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Preparation of *ß*-carotene Enriched Nanoemulsion by Spontaneous Emulsification Using Oleic Acid as Nano Carrier.

Heba H. Salama, M.M. El-Sayed, and M.H. Abd El-Salam*.

Dairy Department, National Research Centre, 33 El Bohouth St. (former El Tahrir st.), Dokki, Giza, Egypt, P.O. 12622.

ABSTRACT

Nanoemulsions based on oleic acid: Tween 80 (O/T) mixtures (2:8, 3:7 and 4:6) were prepared by spontaneous emulsification in 0.05 M phosphate buffer. Laser scattering showed that these emulsions had an z-average diameter of 138.2, 158.6, and 205.6 nm, polydispersity index (PDI) ~ 0.4 and zeta potential of -9.33±8.81, -15.9±8.75 and -18.9±9.70 respectively. The emulsions were stored at room temperature for 4 weeks and the ratio of turbidity of the upper and lower layer was measured as a qualitative index of the stability of the emulsion. The pH and O/T had no significant effect (P>0.05) on the stability of the emulsions. Transmission electron examination of 2: 8 (O/T) emulsion revealed spherical particles sizes ranging from 53.2 to 92.5 nm. The prepared emulsions were used as a nano carrier for *ß*-carotene as model lipophilic bioactive ingredient. The stability and photo stability of *ß*-carotene nanoemulsion were evaluated over a storage period of four weeks.

Keywords: nanoemulsion, spontaneous emulsification, oleic acid, *ß*-carotene, particle size, zeta potential, photo stability

Corresponding author

ISSN: 0975-8585

INTRODUCTION

An emulsion is a stable two phase system of immiscible liquids of which one is dispersed as fine droplets in the other liquid. Emulsions are usually classified according to the sizes of the dispersed droplets which determine mainly the stability, uses and the physicochemical properties of the emulsion. Emulsions with droplet sizes less than 500 nm are termed nanoemulsions, mini emulsions, ultrafine emulsions, submicron emulsions and translucent emulsions [1]. The growing interest in nanoemulsions can be understood from their potential use as stable delivery systems with high bioavailability for food bioactive compounds in functional foods and beverages. Nanoemulsions can be generated by two groups of methods namely: high-energy and low-energy methods [2]. In the high-energy methods a mixture of the two liquids containing small amount of surfactant (<0.1%) is subjected to high pressure (>50 MPa) homogenization or microfluidization, while in the low-energy methods a mixture of oil and surfactants (O/S ratio >0.5) is mixed with the aqueous phase using continuous stirring [3]. The low-energy methods have the advantage of potential scaling up to industrial use without the need for expensive equipment, and to encapsulate sensitive bioactive compounds under mild conditions. Several factors have been reported to affect the physicochemical properties and stability of nanoemulsions prepared by spontaneous emulsification **(SE)** including the type of oil as dispersed phase [4], type and percentage of surfactant and the ratio of oil to surfactant [5]. The most widely oil used in the preparation of nanoemulsions by **SE** has been medium chain triglyceride instead to the common edible oils which are difficult to generate stable nanoemulsions from them. Low-energy methods include **SE** and phase inversion; the former has been used recently to encapsulate several lipophilic bioactive compounds, but designing and factors affecting the prepared nanoemulsions depend on the bioactive compound to be encapsulated. To optimize the system composition and preparation conditions of vitamin E-loaded in **SE** nanoemulsions, Saberi *et al*., [6] studied the effect of surfactant to oil, vitamin E to medium chain triglycerides ratio (oil phase composition), type of surfactant (Tween 20, 40, 60, 80 and 85), and temperature and stirring speed used in the preparation of the nanoemulsion. Nanoemulsions were prepared from mixtures of carvacrol (an essential oil) and medium chain triglycerides (carrier oil) and Tween surfactants by **SE** [7]. The smallest droplets (~ 55 nm) were formed with the use of carvacrol: carrier oil ratio of 1:3 and with the use of Tween 80 as surfactant. Mixing fish oil with lemon oil or medium chain triglycerides at ≥ 50% and ≥40% respectively yielded small droplet size (< 200 nm) by **SE** [8]. A surfactant (Tween 80) to lemon oil ratio of 0.75 was found necessary to get small size droplets. The use of glycerol as co-solvent in the aqueous phase gave better results than ethanol and polypropylene glycol. At glycerol concentration of >40% gave droplet of small size.

The droplet size was also decreased with the increase of the concentration of the surfactant. The droplet size of vitamin E-loaded nanoemulsions prepared under the optimized conditions was smaller than 50 nm. Addition of hydroxypropyl methylcellulose and heat treatment were found to increase the retention and bioaccessability of the hydrophobic nutraceutical "nobiletin" loaded in nano-emulsion prepared by **SE** without affecting the physical properties and stability of the emulsion [9]. A high surfactant to oil ratio (≥ 1.25) was found necessary to prepare fish oil nano-emulsion that remained stable for 14 days at 37ºC, but storage at higher temperatures resulted in some growth of the droplet size [10]. The droplet size and the concentration of the surfactant used had no significant effect on the lipid oxidation. Resveratrol was encapsulated in nanoemulsions delivery system based on mixture of orange oil and grape seed oil prepared using **SE** method [11]. Small droplets (<100 nm) with good stability were obtained with the use of orange oil to grape seed oil ratio of 1:1. Encapsulation improved the chemical stability of resveratrol after exposure to UV-light.

During the search for food grade hydrophobic liquid that can be used in the preparation of emulsions by **SE**, oleic acid (C18:1n-9) was chosen for its physicochemical properties and potential health benefits. Although oleic acid has both hydrophobic and hydrophilic sides, it has a very low hydrophilic /lipophilic balance (**HLB**) ~ 1 and low water solubility but its droplets may show a rather high stability in water [12]. Oleic acid has been considered as an anti-inflammatory fatty acid involved in the activation of different pathways of immune competent cells and diets rich in oleic acid had beneficial effects in inflammatory related diseases [13]. Also, oleic acid was reported to exhibit several positive impacts on various tissues, the most pronounced of which is on the cardiovascular system. Oleic acid was reported to decrease the myocardial infarction rate, platelet aggregation and secretion of thromboxane A2 (TXA2), reduction of the systolic blood pressure and decreasing the level of low density lipoprotein (LDL) cholesterol [14].

Early, oleic/paraffin oil mixture was reported to generate fine vesicles in aqueous alkaline solutions [15] but no attempts have been cited on the use of oleic in the preparation of food grade nanoemulsions. Oleic

acid is almost immiscible with aqueous solutions at low pH value. Changing pH from alkaline to acidic range resulted in changes of oleic acid emulsion from the micellar to the vesicle structure [16]. Recently, oleic has been used as a base for nano emulsification of Lumefantrine, a drug of poor solubility in aqueous solutions [17]. The enhanced dissolution of Lumefantrine has been attributed to its formation of ionic complex with oleic acid. Therefore, oleic can offer the advantage of the formation of nano delivery system for active compounds through hydrophobic interaction or ionic complexation.

The aim of the present paper was (1) to select the optimum conditions for the preparation of stable oleic acid nanoemulsion (2) the use of the prepared nano-emulsion for the delivery of *β*-carotene as a model of lipophilic bioactive compounds.

MATERIALS AND METHODS

Materials

- Oleic acid, polyoxyethylene sorbitan monooleate (Tween 80) and *β*-carotene were obtained from Sigma Inc (St. Luis, Mo)
- Mono and di sodium phosphate (Merck, Germany) were used in the preparation of 0.05 M phosphate buffers pH 5 and 7 respectively.

Preparation of oleic acid nano-emulsions

Oleic acid was mixed with Tween 80 (O/T) at the ratios of 2:8, 3:7 and 4:6 respectively. Five ml of the O/T mixtures were added drop wise to 95 ml of phosphate buffer at 50°C with continuous stirring at 1500 rpm and kept in stoppered tubes until analyzed.

β-carotene was dissolved in O/T mixtures at the ratios of 2, 5, and 10 mg/ml. *β*-Carotene loaded nanoemulsions were prepared from *β*-carotene containing O/T mixture the same as described above.

Storage stability of the prepared emulsions

The storage stability of nanoemulsions containing *β*-carotene was investigated in two-independent factorial design (time x O/T ratio) for each concentration of *β*-carotene. Emulsions were stored at room temperature (25 ± 5°C), for four weeks and analyzed at weekly intervals for creaming by turbidity measurement at 600 nm and for *β*-carotene by absorbance at 450 nm.

Aliquots of 100 µl were taken from the upper layer (2 cm from surface) and middle layer of the emulsion, diluted with 5 ml distilled water and the turbidity of the diluted solutions was measured at 600 nm. The emulsion stability was calculated from the equation

$$\text{Emulsion stability\%} = \frac{\text{Absorbance of the upper layer}}{\text{Absorbance of middle layer}} \times 100$$

Values close to 100% indicate good emulsion stability

Changes in *β*-carotene were followed by measurement of the absorbance of a diluted emulsion solution at 450 nm.

Electron microscopy of nanoemulsions

Freshly prepared nanoemulsion was diluted with distilled water at the ratio of 1:10. A ready carbon coated copper grid was dipped in the diluted emulsion solution, removed and left to dry at room temperature. The size, shape and surface morphology of the emulsion droplets were examined by transmission electron microscope (TEM, JEOL, JEM-1230, Japan) at different magnifications using an electron beam of 100 kV. At least 10 sites were examined in each grid.

Measurement of particle size and zeta potential

The size and zeta potential of the droplets in the emulsions were determined using Zetasizer var. 704 instrument (Malvern Instruments, Malvern, UK). Sample was diluted with ultrapure MQ water before measurement its light scattering for a laser beam (633 nm) at an angle of 173 at 25˚C over time intervals. The changes in laser beam scattering versus time was used to determine the particle size distribution. The mean particle diameter (the scattering intensity-weighted mean diameter, Z-average) and polydispersity index (PDI) were calculated from the particle size distribution. The PDI value provides a measure of the narrowness of the particle size distribution, with values ≤0.1 indicating a very narrow distribution [6].

Zeta potential was measured in the same sample by electrophorsis and results were expressed as mV.

Statistical analysis

Data were analyzed by ANOVA for two independent variables design using Vassarstats computing web site [18].

RESULTS AND DISCUSSION

Effect of pH and oleic/Tween 80 ratio

Emulsions were prepared at oleic: Tween 80 ratios of 2:8, 3:7 and 4:6 respectively at pH 5 and 7 respectively. All preparations gave emulsions of good stability over a storage period of 4 weeks as measured by turbidity (Table1). The opacity of the emulsions increased significantly ($P<0.01$) with the increase of oleic acid in the mixture, mainly due to the increase in the droplet population. Also, the pH affected slightly but not significantly ($P>0.05$) the turbidity of the emulsions. Those emulsions prepared at pH 7 had slightly lower turbidity than that prepared at pH 5 which can be attributed to changes in the structure of the formed emulsions [16]. The ratio of the turbidity of the upper layer to the lower layer of the emulsion was taken as an index for stability of the emulsion. A ratio of 100% was considered to be the maximum stability. Emulsions prepared from different mixtures of oleic/Tween exhibited high turbidity ratios (~90%) over a storage period of 4 weeks and the differences between emulsions prepared at the different pH used were not significant ($P>0.05$). This indicated that all prepared emulsions retained an excellent stability during storage.

Table 1 : Changes in the upper/lower turbidity ratio (%) of emulsions as affected by oleic: Tween 80 ratio and storage period.

Oleic : Tween 80 ratio	pH value	Storage time (week)			
		1	2	3	4
2 : 8	5	90.2	114.2	95.2	97.9
	7	93.7	100.7	98.7	95.2
3:7	5	89.3	91.2	92.3	90.6
	7	91.2	77.7	91.3	61.2
4:6	5	97.3	99.2	99.8	98.2
	7	93.8	98.1	97.9	98.9

Electron microscopic (EM) examination of the prepared emulsion (2:8 oleic/Tween ratio, pH 7) revealed spherical droplets of sizes that ranged from 53.2 to 92.5 nm (Fig 1). Measurement of particle sizes by light scattering revealed average sizes of 138.2 nm, 156.6 nm and 205.6 nm for 2:8, 3:7, and 4:6 emulsions respectively and polydispersity index of 0.39, 0.39 and 0.41 in order (Table 2). This indicates that spontaneous emulsification gave oleic acid emulsions with droplet in the nano size range. Also, the increase of oleic acid in the mixture increases slightly the average size of the particles. Previous studies [7, 8] showed that the increase in the percentage of the oil phase increase the droplet size in agreement with the present findings. It is of interest that the measured sizes by EM were less than that measured by light scattering intensity. Bootz *et al.* [19] found that the sizes of nano particles measured with electron microscope were less than that obtained by

laser light scattering in agreement with the present results. The size distribution of the formed nano-emulsions was also, affected by the oleic: Tween 80 ratio. The droplet sizes of the different emulation followed a normal distribution pattern (Fig 2) with more narrow range of size distribution was observed in the 2:8 emulsion than that made with higher oleic: Tween 80 ratios.

Table 2: Z-average size, polydispersity index (PDI) and zeta potential of oleic acid emulsions and oleic acid emulsions loaded with *β*-carotene (2 mg/ml) of different oleic : Tween 80 ratios.

Oleic : Tween 80 ratio	Plain emulsion			Emulsion loaded with *β*-carotene		
	z-average (nm)	PDI	Zeta potential (mV)	z-average (nm)	PDI	Zeta potential (mV)
2 : 8	138.2	0.39	-9.33±8.81	129.6	0.41	-19.1±5.74
3 : 7	158.6	0.39	-15.9±8.75	272.2	0.37	-24.1±6.55
4 : 6	205.6	0.41	-18.9±9.70	240.6	0.62	-25.8±5.20

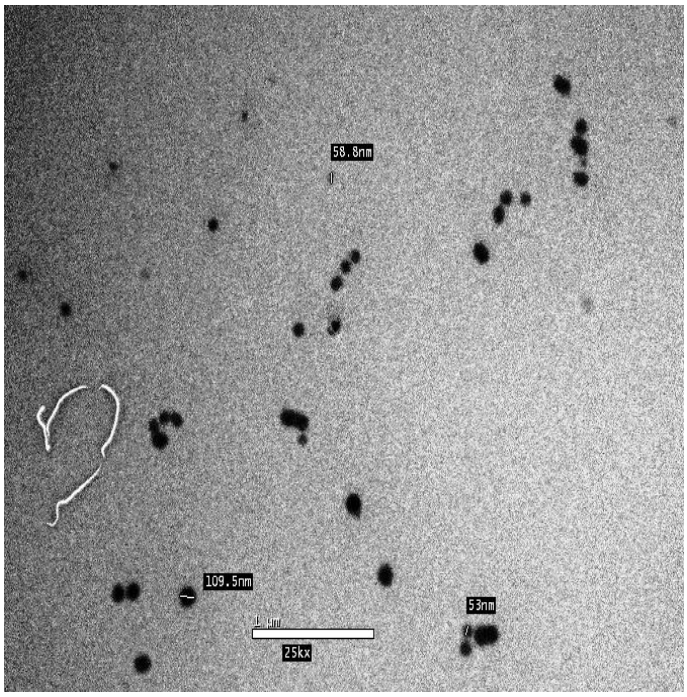

Fig 1: Electron microscope micrograph of oleic acid emulsion (2:8 oleic: tween 80) at pH 7

The zeta potential of the emulsions increased from -9.33 ± 8.81 mV in 2:8 emulsions to -15.8 ±8.75 and -18.9 ±9.70 mV for 3:7 and 4:6 emulsions respectively (Table 2). This indicated that the increase in oleic acid content increased the zeta potential of the emulsion. The oleic acid molecules were probably arranged to keep the carboxyl group at the apex of the droplet while the hydrophobic backbone of the acid formed the droplet core. This may explain the increase in the zeta potential with the increase in the oleic acid content in the emulsion. Fukuda *et al.* [16] reported that oleic acid to form vesicles in aqueous solution at pH <5.

***β*-carotene enriched nanoemulsion**

β-carotene was dissolved in oleic acid: Tween 80 mixtures at the ratios of 2, 5 and 10 mg/ml before the preparation of the emulsions. *β*-Carotene enriched nanoemulsion (2:8) retained smaller droplet sizes after four weeks of storage, while the 3:7 and 4:6 *β*-carotene loaded nanoemulsions exhibited slightly higher droplet size (Table 2) than the unloaded nanoemulsion indicating that *β*-carotene had a slight but not significant (P>0.05) effect on the droplet size. The *β*-carotene loaded emulsions 2:8 and 3:7 showed comparable PDI to that of unloaded emulsions being 0.41 and 0.37 respectively while *β*-carotene loaded 4:6 emulsion showed PDI of 0.6 being more broad size distribution (Fig 3) than the unloaded emulsion (Fig 2). Loading the emulsions

with *β*-carotene also, increased their zeta potential. This suggests that more carboxyl groups of the oleic acid occupied the surfaces of the droplet.

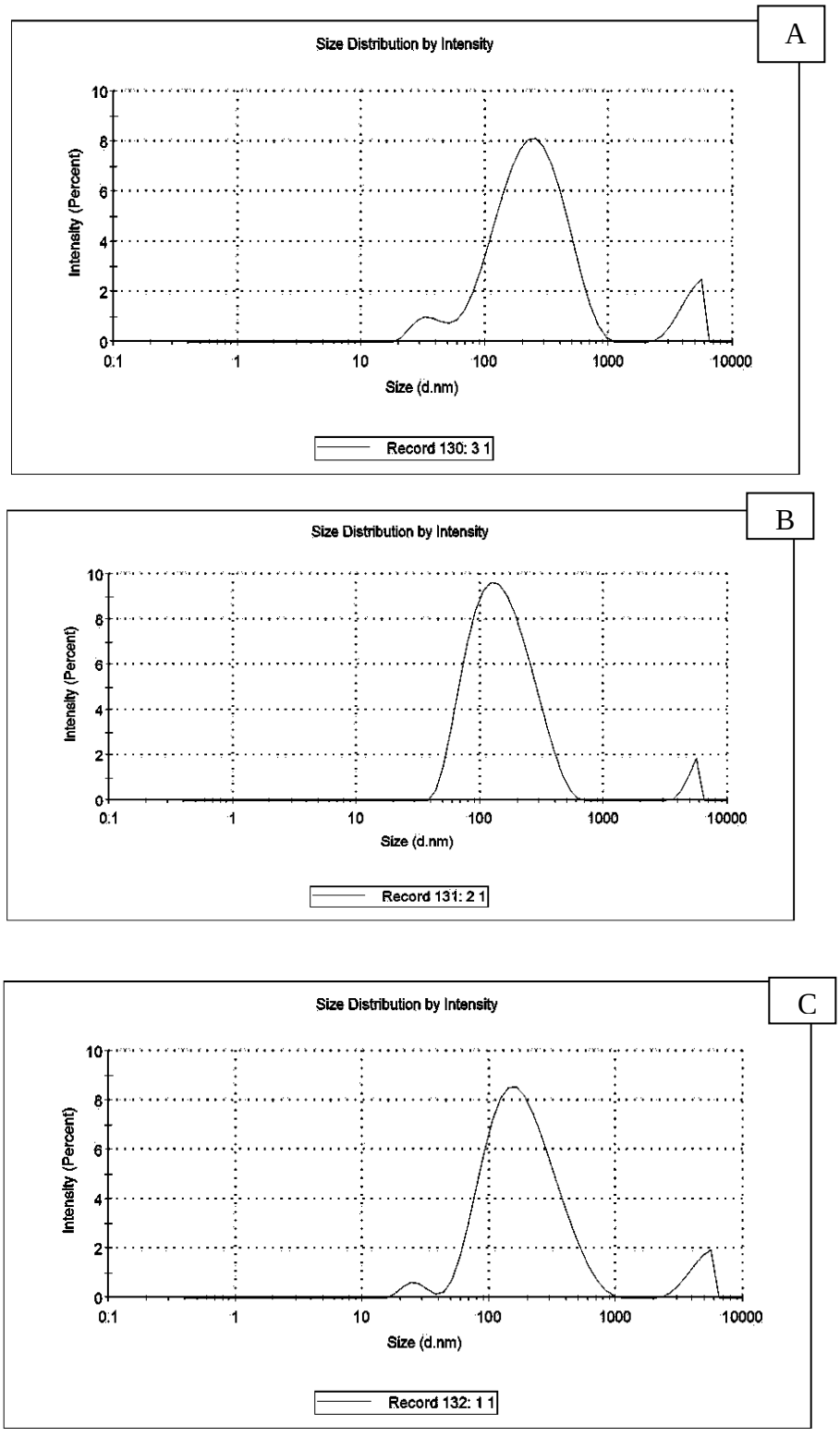

Fig 2: Particle size distribution of 2:8 (A), 3:7 (B) and 4:6 (C) oleic acid emulsion at pH 7

ISSN: 0975-8585

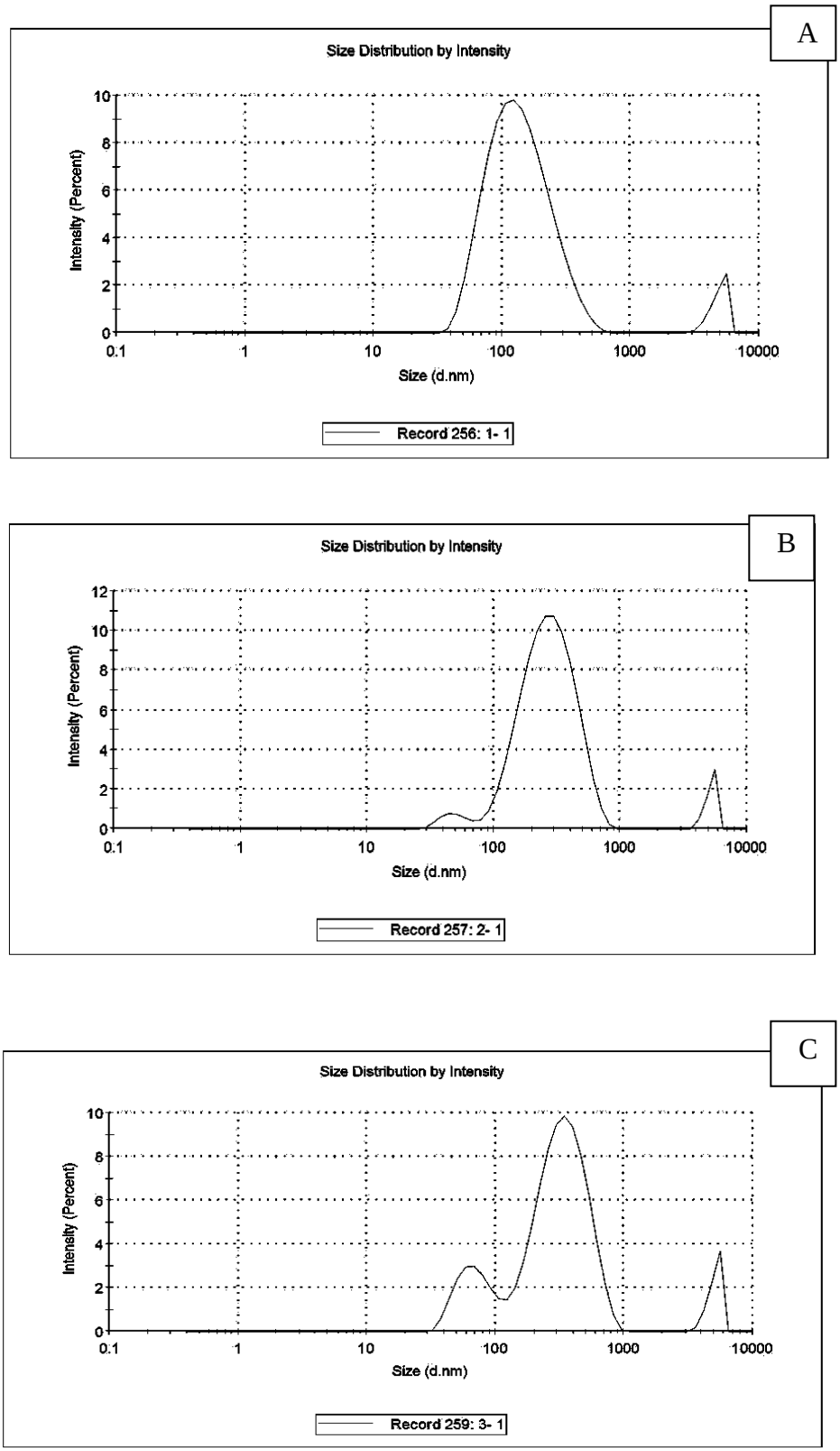

Fig 3: Particle size distribution of loaded 2mg/ml *β*-carotene (A) 2: 8 (B) 3:7 and (C) 4: 6 oleic/tween 80 emulsions respectively at pH 7

Table 3 shows that the turbidity of the emulsions from the different treatments increased with advanced storage. Kamogawa *et al* [20] reported slow growth in oleic acid emulsion during storage which may explain the observed increase of turbidity in the present study. Also, the changes in *β*-carotene content of the emulsion were followed by measuring the absorbance at 450 nm (maximum absorbance of *β*-carotene) (Table

4). The observed increase in absorbance during storage can be attributed to the interference from the turbidity. This indicates that *β*-carotene retained good stability during storage.

Table 3 : Changes in turbidity (as OD at 600 nm) of emulsions loaded with *β*-carotene as affected by oleic/tween 80 ratio and storage period.

Oleic : tween 80 ratio	*β*-carotene (mg/ml)	Storage time (week)			
		1	2	3	4
2 : 8	2	0.129	0.127	0.126	0.067
	5	0.201	0.223	0.185	0.063
	10	0.189	0.168	0.154	0.925
3:7	2	0.316	0.295	0.311	0.151
	5	0.613	0.463	0.432	0.257
	10	0.594	0.584	0.581	0.798
4:6	2	0.490	0.457	0.528	0.438
	5	0.939	0.844	0.859	0.555
	10	1.068	1.877	1.944	1.237

Table 4: Changes in the carotene content (as OD at 450 nm) as affected by oleic: Tween 80 ratio and storage period

Oleic : Tween 80 ratio	*β*-carotene (mg/ml)	Storage time (week)			
		1	2	3	4
2 : 8	2	0.207	0.202	0.209	0.209
	5	0.292	0.222	0.328	0328
	10	1.059	0.190	0.233	0.359
3:7	2	0.517	0.460	0.466	0.483
	5	0.588	0.564	0.655	0.784
	10	1.125	0.760	0.847	0.979
4:6	2	0.939	0.795	0.728	0.760
	5	1.010	1.102	1.145	1.246
	10	1.673	2.281	2.203	1.504

CONCLUSION

Oleic acid/Tween 80 mixtures of ratios ranging from 2:8 up to 4:6 can be used for the preparation of nanoemulsion by spontaneous emulsification. The prepared emulsions had good stability. Loading *β*-carotene in these emulsions indicated that these nanoemulsions can be used effectively as delivery systems for lipophilic nutraceuticals. Further studies are needed to explore the potential use of the prepared oleic acid emulsion loaded with *β*-carotene in some dairy products.

REFERENCES

[1] Fernandez P, Andr´e V, Rieger J and Kühnle, A. Coll. Surf. A: Phys. Eng. Asp., 2004; 251, 53–58.
[2] Anton N, Benoit, JP and Saulnier P. J. Con. Release, 2008; 128: 185–199.
[3] Yang Y, Marshall-Breton C, Leser ME, Sher AA and McClements DJ. Food Hydro., 2012; 29: 398 - 406.
[4] Wooster T J, Golding Ma nd Sanguansri P. Langmuir, 2008; 24: 12758–12765.
[5] Guttoff M, Saberi AH and McClements DJ. Food Chem., 2015; 171: 117–122.
[6] Saberi AH, Fang Y and McClements DJ. J. Coll. Interf. Sci., 2013; 391: 95–102.
[7] Chang Y, McLandsborough L and McClements DJ. J. Agric. Food Chem., 2013; 61: 8906−8913.
[8] Gulotta A, Saberi AH, Nicoli MC and McClements DJ. J. Agric. Food Chem., 2014; 62: 1720-1725.
[9] Chen H, An Y, Yan X, McClements DJ, Li B. and Li Y. Food Hydro., 2015; 51: 395-404.

[10] Walker RM, Decker E A and McClements DJ. J. Food Eng., 2015; 164: 10–20.
[11] Davidov-Pardo G and McClements DJ. Food Chem., 2015; 167: 205–212.
[12] Kamogawa K, Okudaira G, Matsumoto M, Sakai T, Sakai H and Abe M. Langmuir, 2004; 20: 2043-2047.
[13] Carrillo C M, Cavia del M and Alonso-Torre S. Nutr. Hosp., 2012; 27: 978-990.
[14] Karacor K and Cam M. Med. Sci. Discov., 2015; 2: 125-32.
[15] Mansfield W W. Aust. J. Sci. Res., Ser. A: Phys. Sci., 1952; 5: 331-337.
[16] Fukuda H, Goto A, Yoshioka H, Goto R, Morigaki K and Walde P. Langmuir, 2001; 17: 4223-4231.
[17] Patel K, Sarma V and Vavia P. DARU J. Pharm. Sci., 2013; 21: 27.
[18] Lowery 2009; Vassarstats (http://faculty.vassar.edu/lowry/anova2corr.html)
[19] Bootz A, Vogel V, Schubert D and Kreuter J. Eur. J. Pharm. Biopharm., 2004; 57: 369-75.
[20] Kamogawa K, Akatsuka H, Matsumoto M, Yokoyama S, Sakai T, Sakai H and Abe M. Coll. Surf. Phys. Eng Asp., 2001; 180: 41-53.