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RESEARCH ARTICLE

Characterization of β-Carotene Nanoemulsions Prepared by Microfluidization Technique

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Abstract In the present work, β-carotene nanoemulsions, as potential active ingredients for liquid food, were prepared using high pressure homogenization. The influence of different homogenizing conditions (pressure and number of cycles) and emulsifier type and concentrations on particle size parameters and content of β-carotene was investigated. The droplet size of the emulsions was found to decrease from 416.0 to 97.2 nm with increasing microfluidization pressure, number of cycles, and emulsifier concentration. The optimum conditions for preparing β-carotene nanoemulsions were determined to be homogenization pressure of 120 MPa and 3 cycles. The storage study showed that the nanoemulsions were physically stable for about 5 weeks at room temperature (25°C). β-Carotene degradation was considerably slower in WPI-stabilized nanoemulsions than in Tween 20-stabilized ones, which was attributed to the increased surface area. These results have important consequences for the design and utilization of food-grade nanoemulsions.

Keywords: nanoemulsion, mircrofluidizer, β-carotene, emulsion stability, droplet size

Introduction

Bioactive lipids, such as carotenoids, fatty acids, and natural antioxidants, are widely used as active ingredients in various food products. Carotenoids are a class of natural pigments which are mainly found in fruits and vegetables. In recent years, researchers have proposed that carotenoids are beneficial to human health, protecting and preventing against a number of health disorders such as cancer, cardiovascular disease, muscular degeneration, and cataracts. β-Carotene is an important member of the carotenoids family that has received particular attention owing to its high provitamin A activity and antioxidant capacity (1-3). For these reasons, there is a strong interest in using β-carotene and other carotenoids as functional ingredients in food industry. However, β-carotene is insoluble in water and is weakly soluble in oil at room temperature due to its crystalline form, thus making it difficult to incorporate into many food formulations and with poor bioavailability. In addition, β-carotene is sensitive to oxygen, heat, and light, which further limits its appropriate application within the food industry (4,5).

A strategy to improve the solubility and bioavailability of β-carotene involves the preparation of oil-in-water (O/W) emulsions with droplet sizes in the sub-micrometer range. In the last two decades, nanotechnology has rapidly emerged as highly promising and attractive research fields. Nanotechnology offers the potential to significantly improve the solubility and bioavailability of many functional ingredients including carotenoids, polyunsaturated fatty acid, and phytosterols (6-9). Similar to conventional emulsions, nanoemulsions are thermodynamically unstable systems that tend to breakdown over time. Nevertheless, they have some significant advantages over conventional emulsions: they can greatly increase the bioavailability of lipophilic compounds; they have small particle sizes ranging from 50 to 200 nm, and presenting as transparent or translucent; and they are not prone to particle aggregation and gravitational separation. As a consequence of these various benefits, nanoemulsions have become increasingly important vehicles for hydrophobic bioactive substances through the formation of nano-dispersions (10).

Nanodispersions containing lipophilic bioactive substances

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can be produced various emulsification processes such as solvent displacement (2,11), emulsification-evaporation (4,7), and high pressure homogenization (12,13). High pressure homogenization is widely used in the food, pharmaceutical and biotechnology industries to emulsify, disperse, mix, and process various products. Microfluidization technology, a type of high pressure homogenization, is currently used by the pharmaceutical and cosmetic industries as well as the food industry to produce fine emulsions with narrow size distribution. In the microfluidization techniques, shearing caused by velocity gradient, impact by collision and cavitation by sharp pressure drop are the main forces which account for the disruption of oil droplets in forming fine emulsions. Some researchers believe microfluidization to be superior because, size distributions are often narrower and smaller in microfluidized emulsions than those created by traditional emulsifying devices (14-17).

Emulsifiers play a major role in the formation of emulsions in aqueous solutions. They decrease the interfacial tension between the oil and water phases, reducing the amount of energy required to disrupt the droplets and leading to smaller size droplets. Moreover, they form a protective coating surrounding the droplets thus preventing coalescence and aggregation (18). Polyoxythylene sorbitan esters (series of tweens) are non-ionic emulsifiers which adsorbs quickly at the oil-water interface, and have shown good results in small particles formation for various applications, including nanoemulsions (11,19,20). In addition, food proteins, such as whey protein isolate (WPI), soybean protein isolate (SPI) and sodium caseinate (SC), are widely used as food ingredients as they are readily adsorbed at the oil-water interface and provide stability to dispersed systems through a combination of electrostatic repulsion and steric stabilization (21,22).

In this work, β-carotene nanoemulsions were prepared with Tween 20, Tween 80, WPI, SPI and, SC using microfluidization technique. The influence of emulsifier type and concentration, as well as the microfluidization conditions of pressure and number of cycles on the physical properties and stability of the nanoemulsions were investigated. In addition, the oxidation stability of the nanoemulsions during storage were evaluated over a period of five weeks.

Materials and Methods

Materials β-Carotene (30% in corn oil) was purchased from DSM Nutritional Products Ltd. (Basel, Switzerland). Standard β-carotene (>95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and medium chain triglyceride (MCT) oil was provided by SHS International Ltd. (Liverpool, UK). Polyoxyethylene sorbitan monolaurate (Tween 20) and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Junsei (Tokyo, Japan). WPI was obtained from Sungpoong (Asan, Korea), SPI was purchased from VIXXOL Co. (Ansan, Korea) and SC was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Preparation of β-carotene nanoemulsions β-Carotene nanoemulsions were prepared using MCT oil containing β-carotene as the dispersed phase and aqueous emulsifier solutions as the continuous phase. Due to its poor solubility in oil at room temperature, one g of β-carotene (30% in corn oil) was dissolved in 29 g of MCT oil at 140°C for several seconds. The ratio of the dispersed phase to continuous phase was set at 3:97 (w/w) to give a final β-carotene concentration of approximately 0.03%(w/w) in the emulsion. The nanoemulsions were produced by a two stage homogenization procedure to obtain fine droplets of emulsion. The pre-emulsions were obtained with a high-speed mixer (Ultra-Turrax T 18; IKA Works, China) at 11,000 rpm for 3 min. Then, the corse emulsions were further emulsified using a microfluidizer (Model M-110L; Microfluidics, USA). Using the above procedure, nano-emulsions were prepared by varying the emulsifier type and concentration, and homogenization pressure and cycle to investigate their effects on the physical properties of the emulsions. The freshly prepared emulsions were sampled and their particle size, size distribution and ζ-potential were measured immediately. For storage tests, the nano-emulsions were stored in screw-capped amber bottles at room temperature (25°C) for 5 weeks. At the end of each week, the emulsions were sampled and the β-carotene concentration and particle size were determined.

Analysis of particle size and zeta-potential The average particle size, size distribution and ζ-potential of nanoemulsions were determined by dynamic light scattering using a Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK). All samples were diluted approximately 10 times with distilled water prior to each measurement to avoid multiple light scattering effects. The particle size of the nanoemulsions was described by the mean (z-average) diameter, and the size distribution was indicated by the polydispersity index (PdI). Since ζ-potential is directly related to the electrophoretic mobility of the particles, the analyzer calculates ζ-potential from the measured velocity. All measurements were taken in triplicate.

Analysis of β-carotene content β-Carotene content in nanoemulsion was determined by high performance liquid chromatography (HPLC) using a Varian 900-LC series HPLC (Varian, Harbor City, CA, USA) equipped with UV-VIS detector. The sample preparation procedures were

modified from those of Mao *et al.* (16). β-Carotene was first extracted with ethanol and *n*-hexane from the nanoemulsion, and the *n*-hexane solutions were then appropriately diluted with *n*-hexane. The diluted *n*-hexane solutions were sparged with nitrogen gas to remove *n*-hexane, and the remaining solids were dissolved in 1 mL of mobile phase [acetonitrile-ethanol (7:3, v/v)]. Samples were separated on a ZORBAX Eclipse Plus C18 column (4.6×250 mm, 5 μm particle size) (Agilent tech., Palo Alto, CA, USA) maintained at 40ºC. The flow rate was 1.6 mL/min, and 50 μL aliquot of sample was injected. The concentration of β-carotene in the samples was obtained by referring to the standard curve of concentration versus peak area (linear range from 0 to 50 mg/L, R^2=0.9972).

Statistical analysis The data were analyzed by ANOVA using SAS statistical program 9.2 (SAS Institute, Cary, NC, USA). The significant difference (p<0.05) between means was further determined by Duncan's multiple range tests. All the reported values were the means of at least three measurements.

Results and Discussion

Effect of microfluidization conditions on the physical properties of β-carotene nanoemulsions Figure 1 shows a typical profile of particle size distribution in the β-carotene microemulsions and nanoemulsions. A high speed mixer produced bigger droplets (>1 μm) with larger size distribution and a bimodal shape. However, the microfluidizer gave smaller droplets and a narrower distribution because of the more efficient droplet disruption (cavitation along with shear and inertia forces) and the higher energy density (15).

Microfluidizer pressure and cycling number can significantly influence the physical properties of emulsions as the high intensity of the shear forces and turbulence, which are pressure dependent, produced during microfluidization can affect the particle size and size distribution (13,23). The effect of microfluidization pressure on the properties of the β-carotene nanoemulsions were studied at different pressures, from 20 to 120 MPa, by passing through the device for 1 to 3 cycles at each pressure, while maintaining sufficient emulsifier concentration (Tween 20 and 80, 10%) to cover all the droplets produced. As shown in Fig. 2, the mean diameter of the nanoparticles was between 97.2 nm and 228.3 nm, with the PdI values being in the range of 0.19-0.34. The mean size of β-carotene nanoemulsions significantly (p<0.05) decreased with the increment of homogenization pressure over the entire pressure ranges, which agreed with the findings of other researchers (7,13). These results show that the intensity of the high shear forces, and the turbulence and/or cavitation produced during the homogenization process determine particle size. However, the effect of pressure on the particle size distribution did not show a consistent trend. The PdI value measures the spread of the particle size distribution and, thus, a small PdI value indicates a narrow size distribution. In general, the β-carotene nanoemulsions all exhibited a relatively narrow range of size distribution, with PdI values less than 0.34 (0 being the smallest and 1 being the largest possible values).

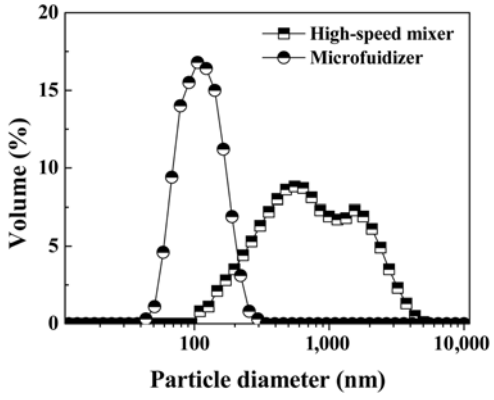

Fig. 1. Particle size distribution of β-carotene nanoemulsions prepared with 10% Tween 20 through high-speed mixer at 11,000 rpm for 3 min and microfuidizer at 120 MPa and 3 cycles.

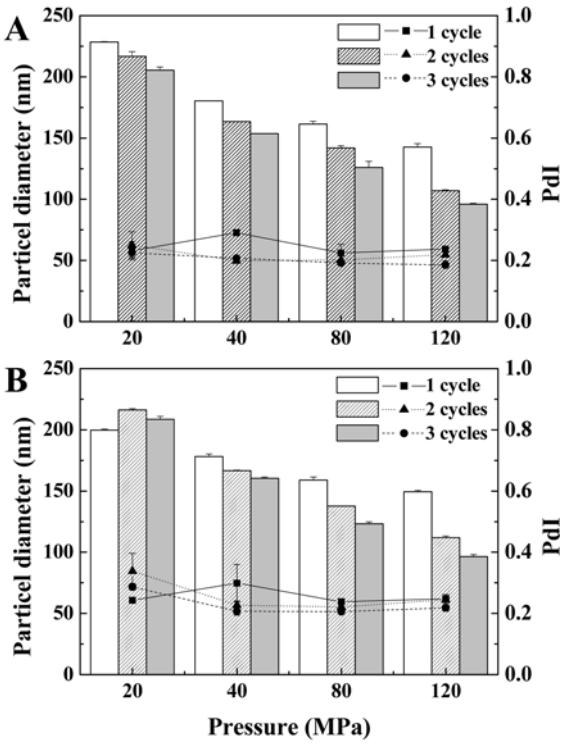

Fig. 2. Effects of microfuidization pressure and cycle on the droplet size of β-carotene nanoemulsions prepared with Tween 20 (A) and Tween 80 (B) at a concentration of 10%(w/w).

The influence of microfluidization cycle on the particle size of nanoemulsions was also studied. As expected, the mean particle diameters significantly ($p<0.05$) decreased with increasing microfluidization cycles applied at each pressure. The number of microfluidization cycles did not have any significant effect on the size distribution. In general, a high homogenization pressure ensures a good emulsification process, and therefore, leads to smaller particles with a satisfactory PdI value (lowere than 0.6). The above results showed that at the homogenization pressure of 120 MPa and 3 cycles, the smallest particles were produced. These microfluidization conditions were therefore used in subsequent experiments to study the effect of other emulsifying conditions on the properties and stability of β-carotene nanoemulsions.

Effect of the type and concentration of emulsifiers on the physical properties of β-carotene nanoemulsions

Table 1 shows the droplet properties of the β-carotene nanoemulsions prepared with various emulsifiers of different concentration at constant microfluidizer conditions (120 MPa and 3 cycles). In this work, the particle size and size distribution of the nanoemulsion were unimodal, and typically extended from 97 to 416 nm, with the PdI value being below 0.6, except for one sample. In general, droplet sizes of nanoemulsions prepared with Tweens were smaller than those stabilized by proteins. Small molecule emulsifiers, such as Tween 20 and Tween 80, can be easily adsorbed onto the droplet surface, with a consequent reduction in the interfacial tension, which favors the formation of small droplets. In contrast, proteins with large molecular structures lower adsorption on the β-carotene particles, resulting in less reduction of interfacial tension. Thus, emulsifier like proteins could lead them to coalescence and creaming.

Of the two different Tween emulsifiers, Tween 20 produced nanoemulsions with smaller particle sizes at all the concentrations (4%-12%). At the emulsifier concentration of 10%, the nanoemulsion emulsified with Tween 20 showed the smallest droplet size of any experimental batch (97 nm) (Table 1). These results can be explained by the different hydrophilicity of the emulsifiers. As shown by Yuan *et al*. (13), emulsifiers with larger hydrophilicity can wrap and stabilize the particles in an O/W emulsion more efficiently, thus, resulting in smaller particles. The hydrophilicity of emulsifiers is usually measured by their hydrophilic-lipophilic balance (HLB) values, with higher numbers indicating larger hydrophilicity (18). The HLB values of Tween 20 and 80 were 16.7 and 15.0, respectively. The higher HLB values of Tween 20 could be responsible for the smaller particle sizes in the emulsions prepared with this emulsifier. Nanoemulsions prepared with Tween 20 was selected arbitrarily for further study of the stability of β-carotene emulsions during storage.

Table 1. Physical properties of β-carotene nanoemulsions prepared with different emulsifiers at different concentration (mean±SD, *n*=3)[1)]

Emulsifier	Property	Concentration (%)				
		4	6	8	10	12
TW20	*D* (nm)[2)]	118.6±6.161	111.7±2.562	105.7±1.497	97.2±2.286	100.9±1.720
	PdI[3)]	0.196±0.017	0.194±0.010	0.194±0.012	0.188±0.011	0.176±0.009
	ζ-Potential (mV)	-9.50±0.944	-8.79±1.196	-8.62±1.395	-8.32±2.056	-7.59±0.777
TW80	*D* (nm)	127.3±1.000	120.7±0.005	136.5±0.424	116.2±2.051	118.2±0.919
	PdI	0.202±0.020	0.220±0.005	0.223±0.012	0.253±0.013	0.204±0.065
	ζ-Potential (mV)	-12.40±0.282	-10.35±0.071	-11.45±0.919	-10.09±0.573	-10.255±0.912
Emulsifier	Property	Concentration (%)				
		0.1	0.5	1	3	6
WPI	*D* (nm)	368.6±13.514	174.3±2.510	162.1±2.427	165.8±1.868	175.3±1.701
	PdI	0.499±0.388	0.791±0.066	0.272±0.008	0.295±0.028	0.245±0.024
	ζ-Potential (mV)	-38.9±2.475	-34.6±1.598	-27.1±0.416	-22.4±0.603	-20.1±0.557
SPI	*D* (nm)	416.0±21.108	265.3±3.157	230.4±4.105	264.1±3.233	309.8±2.663
	PdI	0.671±0.455	0.422±0.107	0.312±0.051	0.346±0.095	0.245±0.016
	ζ-Potential (mV)	-45.9±2.214	-40.7±1.692	-38.6±0.529	-33.3±0.458	-31.5±0.929
SC	*D* (nm)	335.0±2.560	179.9±2.254	171.4±2.722	193.7±1.443	197.0±5.679
	PdI	0.511±0.054	0.225±0.008	0.267±0.017	0.288±0.012	0.157±0.026
	ζ-Potential (mV)	-46.7±0.189	-42.8±1.083	-40.7±1.026	-40.5±0.569	-39.1±0.551

[1)]The emulsions were prepared at the microfluidization pressure and cycle of 120 MPa and 3 cycles, respectively.
[2)]*D* (nm), accumulate mean diameter of the nanoemulsion particles
[3)]PdI, polydispersity index

In general, increasing the Tweens concentration up to 10% resulted in a significant decrease in the particle size. This is because smaller particle sizes have a greater surface area, which would require more emulsifier for that surface area to be covered. However, over 10% of Tweens there were no more decreases in droplet size. This is most likely due to the fact that at certain concentrations of emulsifier, all the droplets in the emulsion were fully covered by the emulsifier, and excessive emulsifier was not be utilized (13). The same results were obtained in the case of nanoemulsion stabilized with protein emulsifiers. The mean droplet diameter decreased steeply with increasing emulsifier concentration, but then reached plateau when the protein concentration was increased further. In general, droplet sizes of nanoemulsions prepared with WPI and SC were smaller than those stabilized by SPI at all the concentrations studied (0.1%-6%). At the protein concentration of 1%, the nanoemulsions prepared with SPI had the largest mean particle size of 230 nm, and with a PdI value of 0.31, whereas WPI produced nanoemulsions with the smallest particle size of 162 nm and a PdI value of 0.27. Nanoemulsions prepared with WPI and SC were selected arbitrarily to study the stability of β-carotene emulsions during storage.

In the emulsion system, ζ-potential is an indirect measure of the electrical charge of the colloidal particles. In general, ζ-potential indicates the moderate stability behavior of dispersion when they are over ±30 mV. The ζ-potentials of the oil droplets coated by Tweens and proteins were negative at all concentrations (Table 1). Although Tween 20 and 80 were non-ionic emulsifiers, the net surface charge of Tweens-stabilized nanoemulsions was also negative. It was thought that the adsorption of OH^- species from the aqueous phase from the oil/β-carotene onto the interface was responsible for the negative surface charge (11,18). Their ζ-potential values were ranged from −7.59 to −12.40 mV, which were much lower negative values than those of protein-stabilized nanoemulsions.

The ζ-potential values of protein-stabilized nanoemulsions were ranged from −20.1 to −46.7 mV. Interestingly, the SC-stabilized nanoemulsions had a higher negative charge than the WPI-stabilized ones, which suggests that the linear charge density of the SC was higher than the WPI. This may have important consequences for the interactions of protein-coated lipid droplets with other charged species in food and beverage systems, such as transition metals that promote lipid oxidation (24). For all protein emulsifiers, there was a decrease in the magnitude of the negative ζ-potential with increasing emulsifier concentration (Table 1), which can be attributed to the pH dependence of the droplet ζ-potential. The pH of the protein-stabilized nanoemulsions was decreased with increasing emulsifier concentration (data not shown). At relatively high H^+ concentrations (low pH), the amino groups are positively charged and the carboxyl groups are neutral, so the net protein charge is positive.

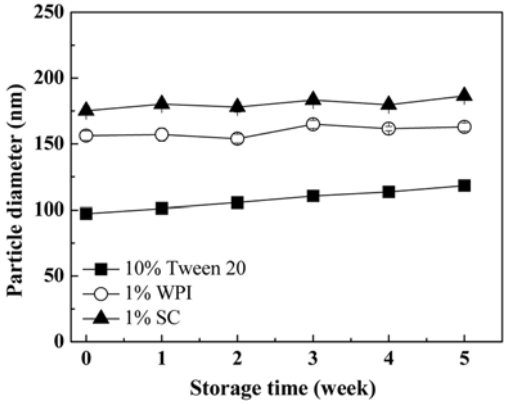

Fig. 3. Stability of β-carotene nanoemulsions prepared with different emulsifiers during storage at room temperature (25°C) for 5 weeks. Nanoemulsions were prepared at 120 MPa and 3 cycles.

Stability of β-carotene nanoemulsions during storage

The stability of nanoemulsions was evaluated based on the changes in particle size and β-carotene retention over a storage time of 5 weeks at room temperature. In general, for all nanoemulsions prepared at 120 MPa and 3 cycles, there was no distinct change in the mean particle size during the storage time, indicating that the nanoemulsions were stable regardless of the emulsifier types. As shown in Fig. 3, the nanoemulsions stabilized by proteins had little higher stability than that stabilized by Tween 20. While the major mechanism preventing particle aggregation in a protein-stabilized dispersion is steric repulsion, the surface charge of the particles also contributes to the dispersion stability (18). The ζ-potential of β-carotene particles prepared with SC and WPI had large surface charges of −40.7 and −27.1 mV, respectively, as compared to that prepared with Tween 20 (−8.3 mV), suggesting that the protein-stabilized nanoemulsions were more stable against aggregation during storage (Table 1).

Due to its highly unsaturated structure, β-carotene is considered to be very sensitive to light, oxygen, and heat during emulsification process and storage (1,25). Although the physical stability of all the β-carotene nanoemulsions was not significantly different during storage (Fig. 3), the influence of the emulsifiers and emulsification conditions on β-carotene stability in the nanoemulsions was rather pronounced. Figure 4 and 5 show the degradation profile of β-carotene as a function of storage time at room temperature. Effects of homogenization pressure and the number of cycles on β-carotene degradation were investigated with nanoemulsions stabilized by 10% of Tween 20 (Fig. 4). The degree of degradation of β-carotene in the nanoemulsions

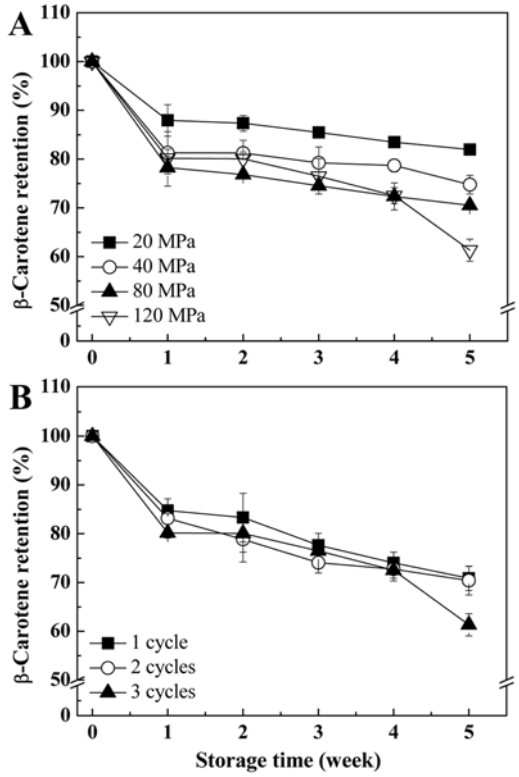

Fig. 4. Degradation of β-carotene in the nanoemulsions prepared with 10% Tween 20 at different microfluidizer pressure (3 cycles) (A) and cycle (120 MPa) (B) during storage at room temperature (25°C) for 5 weeks.

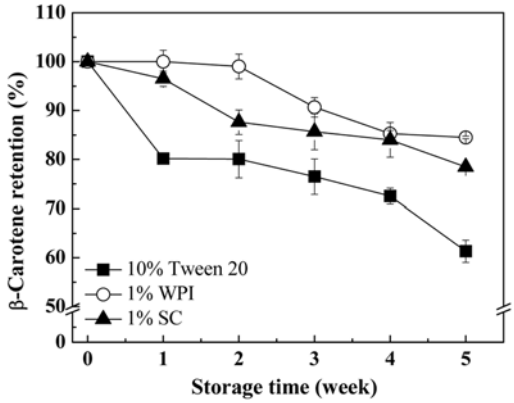

Fig. 5. Degradation of β-carotene in the nanoemulsions prepared with different emulsifiers during storage at room temperature (25°C) for 5 weeks.

was found to increase, with a decrease in mean particle size. The results show that the degree of degradation of β-carotene in the nanoemulsion was found to decrease as microfluidizer pressure and cycle number increased, which agreed with the findings of other researchers (7). Here, β-carotene degradation in nanoemulsion during storage could be explained by two important factors identified by Tan and Nakajima (7). One explanation is the large surface area of the droplets as a result of their size reduction to the nanometer range, and the other is formation of free radicals due to cavitation during the microfluidizer processing. The formation of free radicals promotes oxidation (7,26). Therefore, it is clear that the large surface area of the droplets and the free radical occurred during the high pressure homogenization process could be the cause of the loss of β-carotene in the nanoemulsions.

The stability of β-carotene was also found to be strongly influenced by the emulsifiers used (Fig. 5). All nanoemulsions were prepared at 120 MPa of microfluidizer pressure and 3 microfluidizer cycles. At the end of the storage time, the percentage of β-carotene in the nanoemulsions was reduced to a range of 61%-84%, depending on the type of emulsifier used. β-Carotene in the nanoemulsions prepared with WPI (1%) was the most stable, while the lowest β-carotene retention was observed in Tween 20-stabilized nanoemulsions. Protein-stabilized nanoemulsions had the larger mean particle size, and therefore, a smaller specific surface area than that of Tween-stabilized emulsions. This was one of the reasons why the β-carotene in protein-stabilized nanoemulsions was less susceptible to oxidation than that in Tween-stabilized emulsions.

Several food proteins, such as WPI and SC, have been shown to protect oil in water emulsions from lipid oxidation by altering the droplet interface properties. The food proteins were reported to be an efficient antioxidant protein in milk by absorbing to the droplet surface and exhibiting a synergistic effect of improving antioxidant activity in combination with other antioxidant compounds (27). Especially, the major constituents of WPI are β-lactoglobulin and α-lactalbumin, and both of them contain cysteyl residues, disulphide bonds and thiol functional groups, which can inhibit lipid oxidation by scavenging free radicals (16,28). Therefore, WPI can play the role of an antioxidant, which probably resulted in the less severe degradation of β-carotene in the WPI-stabilized emulsion.

This study has shown that it is possible to produce β-carotene nanoemulsions with a narrow size distribution using a microfluidization technique. The particle size of the nanoemulsions was influenced by emulsifiers and their concentrations, as well as homogenization pressure and the number of cycles. In general, droplet sizes of nanoemulsions prepared with Tweens were smaller than those stabilized by proteins. It was demonstrated that β-carotene nanoemulsions had good physical stability during storage. However, β-carotene nanoemulsions were chemically unstable during storage. β-Carotene in the nanoemulsions prepared with WPI (1%) was the most stable, while the lowest β-carotene retention was observed in Tween 20-stabilized nanoemulsions. These results revealed that β-carotene degradation is very dependent on the droplet size of emulsions. Therefore, in order to commercially apply

this technology to the food and beverage industry, further work is needed to find methods to retard the degradation of β-carotene in nanoemulsion systems.

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