# Enhanced Chemical Stability of Hirsutenone Incorporated into a Nanostructured Lipid Carrier Formulation Containing Antioxidants

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To enhance the chemical stability of hirsutenone (HST), a natural diarylheptanoid, nanostructured lipid carrier (NLC) formulations containing antioxidants were employed. The stability and shelf-life of HST-containing formulations were assessed by using accelerated aging tests. Drug-free NLC formulations, using Compritol 888 ATO as a solid lipid, Labrafil M1944 CS as an oil, and Tween 80 as a surfactant, had a particle size of approximately 177 nm, a polydispersity index of 0.257, and a zeta potential of −15.5 mV. Drug- or antioxidant-loading slightly increased the particle size but did not alter the surface charge of the NLC formulations. HST entrapment efficiencies were 86.83% and 88.19% for antioxidant-free and antioxidant-added NLC formulations, respectively. NLC formulations extended the half-life of HST relative to aqueous solutions, but the stabilizing effect was unsatisfactory. The use of 1% ascorbyl palmitate (AP) and 1% ethylenediaminetetraacetic acid (EDTA) in the NLC formulations resulted in the greatest stabilization of HST, with a half-life of approximately 120 days at 40°C. The stability assessments of different NLC formulations were conducted based on the first-order kinetics and the shelf-life at different temperatures was estimated by using the $Q_{10}$ method. Assuming a $Q_{10}$ value of 2, the shelf-life of AP/EDTA-added NLC formulations at 25°C and 4 °C was 51.2 and 219.5 days, respectively. Although the HST stability was considerably enhanced by NLC formulation and further improved by adding antioxidants, alternative studies to assure the sufficient stability and enable the practical application of NLC formulations in product development are still required.

**Keywords:** Hirsutenone, Lipid carrier, Antioxidant, Shelf-life

## Introduction

Hirsutenone (HST), a major active diarylheptanoid isolated from the bark of *Alnus japonica*, inhibits the nitric oxide synthesis and COX-2 expression.[1–3] Recently, it was introduced as a natural immunomodulator for the treatment of atopic dermatitis (AD).[4] Although the partial disturbances of the skin barrier in AD may increase drug permeation through the skin, an advanced delivery system is still required for developing effective topical preparations.[5] Several techniques, including chemical enhancers, creams, and vesicular carriers, have been investigated as potential enhancers of skin delivery.[6] The topical application of HST-containing cream formulations decreased the eosinophil count and the expression of Th2-related cytokines and IgE inflammatory factors, subsequently inhibited the development of AD-like skin lesions in NC/Nga mice.[7] Kang *et al*. developed a Tat peptide-admixed elastic liposomal system containing HST. This liposomal system significantly improved the skin delivery of HST, greatly improving immune-related responses and therapeutic efficacy.[8]

Despite outstanding pharmacological effects and great potential as a drug product, the use of HST has been hindered by its poor chemical stability. The stability of HST in aqueous solution is very poor, with a half-life of less than 1 week at 20°C. HST is readily hydrolyzed in aqueous solution through the oxidation of its double bond.[9] To stabilize a labile drug from oxidative degradation, various strategies have been investigated, such as the addition of antioxidant, flushing with nitrogen gas, and storage in a frozen state.[10,11] In particular, as an antioxidant, the following excipients such as ascorbic acid (AA), alpha lipoic acid, ethylenediaminetetraacetic acid (EDTA), propyl gallate, ascorbyl palmitate (AP), carotene, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) have been frequently used.[12–16] In addition, various pharmaceutical techniques, including solid dispersion, inclusion complexes, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), liposomes, and micelles, are commonly employed to protect drugs from oxygen and moisture.[17–20] Ahn *et al*. successfully prepared HST inclusion complexes

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with β-cyclodextrin by using a solvent evaporation method; however, the stability of HST was not significantly improved.[21]

Lipid-based solid nanoparticles have exhibited promise as drug delivery systems, either as SLNs or NLCs, with the potential to replace vesicular carriers such as liposomes and micelles. The application of lipid nanoparticles has been widely investigated.[22] In particular, the topical use of SLNs and NLCs has been investigated to enhance skin permeation and hydration via occlusive film formation, modulation of drug release, and localization on the dermal layer.[23] Compared with liquid nanovesicular systems, SLNs and NLCs could reduce the mobility of the encapsulated drug and confer protection to the drug owing to their predominant composition of solid lipids. By virtue of the solid lipid matrix, it is possible to achieve controlled drug release and improve physical and chemical stability. SLNs have some limitations, including drug expulsion during storage and the low drug encapsulation capacity. NLCs, composed of solid lipid and liquid oil, were recently developed to overcome the drawbacks of SLNs.[24] The oil components of NLCs facilitate a higher drug-loading capacity and reduce the problem of drug expulsion. The advantages of NLCs over SLNs include the low moisture content in the particle and reduced tendency for unpredictable gelation.[25] Chemically labile drugs have been successfully incorporated into NLCs to improve the stability. Coenzyme Q10-loaded NLC dispersions demonstrated better long-term physical and chemical stabilities than nanoemulsion formulations.[18] Retinol[26] and ascorbyl palmitate[27] were stabilized by incorporation into NLCs.

Therefore, in this experiment, we prepared HST-containing NLC systems and evaluated their characteristics with regards to particle size, zeta potential, and drug loading. In addition, we screened various lipophilic antioxidants for their potential to enhance the chemical stability of HST. Stability assessments of different NLC formulations were conducted based on first-order kinetics and shelf-lives at different temperatures were calculated by using the $Q_{10}$ method.

## Experimental

**Materials.** HST (purity >95%, as determined by HPLC) was provided from Chung-Ang University Pharmacognosy Laboratory (Seoul, Korea). Compritol 888 ATO (glyceryl behenate) and Labrafil M 1944 CS (Oleoyl macrogol-6 glycerides) were kindly provided by Gattefosse (Saint-Priest, France). Poloxamer 188 and Tinocare GL were provided by BASF (Ludwigshafen, Germany). Miglyol 810N (caprylic/capric triglyceride) and Miglyol 840 (propylene glycol dicaprylate/dicaprate) were provided by SASOL (Witten, Germany). Carbopol 934P NF polymer was purchased from Lubrizol (Wickliffe, OH, USA). Tween 80, urea, BHT, EDTA, EDTA-Na, AP, and α-tocopherol (TC) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals and reagents were purchased from commercial sources and were of analytical grade. Double-distilled water was used for all experiments.

**HPLC Determination of HST Content.** The quantitative determination of HST was performed by using HPLC with an isocratic mobile phase of acetonitrile/0.3% acetic acid in water (25:75, v/v), at a flow rate of 1 mL/min. The HPLC system consisted of a pump (L-2130, Hitachi, Japan), UV detector (L-2400, Hitachi, Japan), data station (LaChrom Elite, Hitachi, Japan), and a 15 cm $C_{18}$ column (Capcell Pak, 4.6 × 150 mm, 5 μm; Shiseido, Japan). The column eluent was monitored at 220 nm and the HST peak was separated with a retention time of 16.0 min. The HST calibration solution was prepared from HST dissolved in the mobile phase at concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL. The least-square regression was linear in the range 0.05–100 μg/mL, with a coefficient of determination ($r^2$) value of >0.99.

**Solubility of HST in Various Oils.** The solubility of HST was evaluated in various oils (Miglyol 810 N, Miglyol 840, oleic acid, grape seed oil, olive oil, and Labrafil M 1944 CS) by using the equilibrium method.[28] An excess of HST (100 mg) was added to the oil (1 mL) in a conical tube and shaken for 24 h. After 24 h, each sample was centrifuged at 12000 ×*g* for 10 min (Micro 17TR, Hanil Science, Korea), and 450 μL of the obtained supernatant was passed through a 0.45 μm PVDF syringe filter. The filtrate was diluted with 450 μL of methanol and the HST content was analyzed by the HPLC method described above.

**Preparation of NLC Dispersions.** NLC dispersions were prepared by using a high pressure homogenization (HPH) method.[25] Briefly, Compritol 888 ATO (solid lipid) and Labrafil M 1944 CS (oil), with a total lipid content of 5 w/v%, were melted together in a water bath maintained at 85°C, and HST was dissolved into the lipid phase. The aqueous phase containing Tween 80 (2.5 v/v%) was also heated in the same water bath. Subsequently, the aqueous phase was added to the melted lipid mixture and the mixture was sonicated in a bath-type sonicator (Model 2210, Branson Ultrasonics Co., Danbury, CT, USA) maintained at 85°C to produce a coarse pre-emulsion. The hot pre-emulsion was passed through a microfluidizer (500 bar, 10 cycles; Microfluidizer® M-110S, Microfluidics, Newton, MA, USA), which produced a clear nanodispersion. The NLC dispersion was cooled at room temperature and stored at 8 °C until use. To produce the HST-loaded NLCs with antioxidants, each antioxidant was added separately to the melted lipid phase, with the subsequent procedures performed using the same method and instruments described above.

**Particle Size and Zeta Potential Measurement.** NLC dispersions were diluted 1000-fold with distilled water and 1 mL of the diluted NLC dispersions was added to disposable capillary cells (Malvern Instruments, Worcestershire, UK). The particle size, zeta potential, and polydispersity index were measured by photon correlation spectroscopy

  

using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) with a 50 mV laser at a scattering angle of 90°. All measurements were performed in triplicate under ambient conditions.

**Encapsulation Efficiency.** The drug encapsulation efficiency (EE) of HST in the NLC formulation was determined by using an ultrafiltration centrifugation method and expressed as a percentage.[29] The quantity of unencapsulated HST was measured by ultrafiltration using centrifugal filter tubes (Amicon Ultra-4; EMD Merck Millipore, Billerica, MA, USA) with a molecular weight cut-off of 30 000 Da. Unencapsulated and total amount of HST were measured by using HPLC, and the ee (%) was calculated from the following equation: $[(A - F)/A] \times 100$, where $A$ is the total amount of HST added and $F$ is the amount of free HST found in the ultrafiltrate after centrifugation. To determine the HST content in the NLC formulations, 100 μL of the NLC dispersion was diluted with 900 μL of methanol and sonicated in a bath-type sonicator at 50°C for 10 min. After cooling, 1 mL of the solution was filtered using 0.45 μm PVDF syringe filter and the concentration of HST in the filtrate was assayed by using HPLC.

**Accelerated Stability Measurement.** The chemical stability of HST in the NLC formulations was evaluated for 4 weeks under the accelerated aging condition (40°C ± 2 °C and 75% ± 5% relative humidity (RH)). The change in the HST content of the solution was considered to represent an index of chemical stability. All samples were placed in a Teflon-capped siliconized glass vial at 40°C and 75% RH. The content of HST in the NLC dispersion was quantified by using HPLC at the predetermined times. Finally, the percentage of HST remaining in the NLC dispersion was calculated. The control experiments used a co-solvent system composed of distilled water and methanol (9:1, v/v) as the vehicle to dissolve HST. The content of HST in the aqueous solution was determined by using the procedure described above. To improve HST stability, the lipophilic antioxidants were loaded into NLC formulations containing HST and in order to find the most effective antioxidant, various lipophilic antioxidants, such as TC, AP, BHT, and EDTA, were added at different concentrations during NLC preparation. The initial HST concentration was 200 μg/mL for all samples and the changes in HST content were monitored by using HPLC.

**Table 1.** Solubility of HST in various oils.

| | Maximum HST concentration (mg/mL) |
|---|---|
| Miglyol 810N | 3.20 |
| Miglyol 840 | 5.57 |
| Oleic acid | 1.28 |
| Grape seed oil | 0.95 |
| Olive oil | 1.67 |
| Labrafil M 1944 CS | 30.21 |

**Statistical Analysis.** All data were reported as the mean ± S.D. Statistical significance was evaluated by Student's *t*-test and considered to be achieved at $P$ values of $<0.05$, unless otherwise indicated.

## Results and Discussion

**Formulation of HST-loaded NLCs (HST-NLCs).** HST-NLCs were formulated with solid lipid, oil, and surfactant. Compritol 888 ATO (glyceryl behenate) was selected as the solid lipid. It has a suitable structure for topical application, is easily compatible with the skin barrier,[30] and shows higher occlusive properties because its lipid matrix has a higher degree of crystallinity than other conventional lipids such as Dynasan 112 and Softisan 154.[31] The solubility measurements of HST in various oils were used to select the oil component. The greater the solubility of HST in oil, the higher the expected drug loading of the NLCs. Among the various oils tested, Labrafil M 1944 CS was selected as a liquid oil because it was able to solubilize the greatest amount of HST (30.21 mg/mL; Table 1). Finally, Tween 80 was selected as the surfactant. Tween 80, a non-ionic emulsifier, improves the physical stability of NLCs through electrostatic repulsion and a steric stabilization effect.[32] In addition, the NLC formulation containing Tween 80 enhanced skin permeation compared with an NLC formulation containing Pluronic F68.[33] Although the total percentage of the lipid phase (Compritol 888 ATO and Labrafil M 1944 CS) was kept at a constant value of 5%, total solid lipids have been mixed with liquid oils, in a preferred ratio of 70:30 up to a ratio of 99.9:0.1.[23] Thus, to encapsulate HST, we selected the lipid-to-oil ratio of 70:30.

**Physical Characteristics of HST-NLC Dispersions.** The properties of the HST-NLC dispersions were characterized by particle size, polydispersity index, and zeta potential (Table 2). The particle size of the drug-free NLC formulation was approximately 177 nm with a polydispersity index of 0.257, which indicated a narrow particle size distribution. A particle size of below 200 nm is considered an ideal size for skin occlusion and dermal delivery: when the occlusive effect was evaluated for particle sizes between 200 and 4000 nm, particles of 200 nm showed the highest occlusive effect.[34] The particle size of HST-NLCs was increased slightly by the entrapment of antioxidant, but remained approximately 200 nm (Figure 1). Drug-loading or antioxidant addition did not affect the surface charge of NLCs, which zeta potentials in the range from −15 to −20 mV. The encapsulation efficiency was high as approximately 86–88%, because HST can be easily partitioned into lipid components owing to its high lipophilicity (log $P$ = 5.0). During the preparation of the NLC formulations, HST was initially dissolved in the melted lipid part before mixing with the water phase. The high solubility of HST in the selected oil (Labrafil M 1944 CS) and the relatively high lipid concentration (5%, w/w) supported this high entrapment efficiency.

  

**Table 2.** Physicochemical characteristics of NLCs.

| | Drug-free NLC | HST-NLC | HST-NLC + 1% AP + 1% EDTA |
|---|---|---|---|
| Size distribution (nm) | 177.59 ± 3.58 | 187.57 ± 1.60 | 200.03 ± 1.94 |
| Polydispersity index | 0.257 ± 0.082 | 0.238 ± 0.113 | 0.388 ± 0.174 |
| Zeta potential (mV) | −15.55 ± 0.06 | −16.70 ± 0.26 | −20.30 ± 1.05 |
| Encapsulation efficiency (%) | — | 86.83 ± 0.56 | 88.19 ± 2.31 |

The data are presented as the mean ± SD ($n$ = 3).

**Chemical Stability of HST in NLC Dispersion.** We investigated the effects of the NLC formulation on the chemical stability of HST at 40°C. It was found that the stability of HST was significantly improved by incorporation into the NLC formulation (Figure 2). In aqueous solution, the content of HST decreased rapidly, but in the NLC formulation, the HST content decreased more slowly. The half-life of HST was 2.96 to 9.50 days for the aqueous solution and NLCs, respectively. As it is known that HST is decomposed by hydrolysis, it is plausible that the solid matrix of NLC partially immobilizes HST, conferring protection from the aqueous environment. Several studies have demonstrated that the incorporation of drug into NLCs could effectively enhance the stability of labile drugs by shielding them from the reactive environment.[18,35] Junyaprasert *et al*. demonstrated that the chemical stability of coenzyme Q10 entrapped in NLCs was higher than that in the nanoemulsion. Under the accelerated aging condition of 40°C, although the NLC formulation improved the chemical stability of HST, the half-life was still too short (less than 10 days) for the development of a practical formulation; thus, further stabilization methods, including antioxidant addition, were considered.

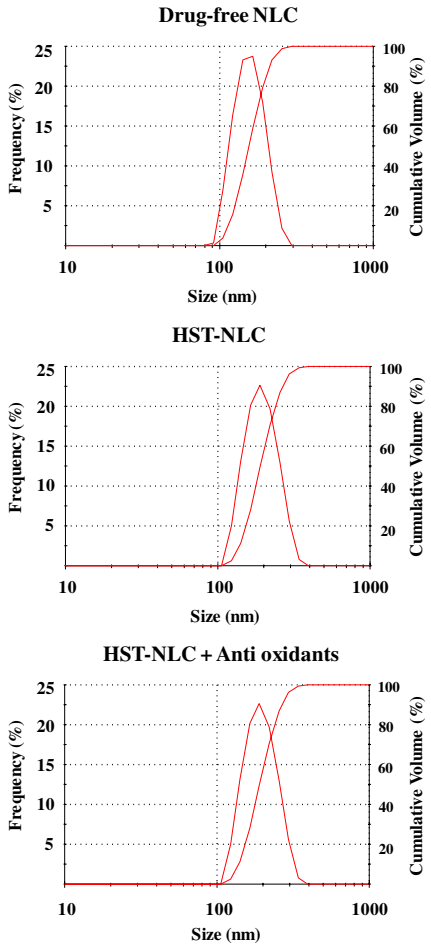


**Figure 1.** The particle distribution graphs of different NLC formulations.

**Antioxidant Screening for HST Stabilization.** The stability of HST in the NLC dispersion in the presence of antioxidants is shown in Figure 3. AP was the most effective agent for the prevention of HST degradation. After 7 days of incubation at 40°C, more than 80% of HST remained intact in the AP-containing NLC formulation, whereas less than 50% of HST remained intact in antioxidant-free NLCs. EDTA also increased the stability of HST, with 70% of HST unaffected after 7 days. However, TC and BHC were not effective for stabilization, with no significant difference between them.

Numerous studies have reported the chemical stabilization of drugs through the employment of antioxidants and/or NLC formulations.[36] HST-loaded ointment with 0.1% EDTA extended the half-life of HST to 119.79 days.[10] Combinations of effective antioxidants were shown to result in greater improvements in stability; for example, the co-loading of BHT and butylated hydroxy anisole (BHA) in SLNs improved the stability of all-trans-retinol[26] and the highest chemical stability of AP was achieved in the presence of BHA, BHT, and vitamin E.[27] To obtain the most effective stabilization of HST for formulation development, the content of AP was increased

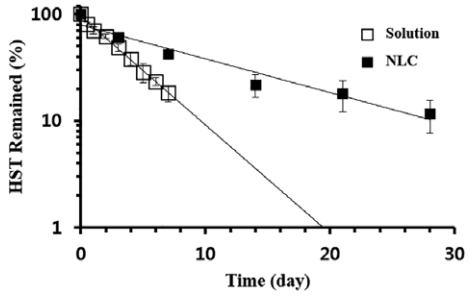


**Figure 2.** Chemical stability of HST in aqueous solution and NLC formulations under the accelerated aging condition of 40°C. The data are presented as the mean ± SD ($n$ = 3).

  

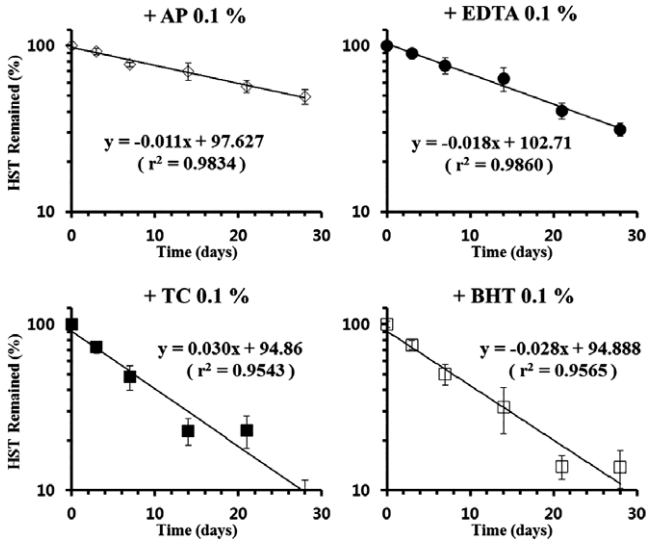


**Figure 3.** The effect of antioxidants on the chemical stability of HST in NLC formulations under the accelerated aging condition of 40°C. The data are presented as the mean ± SD ($n$ = 3).

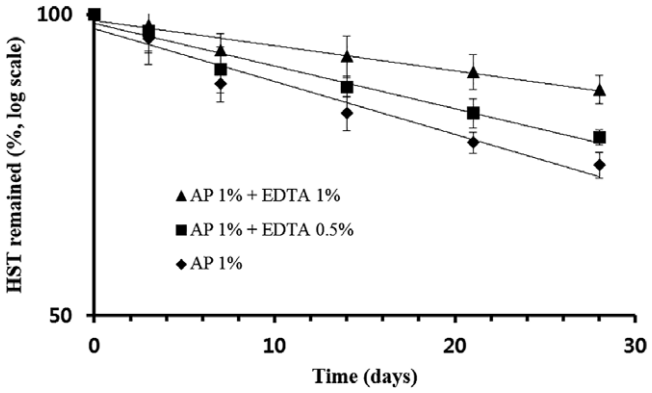


**Figure 4.** Combination effects of the selected antioxidants on the chemical stability of HST in NLC formulations under the accelerated aging condition of 40°C. The data are presented as the mean ± S.D. ($n$ = 3).

and the simultaneous use of EDTA was investigated further. As shown in Figure 4, the stability of HST increased proportionately to the content of AP and EDTA added. The degradation rate constants and half-lives of HST in NLC dispersions with or without antioxidants are listed in Table 3. The half-life of HST in NLC formulations was significantly prolonged by the combined use of AP and EDTA. EDTA is a well-known chelating agent for preventing an oxidative environment formation by chelating transition metal ions with a polydentate ligand; chelators lessen lipid oxidation in oil-in-water emulsions owing to the removal of metal iron around the dispersed droplet.[37] Consequently, the addition of 1% AP and 1% EDTA in the NLC formulation resulted in the greatest stabilization of HST, leading to a half-life of approximately 120 days at 40°C.

**Shelf-Life Estimation by $Q_{10}$ Method.** The $Q_{10}$ rule is generally used to estimate the shelf-life of drugs in solution where the drug degradation process is temperature-dependent. The stability of HST under the accelerated condition at 40°C could be used to predict the stability at room temperature (25°C) or refrigerated temperature (4 °C), as reported previously,[10] using the following equation: $t_{90}(T_2) = t_{90}(T_1) / Q_{10}^{(\nabla T/10)}$, where $t_{90}(T_2)$ is the shelf-life at elevated temperature $T_2$, $t_{90}(T_1)$ is the estimated shelf-life at temperature $T_1$, and $Q_{10}$ is a constant related to activation energy.[38] In general, the value of $Q_{10}$ is typically set at 2, 3, or 4, because these correspond to reasonable activation energies. A $Q_{10}$ value of 2 provides a conservative estimate; these results are most likely accurate. The activation energies for drug decomposition usually fall with a typical value of 19 to 20 kcal/mol which corresponds to a $Q_{10}$ value of 3.[39] In contrast, a $Q_{10}$ value of 4 is less conservative.[40] Thus, in this study, a $Q_{10}$ value of 2 or 3 was applied to estimate the shelf-life of the HST-NLC formulation with different oxidants (Table 4). At a $Q_{10}$ value of 2, the shelf-life of the antioxidant-free HST-NLC formulation at 25°C was 4.07 days; moreover, the addition of TC or BHT resulted in no significant effects. However, in the presence of AP and EDTA, HST-NLC had a longer shelf-life than the antioxidant-free formulation. In particular, HST-NLC formulations containing both 1% AP and 1% EDTA resulted in longest shelf-life, of approximately 220 days at 4 °C, whereas the shelf-life of antioxidant-free

**Table 3.** Chemical stability of HST in aqueous solution and NLC formulations under the accelerated aging condition of 40°C.

| Antioxidant added | Rate constant ($\times 10^{-2}$, day$^{-1}$) | Half-life (day) | Shelf-life (day) |
|---|---|---|---|
| None (solution) | 23.41 | 2.96 | 0.45 |
| None (NLC) | 7.29 | 9.50 | 1.44 |
| AP 0.1% | 2.50 | 27.72 | 4.20 |
| EDTA 0.1% | 4.19 | 16.54 | 2.51 |
| TC 0.1% | 6.88 | 10.07 | 1.53 |
| BHT 0.1% | 6.52 | 10.63 | 1.61 |
| AP 1% | 1.22 | 56.80 | 8.61 |
| AP 1% + EDTA 0.5% | 0.98 | 70.71 | 10.71 |
| 1% + EDTA 1% | 0.58 | 119.48 | 18.10 |

AA, ascorbic acid; AP, ascorbyl palmitate; BHT, butylated hydroxytoluene; EDTA, sodium diedetate; TC, α-tocopherol.

  

**Table 4.** Shelf-life estimation for HST stability in NLC formulations with different concentrations of antioxidants.[a]

| | $Q_{10}$ = 2 | | $Q_{10}$ = 3 | |
|---|---|---|---|---|
| Antioxidant added | 25°C | 4 °C | 25°C | 4 °C |
| None | 4.07 | 17.47 | 7.48 | 75.18 |
| AP 0.1% | 11.88 | 50.93 | 21.82 | 219.22 |
| EDTA 0.1% | 7.09 | 30.39 | 13.02 | 130.80 |
| TC 0.1% | 4.33 | 18.55 | 7.95 | 79.85 |
| BHT 0.1% | 4.55 | 19.52 | 8.37 | 84.03 |
| AP 1% | 24.34 | 104.36 | 44.72 | 449.23 |
| AP 1% + EDTA 0.5% | 30.30 | 129.92 | 55.67 | 559.24 |
| AP 1% + EDTA 1% | 51.20 | 219.52 | 94.07 | 944.93 |

[a] Shelf-life was estimated by the $Q10$ rule, employing the temperatures of T1 (25°C or 4 °C) and T2 (40°C).

HST-NLC was 17.47 days. Nevertheless, this stability was unsatisfactory. Applying a $Q_{10}$ value of 3, the shelf-life of antioxidant-free HST-NLC at 25°C was 7.48 days. The addition of 1% AP and 1% EDTA extended the shelf-life by at least 3 months. At 4 °C, the shelf-life of HST-NLCs in the absence and the presence of antioxidants was calculated as 75.18 and 944.93 days, respectively. Overall, the predicted shelf-lives of all tested formulations.at 25°C or higher temperature was unacceptable. However, refrigerated storage at 4 °C provided an acceptable stability for the HST-NLC formulation. Therefore, with respect to expiration period, the combination of 1% AP and 1% EDTA in HST-NLCs could offer the refrigerated stability for approximately 2 years.

## Conclusion

The NLC formulation composed of Compritol 888 ATO as a solid lipid, Labrafil M1944 CS as an oil, and Tween 80 as a surfactant, extended the half-life of HST relative to the aqueous solution. The use of 1% AP and 1% EDTA in the NLC formulation resulted in the greatest stabilization of HST. However, alternative approaches for further stabilization and practical applications of NLC formulations for future product development are still required.

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