Development of Nanoemulsion Preconcentrate of Capsanthin with Improved Chemical Stability

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ABSTRACT

Capsanthin, like other carotenoids, exhibits poor aqueous solubility, poor stability, and low/variable oral bioavailability that limit its utility as a nutraceutical. In this study, we describe the development of anhydrous nanoemulsion preconcentrate of capsanthin, which upon dilution with water, spontaneously forms nanoemulsion resulting in improved solubility of capsanthin without compromising its chemical stability and antioxidant activity. We chose Food and Drug Administration-approved ingredients to develop capsanthin nanoemulsion preconcentrates. The optimized capsanthin nanoemulsion preconcentrate, upon dilution with water or buffers, yielded the nanoemulsion with size <50 nm and showed ~8-fold higher capsanthin release in 1 h in 0.1 N HCl in vitro compared with pristine capsanthin. The 3-month stability studies at 25°C on the capsanthin nanoemulsion preconcentrate showed that capsanthin retained the physical and chemical stability with no alteration in antioxidant activity indicating that nanoemulsion preconcentrate can be used to effectively deliver capsanthin for health benefits.

Keywords: functional food, carotenoid, antioxidant, nanoemulsion, BHT, vitamin E

INTRODUCTION

Red pepper (*Capsicum annum* L.) is consumed worldwide as a food ingredient. There are two varieties of red pepper namely chili pepper and sweet pepper. The former contains capsaicin as a major constituent, an alkaloid responsible for the pungent nature and later comprising majorly of capsanthin, an intensely red colored xanthophyll carotenoid pigment.¹ Over the years, significant efforts have been made to study various effects (including benefits) of only capsaicin on the human health (>14,000 PubMed search results for capsaicin vs. 137 PubMed results for capsanthin).

Capsanthin, like other carotenoids, contains multiple conjugated double bonds that are responsible for its bright red color and strong antioxidant property. Capsanthin exhibits stronger antioxidant activity compared with other commonly studied carotenoids such as β -carotene.² Studies have shown that capsanthin can inhibit inflammatory and oxidative stress-related processes by interacting with cellular signaling cascades.³ Capsanthin supplementation studies in rats have shown to result in a significant rise in plasma high-density lipoprotein levels in dose-dependent manner.⁴ Studies have shown that capsanthin is also capable of inhibiting adipogenesis in 3T3-L1 preadipocytes and weight gain in obese mice.⁵ Capsanthin, like other carotenoids, has been shown to have the ability to prevent ailments such as cancer and arteriosclerosis.⁶ Thus, capsanthin, in addition to being a natural colorant, clearly has the potential to be a nutraceutical. However, studies in humans have shown that capsanthin, because of its high lipophilicity and poor solubility in gastrointestinal fluids has low and highly variable oral bioavailability.^{7,8} Moreover, capsanthin is prone to oxidative degradation in the presence of heat, humidity, and light,^{9,10} which can compromise its antioxidant activity to a great extent.¹¹ Thus, to fully harness the potential of capsanthin as a functional food and natural colorant, it is quite important to develop a commercially viable delivery system that can improve solubility, dissolution, and absorption of capsanthin without compromising its stability and antioxidant activity.

Nanoemulsion preconcentrate or self-nanoemulsifying formulation (SNEF) is an anhydrous and isotropic mixture of oil, surfactants, cosurfactant or cosolvents, and drug that emulsifies spontaneously to produce fine oil-in-water nanoemulsion when introduced into an aqueous phase under gentle agitation.^{12,13} SNEF, on oral administration, forms nanoemulsion because of gastrointestinal motility. As SNEFs are devoid of water, they generally result in the greater physical and/or chemical stability (of incorporated lipophilic moiety) on long-term storage compared with the nanoemulsions containing water.¹³ Extensive research has been carried to demonstrate the ability of SNEF to improve solubility, stability, dissolution, and oral bioavailability of a variety of therapeutic agents and natural products.^{14,15} SNEFs are advantageous for the delivery of nutraceuticals or coloring

agents as these preconcentrates can be easily mixed with aqueous or oleaginous bases based on the end application. Furthermore, the availability of the SNEF-based products in the nutraceutical and pharmaceutical market demonstrates their commercial viability. In this study, we describe the development of SNEF to overcome the hurdles in the utilization of capsanthin as a nutraceutical.

MATERIALS

Capsanthin was obtained as a gift from Biogenero Labs Pvt. Ltd. (Bangalore, India). Cremophor RH 40[®] (PEG-40-hydrogenated castor oil), Cremophor EL[®] (PEG-35-castor oil), vitamin E (tocopherol acetate), Kolliphor TPGS[®] (vitamin E-PEG-succinate), and Solutol HS 15[®] (PEG-12-hydroxystearate) were obtained as gift samples from BASF Pvt. Ltd. (Mumbai, India). Captex 300[®] (caprylic/capric triglyceride) and Capmul MCM[®] (caprylic/capric mono- and diglyceride) were obtained as gift samples from Indchem International (Mumbai, India). Capryol 90[®] (propylene glycol monocaprylate), Peceol[®] (glyceryl monooleate), Labrafac CC[®] (long chain triglycerides)[™], Lauroglycol 90 (propylene glycol monolaurate), Labrasol[®] (PEG-caprylic/capric glyceride), and Transcutol[®] (diethylene glycol monoethyl ether) were obtained as gift samples from Gattefosse Pvt. Ltd. (Mumbai, India). Tween 20, Tween 80, Span 20, isopropyl myristate, and propylene glycol were purchased from Merck (Mumbai, India). Butylated hydroxy toluene (BHT) was purchased from Analab Finechem (Pune, India). Refined oils such as olive oil, sunflower oil, rice bran oil, almond oil, castor oil, and flax seed oil were purchased from the local grocery store. All the excipients and reagents were of analytical grade and double-distilled water was freshly prepared whenever required throughout the study.

METHODS

Solubility Studies

The solubility of capsanthin in various oils, 10% (w/w) surfactant solutions and cosurfactants was determined by using the shake flask method. In 10 mL glass vial, 5 mL of the selected vehicle like oil, surfactant solution, or cosurfactant was transferred; the excess quantity of carotenoid was added to the vial. The mixtures were first stirred and sonicated to facilitate proper mixing of the carotenoid with the vehicles. The mixtures were shaken for 72 h at 37°C in an orbital shaker (BM-262D; Biomedica, India). The mixtures were centrifuged for 15 min, followed by filtration through Whatman filter paper. The filtrates were suitably diluted with acetone and absorbance was determined by ultraviolet (UV)-visible spectrophotometer at 463.5 nm. The oils included in the study were castor oil, flax seed oil, sunflower oil, rice bran oil, soybean

oil, isopropyl myristate, olive oil, almond oil, Lauroglycol 90, Capryol 90, Labrafac CC, Peceol, and Captex 300. Polysorbates 20 and 80 (Tween 20, Tween 80), sorbitan laureate (Span 20), Cremophor EL, Cremophor RH 40, Solutol HS, Kolliphor TPGS, Labrasol, and Plurol oleique were the surfactants that were studied. Cosurfactants chosen for the study were polyethylene glycol 400 (PEG 400), propylene glycol, Transcutol, vitamin E, and Capmul MCM.

Screening of Surfactants for Emulsifying Ability

Emulsification ability of various surfactants was screened using a simple turbidimetric method reported by Date and Nagarsenker (2007). In brief, surfactant (300 mg) and Captex 300 (300 mg) were mixed together with gentle heating to obtain an isotropic mixture. The isotropic mixture (50 mg) was added to 50 mL double-distilled water in a volumetric flask and the mixture was inverted sufficient times to obtain a fine emulsion. After 2 h, the transmittance of emulsions was measured at 650 nm by UV-160A double-beam spectrophotometer (Shimadzu 1700, Japan) using double-distilled water as blank.

Construction of Ternary Phase Diagram

The ternary phase diagram was constructed for Captex 300, Cremophor RH 40, and Capmul MCM using the method reported by Date and Nagarsenker (2007) with suitable modifications. Ternary mixtures with varying compositions of Captex 300, Cremophor RH 40, and Capmul MCM were prepared. The Captex 300 concentrations were varied from 10% to 50%, Cremophor RH 40 concentrations were varied from 30% to 60%, and Capmul MCM concentrations from 0% to 30%. For any mixture, the total of all component concentrations was always 100%. The first mixture contained 60% surfactant, 30% of the oily phase, and 10% of cosurfactant. In the subsequent experiments, the cosurfactant was increased by 5% for each composition. The oily phase concentration remained constant and the surfactant concentration was adjusted to make a total of 100%. In total, 42 such mixtures with varying oil, surfactant, and cosurfactant concentrations were prepared. Each composition (50 mg) was diluted to 50 mL with distilled water. The transmittance of the resulting dispersions was determined at 650 nm using UV-visible spectrophotometer.

Screening of Antioxidant to Improve Stability of Capsanthin

Accelerated stability studies on the mixture containing capsanthin and components of SNEF were carried out in the presence of different types or amounts of lipophilic

antioxidants. A mixture of Captex 300, Cremophor RH 40, and Capmul MCM was prepared in equal proportion (excipients mixture). Capsanthin, 40 mg and the excipients mixture, 4 g were mixed well in 10 mL amber colored vial using cyclomixer. Vitamin E in different concentrations namely, 3%, 5%, and 10% or BHT 0.5% were added to the admixture of capsanthin and excipients, mixed well and stored at $40^{\circ}\text{C} \pm 2^{\circ}\text{C}/75\% \pm 5\% \text{ RH}$ for 4 weeks. At day 0, weeks 2 and 4, the solutions were subjected to determination of color index in triplicate using ASTA method 20.1¹⁶ in the following manner.

Capsanthin, 10 mg was weighed accurately and diluted with acetone to 10 mL. This was treated as a primary stock solution that was diluted suitably with acetone through serial dilutions to achieve the final concentration of $2.5 \mu\text{g/mL}$. The absorbance of the solution was taken at 463.5 nm using acetone as a reference and the color index was determined using the following formula:

$$\text{Color index} = \text{Absorbance at } 463.5 \text{ nm} \times \text{dilution factor} \times 66,000$$

The color index values of initial, 2- and 4-week samples were compared using one-way analysis of variance followed by Tukey test at $p < 0.05$.

Preparation of SNEF with Capsanthin

Accurately weighed quantities of Captex 300, Cremophor RH 40, Capmul MCM, and BHT (0.5%) (Table 1) were added in a glass vial and mixed using cyclomixer for 15 min. To this admixture, accurately weighed quantity (30 mg) of capsanthin was added and mixed until most of the capsanthin dissolved. This was followed by 5 min of sonication while maintaining the bath temperature at 40°C to ensure complete solubilization of capsanthin. The formulations were observed for isotropicity and were stored at 2°C – 8°C until further use.

Characterization of SNEF of Capsanthin

Determination of self-emulsification time. The emulsification time of SNEF was determined using USP type II dissolution apparatus at 50 rpm (TDT-06L; Electrolab, India). Each formulation, 0.5 g was added to 500 mL purified water at 37°C and stirred at 50 rpm. The time required for complete solubilization of formulation was considered as self-emulsification time.

A small quantity of pristine capsanthin and formulation F6 was added separately to $\sim 25 \text{ mL}$ of purified water to compare their dispersibility.

Transmittance test. In this study, 100 mg of each formulation namely, F1, F2, F6, F7, F8, and F9 was diluted separately with 100 mL of various media like 0.1 N HCl, phosphate buffer pH 6.8, and distilled water. The transmittance of the solutions was measured in triplicate at 650 nm using UV-visible spectrophotometer.

Cloud point measurement. Formulations F1, F2, F6, F7, F8, and F9 were diluted with distilled water in the ratio of 1:100 and kept in a water bath maintained at 25°C . The temperature of the bath was increased gradually at a rate of $5^{\circ}\text{C}/\text{min}$. The temperature at which turbidity in the formulation was observed visually was noted as the cloud point.

Globule size determination. Mean globule size and the polydispersity index of the formulations F1, F2, F6, F7, F8, and F9 were determined by photon correlation spectroscopy (Nanophox NX0088; Sympatec GmbH, Germany). Formulation, 50 mg was diluted with 10 mL double-distilled water, filled in transparent polystyrene cuvette (path length = 1 cm) that was placed in the thermostatic chamber of the instrument maintained at 25°C . The detection was carried out at scattering angle of 90° .

Table 1. Composition of Different Self-Nanoemulsifying Formulation Containing Capsanthin (Selected from the Phase Diagram)

Formulation code	F1	F2	F3	F4	F5	F6	F7	F8	F9
Capsanthin (mg)	30	30	30	30	30	30	30	30	30
Captex 300 (%w/w)	24.5	24.5	24.5	24.5	29.5	29.5	29.5	29.5	29.5
Cremophor RH 40 (%w/w)	60	55	50	45	60	55	50	45	40
Capmul MCM (%w/w)	15	20	25	30	10	15	20	25	30
BHT (%w/w)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

The total weight of each formulation was 530 mg.
BHT, butylated hydroxyl toluene.

Zeta potential determination. Zeta potential of the formulations F1, F2, F6, F7, F8, and F9 were measured by Delsa Nano C (Beckman Coulter). SNEF formulations, 50 mg each was diluted with 10 mL double-distilled water for the measurements. All measurements were carried out at 25°C.

Robustness to dilution. Robustness of SNEF to dilution was studied by subjecting the formulations F1, F2, F6, F7, F8, and F9 to various dilutions like 1:50, 1:100, and 1:500 with various dissolution media namely, distilled water, 0.1 N HCl, and phosphate buffer pH 6.8. The diluted SNEF were stored for 24 h and observed for any signs of turbidity or precipitation.

Determination of capsanthin content. Formulations F1, F2, F6, F7, F8, and F9 each equivalent to 10 mg capsanthin was dissolved in a suitable quantity of acetone. The samples were mixed thoroughly to dissolve carotenoid in acetone, centrifuged at 3,000 rpm for 15 min. The supernatant was filtered using Whatman filter paper, diluted suitably with acetone to make up the volume up to 100 mL, and subjected to determination of absorbance using UV-visible spectrophotometer at 463.5 nm. The blank was prepared in a similar manner using SNEF made without capsanthin.

In vitro capsanthin release studies. Formulations F1, F2, F6, F7, F8, and F9 each equivalent to 30 mg of capsanthin were filled in hard gelatin capsules of size “1.” The *in vitro* carotenoid release test was performed in 900 mL of 0.1 N HCl maintained at 37°C ± 0.5°C using USP type I dissolution apparatus. The baskets were rotated at 50 rpm. Aliquots of 5 mL were collected periodically at 5, 10, 15, 30, 45, and 60-min intervals and replaced with 5 mL of fresh dissolution medium. Aliquots after filtration through 0.45 µm membrane filters were analyzed using spectrophotometer at 463.5 nm. The *in vitro* dissolution study was also performed for 30 mg of plain capsanthin powder.

Determination of Hydrogen Peroxide Scavenging Activity

Capsanthin, 20 mg was weighed and dissolved completely in 20% ethanol and the final volume was made up with distilled water to get 200 µg/mL solution. To 1 mL of this solution, 3.4 mL of 0.1 M phosphate buffer (pH 7.4) and 600 µL of 43 mM solution of hydrogen peroxide (H₂O₂; prepared in the same buffer) were added and mixed well. The absorbance value (at 230 nm) of the reaction mixture was recorded at 0 min and then at every 10 min up to 50 min using a UV spectrophotometer (Shimadzu Corporation, Japan). A separate blank sample (devoid of H₂O₂) was used for background subtraction.¹⁷ The concentration (mM) of H₂O₂ in assay media

was determined using a standard curve that was prepared as described hereunder.¹⁸ H₂O₂ 58 µL was added to a sufficient quantity of phosphate buffer pH 7.4 in 100 mL volumetric flask. The resultant mixture was shaken and volume was adjusted to 100 mL with phosphate buffer pH 7.4. From this stock solution, dilutions namely, 2, 4, 6, 8, and 10 µg/mL were prepared with phosphate buffer pH 7.4. Absorbance values of these resulting solutions were noted using UV-visible spectrophotometer at 230 nm.

Evaluation of Physicochemical Stability of Capsanthin SNEF for 3 Months

Formulation F6 was prepared in bulk and quantity of formulation equivalent to 30 mg capsanthin was filled in hard gelatin capsules of size 1. The capsules were packed in amber-colored airtight glass containers and subjected to accelerated conditions of 25°C ± 2°C, 60% ± 5% RH, and control conditions of 2°C–8°C. Initial samples and samples withdrawn at 1, 2, and 3-month intervals were subjected to all the evaluation parameters stated in the Characterization of SNEF of Capsanthin section. Initial sample and sample withdrawn at 3-month interval were diluted with water to yield the concentration of 200 µg/mL and their H₂O₂ scavenging activity was determined in a similar manner as mentioned in the Determination of Hydrogen Peroxide Scavenging Activity section.

RESULTS

Solubility Studies

We screened various oily vehicles with different chemical compositions such as long-chain triglycerides, medium chain triglycerides, and short- and long-chain fatty acid esters. Among all the oils screened, capsanthin showed maximum solubilization in caprylic/capric medium chain triglycerides, Captex 300 (74.4 ± 1.45 mg/mL) followed by castor oil (55.14 ± 1.62 mg/mL) (Fig. 1A). Captex 300 was hence selected as an oil phase for further studies. Among cosurfactants, Capmul MCM (caprylic/capric mono- and diglyceride) showed highest solubilizing potential for capsanthin (Fig. 1B) and was selected for further studies. We evaluated the solubility of capsanthin in 10% w/w surfactant solution because of high viscosity or solid nature of some of the surfactants. Capsanthin showed the highest solubility in 10% w/w Kolliphor TPGS (vitamin E PEG succinate) solution (Fig. 1B).

Screening of Surfactants for Emulsifying Ability

We evaluated various Food and Drug Administration (FDA)-approved surfactants for their ability to emulsify Captex 300 as the selection of surfactant is governed by their

Fig. 1. Capsanthin solubility in various (A) oils and (B) cosurfactants and surfactants. In case of oils, capsanthin showed highest solubility in Captex 300 (medium chain triglyceride). Hydrophilic cosurfactants such as transcutool and propylene glycol showed negligible solubilizing potential for capsanthin, whereas lipophilic Capmul MCM (medium chain mono- and diglyceride) showed highest solubility. 10% w/w surfactant solutions were used for the studies because of highly viscous or solid nature of some of the surfactants. Data are expressed as mean $\pm$ SD ($n=3$). SD, standard deviation.

emulsification efficiency rather than their mere ability to solubilize the lipophilic moieties. Of all the surfactants tried, Cremophor RH 40 containing emulsions showed highest ($\sim 90\%$) transmittance indicating the best emulsification ability (Supplementary Table S1). Hence, Cremophor RH 40 was selected for further studies.

Ternary Phase Diagram

We used previously described method¹² to construct a ternary phase diagram with slight modifications. We systematically varied the composition of surfactant, oily phase and cosurfactant in the SNEF and evaluated their % transmittance on dilution with water. Compositions having transmittance $>95\%$ were considered desirable and shown as a shaded region in the phase diagram (Supplementary Fig. S1). The nine such compositions in the shaded region that showed visual clarity and high % transmittance values were taken up for further studies.

Screening of Antioxidant to Improve Stability of Capsanthin

We evaluated two commonly used lipophilic antioxidants namely, vitamin E and BHT as they can be easily solubilized in the SNEF. We subjected a mixture of capsanthin and the

components of SNEF to accelerated stability testing at $40^\circ\text{C} \pm 2^\circ\text{C}/75\% \pm 5\% \text{ RH}$. We found that capsanthin undergoes significant degradation ($p < 0.05$) at accelerated storage conditions in 4 weeks (Fig. 2). Unfortunately, vitamin E, at different concentrations (3%, 5%, and 10% w/w) was not able to prevent capsanthin degradation despite its known antioxidant activity. Of interest, BHT (0.5% w/w) could retain the stability of the capsanthin throughout 4 weeks of storage at an accelerated storage condition.

Preparation of SNEF with Capsanthin

A series of SNEFs were prepared with varying ratios of oils (25%–30%), surfactants (40%–60%), and cosurfactants (10%–30%) (Table 1). Studies of capsanthin loading in SNEF showed that addition of ~ 15 mg of capsanthin was achieved easily by slight warming and simple mixing on cyclomixer. Up to 30 mg quantity of capsanthin could be dissolved completely with the aid of gentle sonication, whereas higher quantity showed improper mixing and turbidity. Hence, 30 mg carotenoid was incorporated in all the formulations.

Characterization of SNEF of Capsanthin

We determined the self-emulsification time (measure of how rapidly the formulation will emulsify *in vivo*) of the SNEF

Fig. 2. BHT can prevent degradation of capsanthin. Capsanthin and components of SNEF were mixed and stored in the absence or presence of lipophilic antioxidants, vitamin E, and BHT for 4 weeks at $40^{\circ}\text{C} \pm 2^{\circ}\text{C}/75\% \pm 5\%\text{RH}$ (stress condition) for 4 weeks. Capsanthin underwent significant degradation (measured as a function of color index) in 4 weeks in the absence of antioxidant or in the presence of different concentrations of vitamin E. The presence of BHT prevented the degradation of capsanthin. Data are expressed as mean $\pm$ SD ($n=3$); * $p < 0.05$, ** $p < 0.005$. BHT, butylated hydroxy toluene; SNEF, self-nanoemulsifying formulation.

listed in Table 1 and the formulations (F1, F2, F6, F7, F8, and F9; Table 1) showing self-emulsification time <1 min were selected for characterization studies such as transmittance test in various buffer media, cloud point, average globule size, polydispersity index, zeta potential, and assay. The summary of the results is given in Table 2. Capsanthin in neat state was

difficult to disperse in water and settled at the bottom, whereas the SNEF mixed instantaneously in water gave a transparent, orange solution (Supplementary Fig. S2). Dilution of SNEF with different dissolution media like 0.1 N HCl, distilled water, and phosphate buffer pH 6.8 yielded nanoemulsions with transmittance $>95\%$ which did not show any turbidity or separation visually after 24 h of storage. This confirmed their physical stability and compatibility with different pH conditions (Table 2). Cloud point in the range of 70°C to 81°C indicated thermal stability of SNEF during the normal storage conditions. All the formulations except formulations F2 and F9 showed globule size <100 nm. Such a small droplet size could be the result of the optimum ratio of surfactant and cosurfactant being available to stabilize the oil–water interface. The average globule size of the F6 formulation was found to be minimum (~ 41 nm) among all the formulations (Table 2 and Supplementary Fig. S3) and its zeta potential value was -9.8 mV (Supplementary Fig. S4). The capsanthin content of the formulations was in the range of 95% – 105% . This indicated that the method of preparation of formulation did not affect the drug stability.

In vitro capsanthin release studies. *In vitro* carotenoid release studies were performed in 0.1 N HCl (similar to stomach content) using type I dissolution apparatus, to compare the release of carotenoid from different SNEFs. To ensure ease of handling and uniformity of weight, SNEFs were filled in hard gelatin capsule shells and then placed in basket type apparatus to perform the release studies. All the SNEFs showed $>60\%$ release of the capsanthin in 0.1 N HCl, whereas $<10\%$ pure

Table 2. Evaluation of Physicochemical Properties of Various Self-Nanoemulsifying Formulation Containing Capsanthin

Evaluation parameter	Formulation					
	F1	F2	F6	F7	F8	F9
% Transmittance-distilled water	98.9 ± 0.1	99.5 ± 0.3	98.3 ± 0.6	98.4 ± 0.4	98.0 ± 0.2	96.7 ± 0.5
% Transmittance-0.1 N HCl	99.5 ± 0.7	99.7 ± 0.5	99.8 ± 0.2	99.7 ± 0.3	92.9 ± 0.04	97.7 ± 0.5
% Transmittance-phosphate buffer pH 6.8	99.0 ± 0.9	98.9 ± 0.6	99.7 ± 0.2	94.9 ± 0.3	98.1 ± 0.9	96.8 ± 1.0
Cloud point ($^{\circ}\text{C}$)	79.0 ± 0.8	71.5 ± 2.8	81.0 ± 1.6	74.0 ± 2.0	76.5 ± 1.2	72.0 ± 1.6
Mean globule size (nm)	46.37	137.24	41.77	99.17	70.86	234.81
PDI	0.35	0.25	0.31	0.08	0.34	0.29
Zeta potential (mV)	-9.72	-2.38	-9.81	-2.93	-7.44	-1.53
Capsanthin content (%w/w)	99.22 ± 0.45	97.36 ± 0.57	102.04 ± 0.56	96.36 ± 0.69	98.24 ± 0.66	99.06 ± 0.74

Data are expressed as mean $\pm$ standard deviation ($n=3$).
PDI, poly dispersity index.

Fig. 3. SNEFs improve dissolution rate of capsanthin *in vitro*. *In vitro* dissolution of various capsanthin SNEFs and pure capsanthin was carried out in 0.1N HCl United States Pharmacopeia (USP)-Type 1 dissolution apparatus. All the SNEFs showed improved capsanthin release *in vitro* compared with pure capsanthin powder. Formulation F6 showed highest *in vitro* capsanthin release. Quick *in vitro* capsanthin release may translate to rapid bioaccessibility of capsanthin *in vivo*. Data are expressed as mean $\pm$ SD ($n=3$).

capsanthin was released during *in vitro* release studies (Fig. 3). Presence of capsanthin in solubilized form and the presence of surfactants in the formulations could have attributed to greater rate and extent of dissolution of capsanthin from SNEF compared with the pure drug. The highest capsanthin release of 78.57% was obtained from formulation F6, which could be attributed to its lowest globule size presenting higher surface area for dissolution.

Determination of H_2O_2 Scavenging Activity

The H_2O_2 (a source of free peroxide radical) scavenging ability of capsanthin solution and capsanthin SNEF (F6) was carried out to determine their antioxidant potential *in vitro*. The capsanthin hydroalcoholic solution, at the end of 40 min, showed 75.10% scavenging of H_2O_2 (Fig. 4). Of interest, capsanthin SNEF showed similar H_2O_2 scavenging activity at the end of 40 min. Of note, there was no difference in the H_2O_2 scavenging activity of capsanthin SNEFs prepared fresh and stored for 3 months at $25^\circ\text{C} \pm 2^\circ\text{C}$, $60\% \pm 5\%$ RH.

Evaluation of Physicochemical Stability of Capsanthin SNEF for 3 Months

The recommended storage temperature of capsanthin is 2°C to 8°C .¹⁹ Nevertheless, we stored capsanthin SNEF (F6) at $25^\circ\text{C} \pm 2^\circ\text{C}/60\% \pm 5\%$ RH for 3 months and evaluated its

Fig. 4. Capsanthin SNEFs retain antioxidant activity of capsanthin even after 3 months of storage. The antioxidant effect of capsanthin hydroalcoholic solution, capsanthin SNEF prepared fresh and stored at $25^\circ\text{C} \pm 2^\circ\text{C}$, $60\% \pm 5\%$ RH for 3 months was evaluated using hydrogen peroxide (H_2O_2) scavenging assay. Capsanthin SNEF showed similar H_2O_2 scavenging activity as that of capsanthin hydroalcoholic solution indicating that SNEF allowed for immediate accessibility of capsanthin because of solubility enhancement and rapid release. Furthermore, capsanthin SNEF stored for 3 months also showed similar antioxidant activity as that of freshly prepared SNEF indicating retention of antioxidant activity of capsanthin during the 3 months of storage. Data are expressed as mean $\pm$ SD ($n=3$).

physicochemical stability at various intervals (Table 3). The formulation retained its stability with no significant changes in physical aspects, drug content, and *in vitro* release characteristics and it also retained its antioxidant activity (Fig. 4)

DISCUSSION

Carotenoids are the class of natural pigments that have shown potential applications in cosmetic, nutraceutical, and pharmaceutical field owing to their characteristics such as strong coloring capability, excellent antioxidant activity, and potential to prevent cancer incidences.^{20,21} Unfortunately, high lipophilicity and poor chemical stability in the presence of air, heat, and light are the major factors that limit the utility of carotenoids. High lipophilicity and poor solubility in the gastrointestinal fluids are the major reasons for the low oral bioavailability of carotenoids that eventually compromises the capability of the carotenoids to act as an effective

Table 3. Evaluation of Physicochemical Stability of the Optimized Capsanthin Self-Nanoemulsifying Formulation (F6) Stored at 25°C

Evaluation parameter	Duration			
	Day 0	1 Month	2 Months	3 Months
% Transmittance-distilled water	99.08±0.21	99.01±0.4	98.95±0.16	98.73±0.33
% Transmittance-0.1 N HCl	99.56±0.18	99.01±0.12	98.91±0.12	98.86±0.08
% Transmittance-phosphate buffer pH 6.8	99.64±0.08	99.27±0.11	99.06±0.12	98.83±0.09
Average globule size (nm)	42.34	43.51	44.21	43.28
PDI	0.31	0.28	0.29	0.3
Zeta potential (mV)	-9.01	-9.12	-9.98	-11.99
Capsanthin content (%w/w)	103.15±0.62	102.65±0.71	101.26±0.56	100.04±0.89
% Capsanthin release in 0.1 N HCl at 1 h	77.97±1.24	76.37±1.57	75.64±3.3	74.15±2.23

The capsanthin self-nanoemulsifying formulation did not show any significant change in the various physicochemical parameters at the end of 3 months indicating good stability.

nutraceutical. The delivery system capable of improving solubility and absorption of carotenoids without compromising its stability would be of great value to improve the utility of carotenoids as a nutraceutical. Studies have shown that the oral bioavailability of carotenoids increases with the co-administration of fat indicating the potential of lipid-based delivery systems.²² Nanoemulsions are a lipid-based drug delivery system that has been successfully utilized to improve oral delivery of a variety of lipophilic compounds including natural products. SNEFs are anhydrous nanoemulsion preconcentrates that contain the optimal ratio of surfactant, oil, cosurfactant, and lipophilic moiety that is solubilized in the preconcentrate. SNEFs are prepared by simply mixing these components with the help of suitable mixing equipment that indicates their commercial viability.

Previously, carotenoids have been incorporated into nanoemulsions to improve their oral bioavailability.²³ In addition, to date, some investigations have been carried out to explore the potential of self-nanoemulsifying systems to improve oral delivery of β -carotene,²⁴ lutein,^{25,26} astaxanthin,²⁷ and lycopene.²⁸ Thus far, the *ex vivo* and/or *in vivo* data have clearly demonstrated that the nanoemulsions/SNEFs are able to significantly improve oral bioavailability of carotenoids in animals and humans. To date, reports on the development of delivery systems for capsanthin are scarce compared with the other xanthophyll carotenoids such as astaxanthin. Investigators have developed capsanthin-cyclodextrin complexes²⁹ and capsanthin-chitosan-soybean protein isolate microcapsules to improve the stability of capsanthin.³⁰ An

et al. have reported the development of the nanoemulsions for capsanthin using low-energy method.¹¹ However, they did not develop anhydrous self-nanoemulsifying concentrates and did not demonstrate long-term stability of capsanthin in nanoemulsions.¹¹ After a thorough analysis of the published literature on the delivery systems for carotenoids including capsanthin, we concluded that “development of SNEF” would be an efficient and commercially viable approach to improve oral delivery of capsanthin. We noticed that the published literature does not report the systematic development of SNEF for carotenoids. The missing components from the reported literature are the solubility data of the carotenoids in various oils, surfactants, and cosurfactants, systemic screening of the SNEF components using a simplified turbidimetric analysis and mainly the long-term storage stability of carotenoids in the nanoemulsions and/or SNEFs. We tried to address all these gaps in the published literature in the present investigation while focusing on the development of capsanthin SNEF. Our goal was to identify an optimal nanoemulsion preconcentrate that can solubilize high amount of capsanthin. As emulsion droplet size has been shown to be inversely proportional to the carotenoid bioavailability,³¹ we also wanted to develop SNEF that can yield nanoemulsion with small globule size (<100 nm).

We carried out extensive studies to evaluate the solubilizing potential of various FDA-approved oily vehicles, surfactants, and cosurfactants. Identifying the suitable oil, surfactant/cosurfactant having the maximal solubilizing potential for the compound under investigation not only achieves optimum

compound loading but also minimizes the final volume of SNEF.³² Our solubility studies showed that the chemical composition and polarity of the vehicles significantly influenced the solubility of capsanthin. Capsanthin showed low solubility in long-chain or short-chain fatty acid esters containing oily vehicles and polar cosurfactants such as propylene glycol and Transcutol. Capric/caprylic mono-, di-, or triglyceride containing oily phases (Captex 300 and Capmul MCM) showed a good solubilization potential for capsanthin and were selected for further experiments. It should be noted that Captex 300 and Capmul MCM are both fractionated from coconut oil and are approved by the FDA for oral delivery.

We have previously reported a simple turbidimetric method that can be used to systematically screen a variety of surfactants for their ability to emulsify the selected oily phase. In this method, we measure the % transmittance of the surfactant and oil mixture after its dispersion into the water. The higher % transmittance value indicates better emulsification capability of the surfactant¹² and potentially corresponds to the lower globule size of the (nano) emulsion. Using this method, we identified that Cremophor RH 40 (PEG-40 hydrogenated castor oil) has the best ability to emulsify the Captex 300. We also confirmed that the addition of cosurfactant (Capmul MCM) to the mixture of Cremophor RH 40 and Captex 300 further improved % transmittance value (data not shown) confirming its role as a cosurfactant. Of note, we have previously shown the capability of caprylic/capric mono- and diglycerides to act as a cosurfactant.¹² As the stability of carotenoids including capsanthin is relatively low on storage, we decided to evaluate the potential of antioxidants to improve the storage stability of capsanthin in the presence of the components of SNEF (Cremophor RH 40, Captex 300, and Capmul MCM). Color index is an acceptable method for the determination of carotenoids and it can also be used to monitor the stability of carotenoids.^{33,34} Hence, we stored SNEF components and capsanthin with or without antioxidant for 4 weeks at $40^{\circ}\text{C} \pm 2^{\circ}\text{C}/75\% \pm 5\%$ RH for 4 weeks and monitored the stability of capsanthin by measuring color index. It is noteworthy that these stressed storage conditions were chosen to induce forced degradation and determine the stability of capsanthin. In reality, the recommended storage conditions for carotenoids are refrigeration.¹⁹ Capsanthin+SNEF components mixture showed significant degradation of capsanthin at weeks 2 and 4 indicating the need for the use of antioxidant. Hence, we explored the potential of two FDA-approved lipophilic antioxidants namely, vitamin E and BHT to stabilize capsanthin. Vitamin E was not able to prevent the degradation of capsanthin even when used at a high concentration of 10% w/w and these results are in line with

the previously published data on the stability of carotenoids containing vitamin E as an antioxidant.³⁵ On the contrary, only 0.5% BHT was able to prevent the degradation of capsanthin for at least 4 weeks. Although vitamin E and BHT are both lipophilic reactive oxygen species scavengers, only BHT showed greater antioxidant activity indicating the importance of the chemical structure of antioxidants.^{23,36}

Once all the SNEF components were identified, we constructed the ternary phase diagram to identify the compositions that can yield >95% transmittance value (clear/translucent nanoemulsions) after dilution with water. We selected a total of nine compositions for further studies (Table 1) and tried to incorporate 30 mg of capsanthin. The selected SNEF formulations were screened based on emulsification time upon dilution with water. Lower self-emulsification time is a desirable property of SNEF because it ensures rapid and spontaneous emulsification of formulation *in vivo*, in turn, enhancing the rate of release and absorption of the active ingredient. Formulations showing <1 min of the self-emulsification time were chosen for further studies (Table 2). After additional characterization of the chosen formulations, we selected formulation F6 for our further studies. It showed the smallest globule size (~ 41 nm) and highest release of solubilized capsanthin *in vitro*. The negative zeta potential of the F6 (-9.8 mV) could be because of the small amount of the free fatty acid present in the caprylic/capric acid mono-, di-, and triglycerides used in the formulation.

It is very important that the delivery system designed for capsanthin should not compromise its inherent antioxidant activity. Our *in vitro* H_2O_2 scavenging activity assay showed that capsanthin hydroalcoholic solution is capable of $\sim 70\%$ scavenging of H_2O_2 (peroxide radical) confirming its inherent antioxidant activity. Of interest, capsanthin SNEF (F6) dispersed in water showed similar antioxidant activity as that of capsanthin hydroalcoholic solution. The initial lag time observed for F6 can be attributed to the time required for the sufficient release of capsanthin from SNEF. It is noteworthy that SNEF presented highly lipophilic capsanthin in a solubilized form that enabled exertion of the antioxidant activity. This indicated that the capsanthin SNEF can allow for rapid bioaccessibility of capsanthin on oral administration. Finally, our stability studies on capsanthin SNEF stored at 25°C showed that the chemical content, physicochemical properties (globule size, *in vitro* capsanthin release) (Table 3), and antioxidant activity of capsanthin did not change during the course of 3 months. (Fig. 4) Thus, we successfully developed a commercially viable approach to improve the oral delivery of capsanthin without compromising its chemical stability and inherent antioxidant activity. We believe that the developed

capsanthin SNEF can have a variety of nutraceutical and cosmeceutical applications.

CONCLUSION

SNEF of capsanthin was developed by simply mixing optimized concentration of Captex 300 (medium chain triglyceride), Cremophor RH 40, Capmul MCM (medium chain mono- and diglyceride), and BHT as an antioxidant. The optimized formulation showed ~8-fold enhancement in dissolution as compared with pure carotenoid. The 3-month stability studies of capsanthin SNEF confirmed its physical and chemical stability and retention of the antioxidant property. Such a formulation could serve as a natural food colorant that can be easily mixed with water-based beverages and foods and oil-based foods. The formulation could serve as a nutraceutical supplement upon confirming its oral bioavailability *in vivo*.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Table S1

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Abbreviations Used

BHT = butylated hydroxyl toluene
H₂O₂ = hydrogen peroxide
SNEF = self-nanoemulsifying formulation