碩士學位論文

Emulsion Characteristics and Oxidative Stability of Horse Oil-in-Water Emulsion as Impacted by HLB System and α-Tocopherol Addition

濟州大學校 大學院

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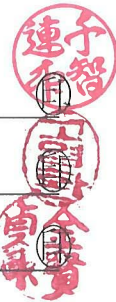
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ABSTRACT

Horse oil, a popular material used in cosmetic products, has some defects in an intense oily feel and unpleasant odor when applied to the skin. To avoid these problems, the optimal condition was determined to prepare horse oil-in-water (HO/W) emulsion stabilized by different HLB system. Span 60 and Tween 60 were used to achieve the predetermined HLB values ranging from 10 to 14 and the surfactant concentrations were adjusted to 10-20%. Fifteen formulated HO/W emulsions were characterized by measuring mean particle diameter (nm), zeta-potential (ZP, mV), polydispersity index (PDI), and encapsulation efficiency (EE, %) during storage of 15 days at 40°C. The mean particle diameter of the HO/W emulsion decreased with the increase of HLB value and surfactant concentration. The HO/W emulsions formulated with HLB 11 and 12 showed the highest ZP value. Particles of the HO/W emulsion formulated with HLB 12 and surfactant concentration at 15% were distributed in the size of below 500 nm. The particle diameter and EE (%) of the HO/W emulsions with HLB 11 or 12 and surfactant concentration at 15 or 20% were not significantly changed during storage. These results suggested that the characteristics of HO/W emulsions were dependent on HLB values and surfactant concentration so that affected to emulsion properties during storage.

Emulsion is a key type of materials used to make healthcare or cosmetic products; however, the emulsion made with horse oil has not much data or research at present. In this study, the horse oil-in-water (HO/W) emulsion was prepared and α-tocopherol was added at 0, 100, 200, and 500 ppm to enhance the oxidative stability of it. The mean particle diameter of the HO/W emulsions was varied by changing the concentration of the α-tocopherol. Zeta-potential values of the HO/W emulsion were increased with the addition

of α-tocopherol; however, they were decreased during storage at 40℃ for 30 days. The particle size distribution of the HO/W emulsion with α-tocopherol remained relatively the original size compared to the emulsion without α-tocopherol. For the oxidation of the HO/W emulsion, the peroxide values of the emulsion without α-tocopherol and with α-tocopherol at 500 ppm were greatly increased from 2.96 and 2.89 to 13.76 and 12.46 mmol/kg oil after 30 days. The HO/W emulsions added with α-tocopherol at 100 and 200 ppm were kept in lower peroxide value than other emulsions during storage. The TBARS values of the HO/W emulsion without α-tocopherol and with α-tocopherol at 500 ppm were higher than other emulsions during storage. These results indicated that the addition of α-tocopherol from 100 to 200 ppm to the HO/W emulsion effectively improved the emulsion stability along with the oxidative stability during storage.

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PART Ⅰ

Formulation and Stability of Horse Oil-in-Water Emulsions by HLB System

1. Introduction

Horse oil extracted from horse fatty meats has been widely used to cure skin as a folk medicine in ancient Asia (Jang et al., 2014). Along with this benefit, horse oil has been reported to show anti-inflammation, anti-bactericidal, and skin moisturizing effects (Choi et al., 2014). As these benefits of horse oil are known, the number of cosmetic industries to manufacture horse oil and suppliers of horse meat by-products have been increased (Kim et al., 2016; Lee et al., 2013). However, horse oil has a problem with the physical property because it can be liquid or solid depending on the ambient temperature since the ratio of unsaturated and saturated fatty acids is 6:4 (Park et al., 2019). This makes consumers unpleasant to use horse oil as skin care products.

The type of emulsion can be a solution to avoid this physical property, which maintains a sol form at room temperature. Emulsions have been applied for various cosmetic products, such as body and face wash, essence, cream, and lotion depending on their viscosity or appearances (Ahn et al., 2004; Lim, 2004). Oil-in-water (O/W) emulsions are typical emulsions that the oil phase is dispersed in aqueous phase. These emulsions contain particles with a mean particle diameter of more than 1 μm and are thermodynamically unstable system that is liable to destruct (Mun et al., 2006). Nano-sized emulsions are the oil-in-water colloidal dispersions, usually in 20 - 500 nm

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particle size distributed in the system with good physical stability (De Oca-Ávalos et al., 2017; Porras et al., 2004). Generally, nano-emulsions are prepared by high energy emulsification methods using high-speed homogenizer and high-pressure homogenizer to produce nano-scale particles (Mulia et al., 2018.; Wang et al., 2018). Nano-particles are formed when correct compositions of surfactant hydrophilic-lipophilic balance (HLB), oil phase, and surfactant concentration are mixed in the right sequences (Llinares et al., 2018). Additionally, nano-emulsions possibly lead to enhance the therapeutic efficacy by maximizing the useful component penetration into the skin with maintaining the emulsion stability (Jaiswal et al., 2015; Kale and Deore 2017).

The oil-in-water emulsion with less than 100 nm of particle size was formed by formulation of nonionic surfactant mixtures, a combination of hydrophobic (Span 20, 40, 60, and 80) and hydrophilic (Tween 80) surfactants (Cho et al., 2008). According to Griffin (1949), a stable emulsion can be formulated with surfactants or blends of surfactants, which were compatible with the required HLB value of the oil phase used. The utilization of surfactant mixtures increases the repulsive interactions of particles in the emulsion and furthermore improves their flocculation stability (McClements and Jafari, 2018). The required HLB value is an essential parameter related to the emulsion formulation because it allows the selection of suitable surfactants or even a surfactant mixture required to produce a stable emulsified system (Egito et al., 2018). The HLB method is useful as a rough guide for surfactant selection and the calculation values can be used to determine hydrophilic or lipophilic of the emulsifiers in oil-in-water or water-in-oil emulsion. Non-ionic surfactants are generally recognized as being safe and biocompatible and are not affected by pH changes in media (Sanjeewani and Sakeena, 2013).

Currently, there are not many studies or data on formulating emulsion with

horse oil. Therefore, this study was conducted to prepare horse oil-in-water emulsions based on two mixed non-ionic surfactants by adjusting HLB system and to determine the characteristics and emulsion stability of the prepared HO/W emulsions during storage.

2. Materials and Methods

2.1. Materials and chemicals

Horse fatty meats were purchased from Sansaemi Agricultural Co. (Jeju, Korea). Sorbitan monostearate (Span 60) and polyethylene glycol sorbitan monostearate (Tween 60) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals used in the current study were analytical grade.

2.2. Extraction of horse oil

Horse oil was extracted from horse fatty meats by the method of Cho and Kim (2019). In detail, horse fatty meats were chopped with a meat grinder (MN-22S, Hankook Fujee Industries, Hwaseong, Korea) and mixed with water (1:1, w/w). The primary rendering of horse fatty meats was carried out using a vacuum low temperature extractor (Cosmos-660, Kyungseo E&P, Incheon, Korea) at 70°C and -0.1 MPa for 3.5 h. After extraction, the oil part was separated and the remained fatty meats were refined again with water (1:1) for 3.5 h. The first and second refined horse oil were collected and extracted with water (1:1) for another 3.5 h. The refined horse oil was finally gathered, filtered, and neutralized with 0.2% sodium hydroxide to remove free fatty acids. The impurities of the neutralized horse oil were removed by placing them in a stainless steel at 100°C for 30 min. The final refined horse oil was stored in a freezer until use.

2.3. Preparation of horse oil-in-water (HO/W) emulsion

The horse oil-in-water (HO/W) emulsions were prepared based on the HLB (hydrophilic lipophilic balance) system, which binary nonionic surfactants with low and high HLB values were combined. As binary surfactants, Span 60 and Tween 60 were used to achieve the predetermined HLB values of 10, 11, 12, 13, and 14 (equation (1)). The surfactant blends were directly added to horse oil at the concentration of 10, 15, or 20%. The mixture of horse oil (30%, w/w) and surfactant was pre-heated at 60°C and homogenized at high speed using a homogenizer (T25D, Ika, Staufen, Germany) at 10,000 rpm for 1 min. Ultrapure water (50, 55, or 60%) heating at 60°C was poured to the homogenized mixture for 5 min at 60°C and the final mixture was stirred at room temperature for 20 min. Fifteen formulations of the HO/W emulsion (E1 to E15) were prepared and the composition of them is shown in Table 1. After emulsifying, the formulated emulsions were poured into 60 mL-wide neck glass bottles and then stored at 40°C in the dark for storage test.

$$\% \, \text{Span} \, 60 = \left[\frac{(\text{X} - \text{HLB}_{\text{Span60}})}{\text{HLB}_{\text{Tween60}} - \text{HLB}_{\text{Span60}}} \right] \times 100 \qquad (1)$$

% Tween 60: 100% - % Span 60

X: the target HLB value of 10, 11, 12, 13, and 14

2.4. Characterization of the HO/W emulsion

The mean particle diameter, zeta-potential (ZP), polydispersity index (PDI), and particle size distribution of the HO/W emulsions were determined using a light scattering analyzer (Delsamax pro, Beckman Coulter, Brea, CA, USA). For the measurement, the HO/W emulsions were diluted to 500-fold with distilled water to prevent multiple scattering. The pH values were measured

at 25°C by a pH meter (SevenExcellence, Mettler‒Toledo, Schwerzenbach, Switzerland). Encapsulation efficiency (EE) was measured by the method of Davidov‒Pardo and McClements (2015) as details are followed. The HO/W emulsions were diluted in hexane (1:3), vortexed for 2 min, and centrifuged at 2,700 × g for 15 min (1248R, Labogene Co., Daejeon, Korea). The supernatant of the solution was collected and the absorbance at 272 nm was measured using a UV‒visible spectrophotometer (OPTIZEN 2120UV, Mecasys, Daejeon, Korea). The absorbance of the horse oil concentrations ranging from 0 to 10% was obtained at 272 nm for the calibration curve. The EE (%) of the HO/W emulsion was calculated by the following equation:

$$EE\,(\%) = \left[\frac{(A-B)}{A}\right] \times 100 \qquad (2)$$

A: Amount of horse oil initially added to the emulsion.

B: Amount of horse oil escaped the emulsion.

2.5. Statistical analysis

All experiments in this study were performed at least in triplicate. The results are given as means± standard deviation. Statistical comparisons were performed by analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Significant difference at $p < 0.05$ were considered.

Table 1. Composition of the HO/W emulsions formulated with horse oil content of 30% (w/w), HLB values of 10 to 14, and surfactant concentrations of 10, 15, and 20%

HO/W emulsions	HLB value	Surfactant (%, w/w)	Distilled water (%, w/w)
E1		10	60
E2	10	15	55
E3		20	50
E4		10	60
E5	11	15	55
E6		20	50
E7		10	60
E8	12	15	55
E9		20	50
E10		10	60
E11	13	15	55
E12		20	50
E13		10	60
E14	14	15	55
E15		20	50

3. Results and Discussion

3.1. Characteristics of HO/W emulsions

Fifteen HO/W emulsions were formulated with various HLB values and surfactant concentrations (Table 1). These formulations were prepared based on the assumption that the particle size of the HO/W emulsions were nano-sized and the emulsions were stable at the optimum HLB value and surfactant concentration of nonionic surfactant blends.

The characteristics of fifteen HO/W emulsions prepared with 10 to 14 of HLB values and 10, 15, and 20% surfactant concentrations were shown in Table 2. Briefly, the mean particle diameter, zeta-potential (ZP), polydispersity index (PDI), and pH value of the fifteen formulations were significantly different ($p<0.05$) depending on the HLB values and surfactant concentrations (w/w) of the HO/W emulsions. The mean particle diameters of the HO/W emulsions were decreased as increasing surfactant concentration from 10 to 15 and 20% (Table 2). Ramisetty et al. (2015) reported that the decrease in particle size of emulsion has been observed as increasing the surfactant concentration. Small sizes of particles in the emulsion made increase the emulsion stability and decrease the sedimentation or creaming to gravitational separation (Pathak et al., 2017). The high particle sizes of the HO/W emulsions formulated with 10% surfactant concentration might be caused by insufficient surfactant amounts to cover the interface of oil and water. Additionally, the particle sizes lowered with increasing HLB values ranging from 10 to 12 (Table 2). These were similar to the results of lippia and peppermint oil in water emulsions as reported by Orafidiya et al. (2002). These results represented that increasing the hydrophilicity of the surfactant blends led to the decrease of the particle size. The hydrophilic surfactants

with a larger head group compared with lipophilic surfactants generated larger particles. The mean particle diameter of E1, E2, E3, E4, E7, and E10 formulations did not belong to nano-sized (diameter < 500 nm) emulsion (Table 2).

The ZP value is a useful parameter for studying the emulsion stability because it reflects the electrostatic interaction of moving particles (Gutierrez G et al., 2008). High ZP values were reported that particle repulsion interactions can protect the emulsion from particle aggregation and eventually the interfacial membranes can prevent particle flocculation or coalescence (McClements, 2005). The particles of the HO/W emulsions were found to be negatively charged (Table 2). The ZP values of E5 and E6 formulations were the highest from -32.03 to -32.30 mV and E8 and E9 formulations were followed from -29.38 to -29.76 mV. There were no significant differences in the ZP values of the emulsions formulated with 15 and 20% surfactant concentrations except E6, E11, E14, and E15 formulations. The ZP values of the HO/W emulsions increased as increasing the HLB values of 10 to 11 or 12, which the ratio of Tween 60 (HLB 14.9) increased. The emulsion with high HLB value from the high concentration of Tween series surfactants is known to form the oxonium ions made by hydrogen bonds with hydroxyl groups and ether-oxygen in polyoxyethylene chain (Hong et al., 2018). The presence of oxonium ion can increase the ZP value; however, we observed that ZP values decreased when the HLB value was excessed to 12. The reason for this is that as HLB value increases, the solubility of particles increases so dispersed particles move to continuous phase. Eventually, the dispersed particles moved in continuous phase, coagulated them, and emulsion stability was decreased.

The PDI values of E2, E3, E5, E8, E9, and E12 formulations were 0.16 to 0.23 (Table 2), which indicated close to monodispersity of particles in the HO/W emulsion formulated with 15 and 20% surfactant concentration. The

PDI values above 0.3 are indicative of a polydisperse system. The polydispersed emulsion required high energy at the emulsification process and the less than 0.3 PDI meant sufficient energy was provided in the emulsification process (Izquierdo et al., 2002).

All of the HO/W emulsions were observed as alkalescency (Table 2). The pH value of all emulsion formulations increased with decreasing HLB value because of hydrophilic emulsifiers which contain carboxyl groups. The study of Macedo (2006) showed the similar trend that pH increases (in the case of an increasing ratio of Span 80) or decreases (in the case of an increasing ratio of Tween 20) according to the HLB value of the respective surfactant. Hsu et al. (2003) reported that the ZP value of particles is highly dependent on pH value and ZP value increased with the increase of pH. These results showed that the optimum value for HLB of the HO/W emulsion was determined to 11 or 12 with the surfactant concentration at 15%.

Table 2. Characteristics of the HO/W emulsions formulated with various HLB values and surfactant concentrations

HO/W emulsions	Mean diameter (nm)	Zeta-potential (mv)	Polydispersity index	pH
E1	1033.28±41.27[a]	−19.96±2.85[c]	0.19±0.04[c]	7.63±0.01[c]
E2	886.15±46.67[b]	−16.35±2.84[b]	0.16±0.06[c]	7.66±0.02[b]
E3	676.02±44.74[c]	−21.50±3.54[c]	0.20±0.08[c]	7.74±0.00[a]
E4	882.93±58.54[b]	−26.60±1.00[e]	0.14±0.10[c]	7.45±0.02[gh]
E5	386.83±4.70[ef]	−32.30±0.46[g]	0.23±0.01[c]	7.49±0.01[e]
E6	353.52±4.49[fg]	−32.03±0.92[g]	0.48±0.14[ab]	7.56±0.01[d]
E7	577.65±11.73[d]	−24.05±1.18[d]	0.44±0.00[b]	7.41±0.00[i]
E8	255.93±4.06[h]	−29.38±0.24[f]	0.21±0.00[c]	7.47±0.02[ef]
E9	240.52±3.29[h]	−29.76±0.42[f]	0.20±0.02[c]	7.46±0.01[fg]
E10	610.65±28.38[d]	−8.94±1.35[a]	0.56±0.01[a]	7.21±0.02[m]
E11	334.62±9.27[g]	−21.13±0.50[c]	0.52±0.12[ab]	7.34±0.01[j]
E12	222.17±4.74[h]	−23.82±0.68[d]	0.20±0.02[c]	7.43±0.02[hi]
E13	396.77±11.79[ef]	−19.59±0.83[c]	0.57±0.00[a]	7.24±0.02[l]
E14	246.70±4.89[h]	−21.37±0.32[c]	0.57±0.00[a]	7.26±0.01[l]
E15	220.28±3.72[h]	−20.72±0.51[c]	0.55±0.05[a]	7.28±0.01[k]

Each value is expressed as mean±standard deviation of triplicate determination.

Values with different letters in a column are significantly different by Duncan's multiple range test ($p < 0.05$).

3.2. Particle size distribution

Nano-emulsions are characterized by particle size and its distribution in the dispersed phase. Fig. 1. shows the particle size distribution of the HO/W emulsions formulated by HLB values ranging from 10 to 14 and 15% surfactant concentration (E2, E5, E8, E11, E14). The mean particle diameter of E2, E5, and E8 was 961, 522, and 343 nm (main particle diameter), respectively. The E11 and E14 obtained particles relatively wide ranges of 123 to 712 nm and 163 to 712 nm (two main particle diameter shown in Fig. 1). The particle size distribution of E2, E5, and E8 formulation with the HLB value of 10, 11, and 12 were towards low size. As shown in Fig. 1, the particle size distribution of E2, E5, and E8 emulsions become narrow as its HLB value increased from 10 to 11 or 12. The mean particle diameter of those emulsions declined from 886 to 256 nm as the HLB value increased from 10 to 11 or 12 (Table 2).

The narrow range and the high intensity in the particle size distribution means high protection from Ostwald ripening of particles in emulsions (Klaus et al., 2012). Because the relatively small particles in HO/W emulsion were more hydrophilic than large particles, they were merged to aqueous phase and coagulated with large particles. The particle size of this type of emulsion increases during storage and finally separates by creaming. Llinares et al. (2018) reported that the particle size distribution of rosemary oil-in-water emulsion tended to decrease as the HLB value increased, which results were similar to the current study. There was a shift toward high particle size distribution in the emulsions with the HLB values increasing from 12 to 14 as shown in an increase of polydispersity (Table 2). The E11 and E14 formulations had large polydispersity than E2, E5, and E8 formulations. The reason is that Tween 60 molecules (hydrophilic surfactant) were located at the oil-water interface and had large areas compared to Span 60 molecules.

As discussed above, the E8 formulation with HLB 12 and surfactant concentration at 15 % was nano-sized emulsion showing the particle distribution below 500 nm and narrow size distributions.
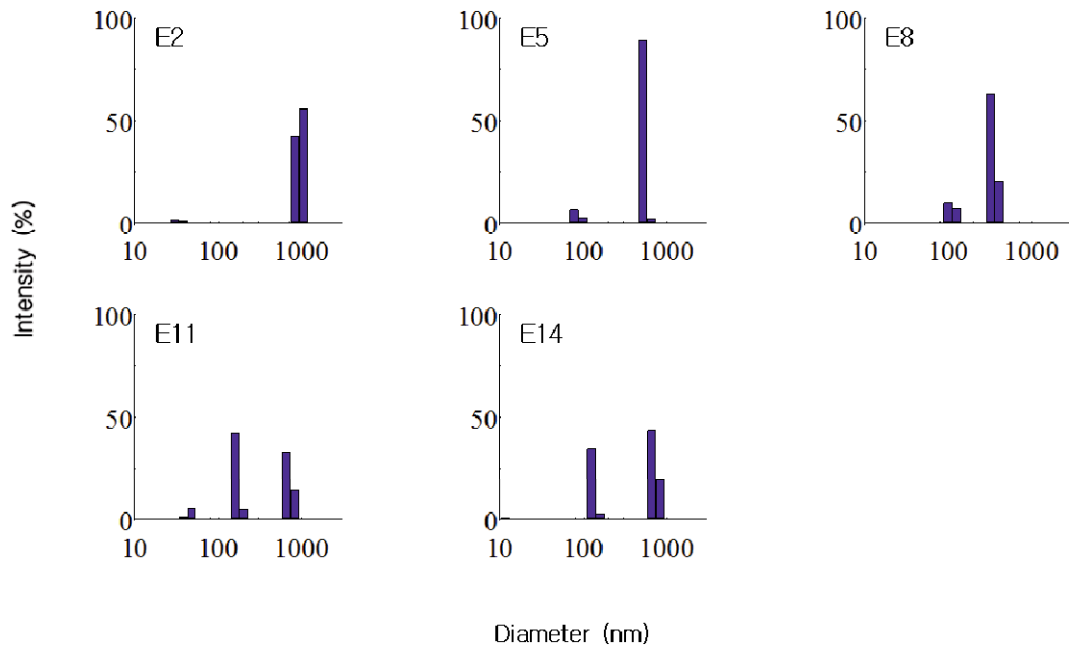
Fig. 1. Particle size distribution of the HO/W emulsions prepared with various HLB values. The HO/W emulsions of E2, E5, E8, E11, and E14 are made with 15% surfactant and HLB values of 10, 11, 12, 13, and 14, respectively.

3.3. Emulsion stability

Changes in particle sizes of selected HO/W emulsions formulated with HLB 10 to 14 and surfactant concentration of 15 and 20% during storage at 40°C for 15 days were shown in Fig. 2. The E2, E3, E5, and E6 emulsions (HLB 10 and 11) had large particle sizes at the beginning and the particles became smaller after 15 days of storage compared to those of 0 day. The particle size of E2 and E3 formulation decreased during storage, which occurred to sedimentation due to the high massive particles. Only the mean particle sizes of E8 and E9 formulation were not significantly changed during storage. The particle sizes of E11, E12, E14, and E15 formulations were increased with the increase of storage day at 40°C. These differences are attributed to the different HLB values. When the HLB value increased, emulsion destabilization was appeared because of the dispersed particles with highly hydrophilicity. The wide high-intensity particle size distribution of those formulations were observed (data not shown). As the concentration of Tween 60 ratio in surfactants blends increases, hydrophile of dispersed phase increases, shifts to continuous phase, and coagulates other particles in the dispersed phase (Yamashita et al., 2017). In principle, the Ostwald ripening is a spontaneous process implemented by chemical potential differences among different particle sizes at nano-sized (Tadros et al., 2004). Specifically, larger particle grows at the interchange of smaller ones which have a higher solubility than the large ones (Kabalnov and Shchukin, 1992 ; Djerdjev et al., 2008 ; Zhou et al., 2018). Consequently, the high concentration of tween60 (high HLB value) in oil in water will be lead to emulsion destabilization. When comparing E11 and E12 formulation, the particle sizes of E12 emulsion were not increased during storage compared to E11 because of surfactant concentration. There must be enough surfactants blends left after complete coverage of the oil-in-water interface to form the micelles in water phase, which will withdraw horse oil

molecules diffusing in the water phase to lower the Ostwald ripening rate. As the concentration of surfactants blends increases, the number of micelles in the water phase be generated, which is the cause for the reduced Ostwald ripening rate. Liu et al. (2006) reported that the main destabilized mechanism for the nano-emulsions has been found to be Ostwald ripening, with a reduced ripening rate as the nonionic surfactant concentration rises.

Changes in encapsulation efficiency (EE) of HO/W emulsions with HLB 10 to 14 and surfactant concentration 15 and 20% during storage were shown in Fig. 3(A) and 3(B), respectively. The EE (%) of the emulsions with 10% surfactant concentration (E1, E4, E7, E10, E13) was not shown since phase separation was observed during storage of 15 days at 40°C. The EE (%) of HO/W emulsions in E2, E5, E11, E14 formulation were 85.9, 88.9, 89.7, and 85.6% at 0 day, respectively, and after 1 day they were increased to 91.7, 92.6, 92.8, and 90.9%. However, only EE (%) of E8 formulation did not increase and not significantly change from 94.8 to 92.5%. This data indicated that not enough emulsification process except the E8 formulation. The EE (%) of E2, E5, and E8 formulation were not significantly changed during storage; however, the EE of E11 and E14 formulation decreased. Fig. 3B showed EE (%) of E3, E6, E9, and E12 formulations were not significantly changed during storage. The EE (%) of E3, E6, and E9 formulations were maintained at the high value (EE > 90%) for 15 days. Capan et al (1999) reported that the wrong emulsion stability led to low EE (%) which occurs in the interfacial arears between dispersed phase and continuous phase. These result showed that HO/W emulsions with oil content 30% can be encapsulated at the HLB value below 13 without increasing the surfactant concentration.
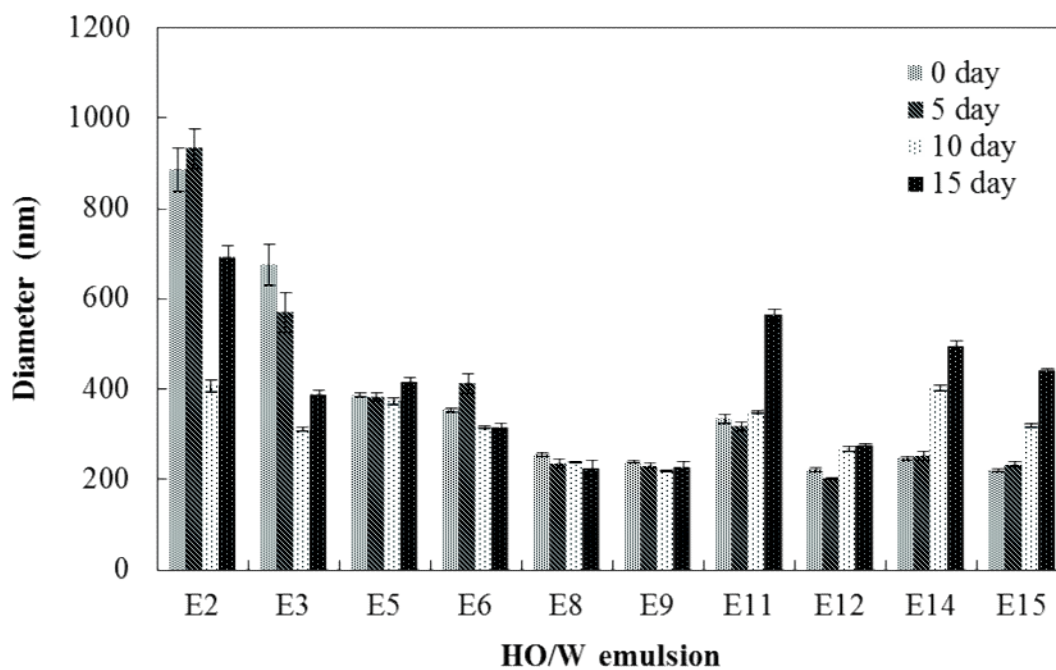
Fig. 2. Changes in particle sizes of the HO/W emulsions formulated with HLB 10 to 14 and surfactant concentration at 15 and 20% for the storage of 0, 5, 10, and 15 days at 40℃ in the dark.
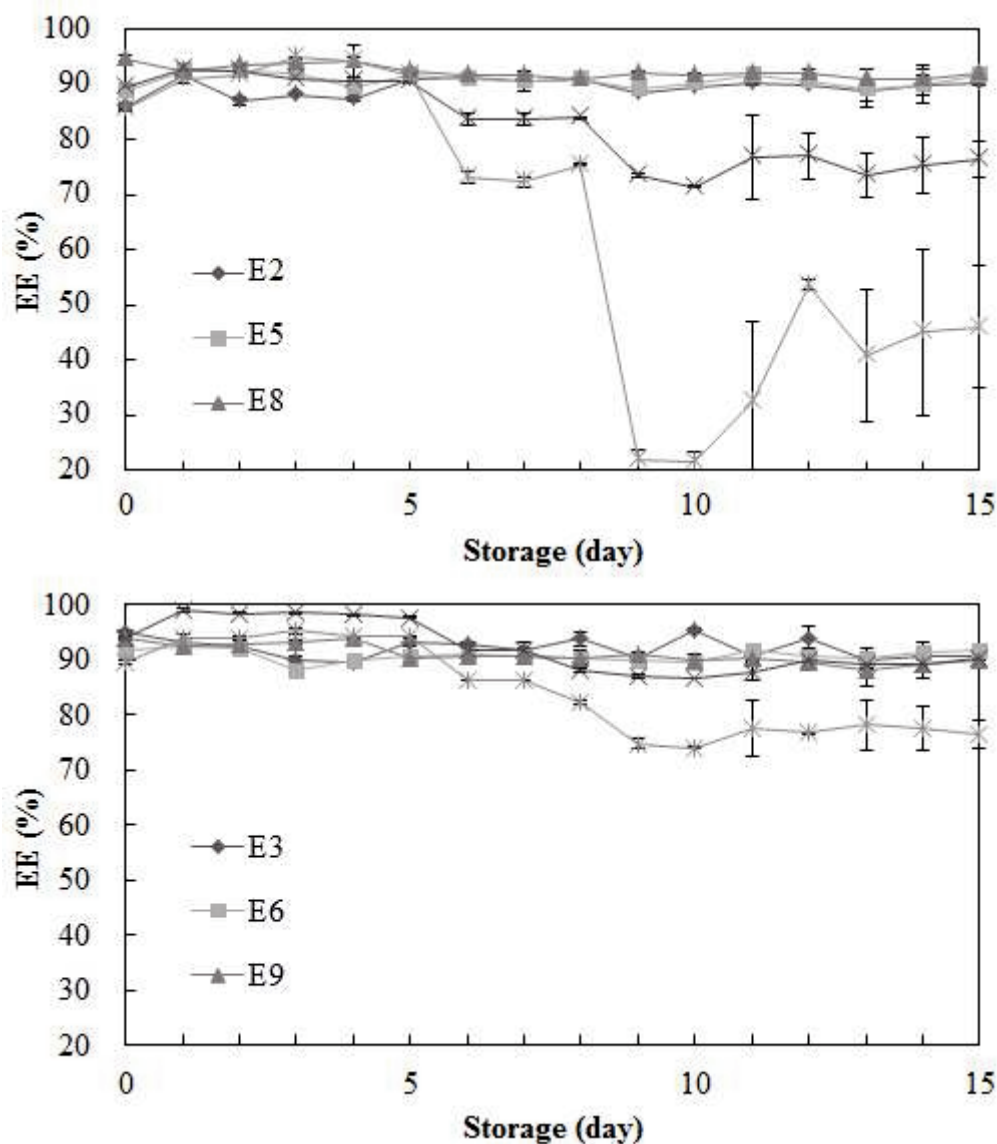
Fig. 3. Changes in encapsulation efficiency (EE,%) of the HO/W emulsions formulated with various HLB values and 15% (A) and 20% (B) surfactant concentration for the storage of 15 days at 40℃ in the dark. The HO/W emulsions of E2, E5, E8, E11, and E14 are made with 15% surfactant and HLB values of 10, 11, 12, 13, and 14, respectively (A). The HO/W emulsions of E3, E6, E9, E12, and E15 are made with 20% surfactant and HLB values of 10, 11, 12, 13, and 14, respectively (B)

4. Conclusion

The HO/W emulsions with different HLB value and concentration of surfactants blends were prepared with horse oil content 30%, ultrapure water at 50, 55, and 60%, and binary nonionic surfactants (Span60 and Tween60) at 10, 15, and 20%. When formulating the stable HO/W emulsion with 30% horse oil, surfactant concentration at 10% were not suitable. The HO/W emulsion formulated with the HLB value of 12 and surfactant concentration at 15% (E8 formulation in the current study) was a stable nano-particle size emulsion. The emulsion stability of E8 formulation were not significantly changed during storage at 40°C for 15 days. These results suggested that the characteristics of the HO/W emulsions were dependent on HLB values and surfactant concentrations so that affected to emulsion properties during storage. Moreover, it is possible to guide formulation of HO/W emulsions, which will be able to select HLB value and concentration of various surfactant in cosmetics industries.

PART II

Oxidative Stability of Horse Oil-in-Water Emulsion during Storage: Impact of α-Tocopherol Concentration

1. Introduction

Horse oil rendered from horse fatty meats is known as a cure oil that has the fatty acid composition close to human dermal oil, thus excellent permeability of moisturizing components into human skin (Jang et al., 2014). Particularly, palmitoleic acid (C16:1) of the horse oil, which contains twice as much as beef-tallow and lard, has anti-inflammatory and anti-bactericidal activities (Choi et al., 2014; Han et al., 2016). However, horse oil which contains relatively high contents of unsaturated fatty acids is possibly oxidized faster than other animal fats (Kim, 2015; Park et al., 2019) and gives a sensory disadvantage to use it because of off-flavor. Additionally, the physical type of horse oil can be solid or liquid depending on ambient temperature because solid fat (saturated fatty acids) and liquid oil (unsaturated fatty acids) are similarly existed.

The oil-in-water emulsion can be a solution to maintain a sol form at room temperature. Emulsification of oil-in-water (O/W) is the most effective strategies to improve the effectiveness as skin-care products, texture to be smooth, and flavor to cover the off-flavor (Kim et al., 2016; Lee et al., 2013). In our previous study, the horse oil in water (HO/W) emulsion was formulated and optimized with the blends of Tween 60 and Span 60 surfactants and it was characterized as nano-sized emulsion (Part I). This type of HO/W emulsion can be easily oxidized since the horse oils in the

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emulsion are dispersed in aqueous phase (Nelson and Labuza, 1992).

The use of antioxidant is the most commonly used method to retard lipid oxidation in food or cosmetic products. The chemical reaction of antioxidants at emulsion system is dissimilar to bulk oil system (Frankel et al., 1994). As described by "antioxidant polar paradox", lipophilic antioxidants are more effective in water soluble substance (e.g., O/W emulsion) while hydrophilic antioxidants more effectively protect the lipid oxidation in fat soluble substance (e.g., bulk oil). The hydrophilic antioxidants tend to locate at the oil and air interfaces and the micelles that lipid oxidation rates are high are reversed (Huang et al., 1996). On the other hand, lipophilic antioxidants less protect lipid oxidation than hydrophilic antioxidants because they are dispersed throughout the oil phase. Lipophilic antioxidants preferentially locate at the oil and water interfaces in O/W emulsion and protect lipid oxidation more effectively than hydrophilic antioxidants that dispersed through the aqueous phase (Klinkesorn et al., 2005; Porter et al., 1989).

α-Tocopherol is mainly used as an antioxidant in fat soluble products and helps inhibit free radicals preventing formation of intermediates hydroperoxide in the chain reaction (Nada et al., 2012). However, α-tocopherol was reported to have antioxidant activity at low concentrations but prooxidant activity at high concentrations (Cillard and Cillard, 1980).

In this study, the characteristics and emulsion stability of the HO/W emulsion were investigated according to the addition of α-tocopherol at different concentration. Additionally, the oxidative stability of bulk oil system and emulsion system were compared during storage.

2. Materials and Methods

2.1. Materials and chemicals

Horse fatty meats were purchased from Sansaemi Agricultural Co. (Jeju, Korea). Sorbitan monostearate (Span 60), polyethylene glycol sorbitan monostearate (Tween 60), α-tocopherol, barium chloride, iron(II) sulfate heptahydrate, 1,1,3,3-tetraethoxypropane (TEP), and cumene hydroperoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Butyl alcohol (1-butanol), isopropyl alcohol (2-Propanol), methyl alcohol, ethyl ether, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA) of analytical grade were used in the current study.

2.2. Extraction of horse oil

Horse oil was extracted from horse meats following the method of Cho and Kim (2019). Horse fatty meats were chopped with a meat grinder (MN-22S, Hankook Fujee Industries, Hwaseong, Korea) and mixed with water (1:1, w/w). The primary extraction of horse fatty meats was conducted using a vacuum low temperature extractor (Cosmos-660, Kyungseo E&P, Incheon, Korea) at 70°C and -0.1 MPa for 3.5 h. After extraction, the horse oil part was separated and the remaining fatty meats were refined again with water (1:1) for 3.5 h. The first and second refined horse oil were collected and extracted with water (1:1) for another 3.5 h. The impurities of the horse oil were removed by placing them in a stainless steel container at 100°C for 30 min. The refined horse oil was filtered by non-woven fabric(Hana Industry, Gimpo, Korea). To the final horse oil, α-tocopherol at 0, 100, 200, and 500 ppm was added and frozen until use.

2.3. Preparation of HO/W emulsion added with α-tocopherol

The HO/W emulsion was prepared with 30% horse oil containing 0, 100, 200, and 500 ppm of α-tocopherol, 55% ultrapure water, and 15% blended surfactants. The blends of Span 60 (HLB 4.9) and Tween 60 (HLB 14.9) surfactant were prepared as emulsifying agents. The previous study showed that HLB value 12 and surfactant blend at 15% were the optimized condition for the HO/W emulsion formulation (Part I). For adjusting HLB value 12, the blending ratio of Span 60 and Tween 60 was calculated according to the following equation:

$$\% \, Span \, 60 = \left[\frac{(X - HLB_{Span60})}{HLB_{Tween60} - HLB_{Span60}} \right] \times 100 \qquad (3)$$

% Tween 60: 100% - % Span 60
X: the target HLB value of 12

The surfactant blend was directly added to horse oil. The mixture of horse oil and surfactant blend was pre-heated at 60°C and homogenized using a homogenizer (T25D, Ika, Staufen, Germany) at 10,000 rpm for 1 min. Ultrapure water (55%) heating at 60°C was poured to the homogenized mixture at 60°C for 5 min and the mixture was stirred at room temperature for 20 min. Four types of HO/W emulsion (α-T0, α-T100, α-T200, and α-T500) were prepared with 0, 100, 200, and 500 ppm α-tocopherol concentration. After emulsifying, the formulated emulsion was poured into a 60 mL-wide neck glass bottle and stored at 40°C in the dark for storage test. For the comparison, horse oil as a bulk oil system was prepared.

2.4. Characterization of the HO/W emulsions

The mean particle diameter, zeta-potential (ZP), polydispersity index (PDI), and particle size distribution of the HO/W emulsions added with α-tocopherol were determined using a light scattering analyzer (Delsamax pro, Beckman Coulter, Brea, CA, USA). For the measurement, the HO/W emulsions were diluted to 500-fold with ultrapure water and vortexed for 1 min to prevent multiple scattering. Encapsulation efficiency was measured by the method of Davidov-Pardo and McClements (2005). One mL of the HO/W emulsion was mixed with 3 mL of hexane for 2 min and centrifuged at 2,700 × g for 15 min (1248R, Labogene Co., Daejeon, Korea). The upper organic layer that contained horse oil in hexane was collected and the absorbance at 272 nm was then measured using a UV-visible spectrophotometer (OPTIZEN 2120UV, Mecasys, Daejeon, Korea). The absorbance of the horse oil concentrations ranging from 0 to 10% was obtained at 272 nm for the calibration curve. Encapsulation efficiency (EE, %) of the HO/W emulsion added with α-tocopherol was calculated by the following equation: (1)

2.5. Lipid oxidation measurement

For lipid oxidation analysis, hydroperoxide concentration and TBARS were measured. Lipid hydroeproxides were measured by the method of Elias et al. (2006). First, hydroperoxides were extracted from the HO/W emulsions or bulk horse oil using a 1.5 mL of isooctane-propanol (3:1, v:v) followed by vortexing three times for 10 sec each and centrifuging for 5 min at 2500 x g. The supernatant of the mixture (0.2 mL) was mixed with methanol – 1-butanol mixture (2:1, 2.8 mL), ammonium thiocyanate (3.97 mol/L, 15 µL), and ferrous iron solution containing 0.132 mol/L $BaCl_2$ and 0.144 mol/L $FeSO_4.7H_2O$ (30 µL). The solution was vortexed and the absorbance at 510 nm was measured after 20 min. Lipid hydroperoxide concentrations were

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determined from a cumene hydroperoxide standard curve.

Changes in the secondary oxidation products of the HO/W emulsions or bulk horse oil with α-tocopherol were determined by measuring TBARS (thiobarbituric acid reactive substances) according to the method of McDonald and Hultin (1987). Two mL of TBA reagent was taken in a 15 mL-conical and 1 ml of emulsion of oil was added. The blank was prepared with 1 ml of distilled water instead of the sample. The 15 mL-conical tubes were closed and mixed thoroughly by vortexing for 10 sec thrice and incubated in a boiling water bath (90℃) for 15 min. They were cooled by placing in a cool water bath for 10 min and centrifuged at 2,700 × g for 15 min. The absorbance of the supernatant was measured at 532 nm. TBARS concentration was determined according to a standard curve made from 1,1,3,3-tetraethoxypropane (TEP). TBARS were expressed as mmol/kg oil.

2.11. Statistical analysis

All experiments were performed at least in triplicate. The results are given as means± standard deviation. Statistical comparisons were performed by analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Significant difference at $p < 0.05$ were considered.

3. Results and Discussion

3.1. Effect of α-tocopherol on the characteristics of HO/W emulsion

The mean particle diameter and PDI value of the O/W emulsions are important parameters effecting skin permeation and emulsion stability for cosmetic products (Ishikawa et al., 2012). The influence of α-tocopherol on the mean diameter, ZP (mV), PDI, and EE (%) of the HO/W emulsions was shown in Table 3.

The initial mean particle diameter of the HO/W emulsion without α-tocopherol (α-T0) was 264.9 nm. The initial mean particle diameter of HO/W emulsion added with α-tocopherol 100 ppm (α-T100) decreased to 243.2 nm but increased to 273.3 and 299.3 nm added with α-tocopherol concentration at 200 and 500 ppm (α-T200, α-T500). Relkin et al. (2009) reported that mean diameter increased when α-tocopherol at 4% was added to high melting temperature triacylglycerols emulsion, because the interfacial of high melting temperature triacylglycerols leading to more crystallization-induced particle coalescence. In the current study, α-tocopherol at 100 ppm increased particle diameter in HO/W emulsion to make particles relative small but α-tocopherol above 200 ppm showed the effect to increase particles. These phenomena were observed during storage of 15 day and 30 day. The mean particle diameter of α-T0 emulsion increased about 29%, from 264.9 to 341.6 nm after 30 days of storage. However, the mean particle diameter of α-T100, α-T200, and α-T500 emulsion increased about 11, 15, and 14% after 30 days of storage compared to 0 day. These results indicated that α-tocopherol helped to protect HO/W emulsion from particle coalescence. Especially, the mean particle size of the HO/W emulsion with α-tocopherol at 100 ppm stayed small compared to other emulsions during storage.

The ZP value is a useful parameter for studying the emulsion stability because it reflects the electrostatic interaction of moving particles (Gutiérrez et al., 2008). The ZP means the formation of a colloidal crystal that is generally produced in liquid-liquid or solid-liquid systems with force that determines the charge between two particles that keeps them separated from another (Olson 2012). Thus, the greater absolute value of ZP is, the more stable the particles of emulsion is. In this study, initial ZP value was within -27.55 to -30.39 mV (Table 3). Since α-tocopherols are hydrogen donors and negatively charged, the absolute ZP value of HO/W emulsions increased with the addition of α-tocopherol. The ZP value of α-T100 HO/W emulsion was the greatest compared to α-T200 and α-T500 emulsions. After 15 day, the ZP values were decreased in all HO/W emulsions and the values were not significantly changed after 30 days storage. The α-T100 emulsion had a minimum change of absolute ZP value during storage of 30 days compared to other emulsions. The addition of α-tocopherol led to increase emulsion stability which was dependent on the concentration. Sahari et al. (2017) reported that liposome added with α-tocopherol showed higher absolute ZP value and maintained the mean particle size in less changes than that without α-tocopherol during storage at 40℃.

The PDI values of α-T0, α-T100, α-T200, and α-T500 emulsions were 0.41 to 0.57, which indicated close to polydispersed particles in the HO/W emulsions. When α-tocopherol concentrations were increased, PDI value increased. These results indicated that energy provided for the emulsification process was insufficient. Cieśla et al., (2017) reported similar results with the current study that increasing vitamin E content contributed to high PDI values in O/W emulsions system.

EE (%) is the ratio of the concentration of the horse oil escaped in the emulsion to the concentration used to make the emulsion. The escaped horse oil is considered as the oil floating in aqueous phase without being captured

by the surfactants in O/W emulsions. According to Carpan et al. (1999), the low EE (%) emulsion is wrong formulated and this affected to the interfacial behavior between oil phase and aqueous phase. Initial EE (%) of α-T0, α-T100, α-T200, and α-T500 HO/W emulsions were 90.00, 89.31, 88.29, and 88.26%, respectively (Table 3). And they were decreased to 79.62, 79.19, 80.37, and 79.15%, respectively, after 30 days of storage at 40°C. The reduction of EE (%) can probably be attributed to the loss of surface tension during storage at relatively high temperature (Chen and Tao 2005).

Table 3. Characteristics of the HO/W emulsions with α-tocopherol at 0, 100, 200, and 500 ppm during storage at 40℃

Storage (day)	HO/W emulsion	Emulsion characteristics			
		Mean diameter (nm)	Zeta-Potential (mv)	Polydispersity Index (PDI)	Encapsulation efficiency (EE, %)
0	α-T0	264.9±7.3[bE]	-27.55±0.30[aBC]	0.41±0.17[bB]	90.00±0.23[aA]
	α-T100	243.2±5.2[cF]	-30.39±0.24[cF]	0.57±0.00[aA]	89.31±0.19[abAB]
	α-T200	273.3±5.8[bE]	-29.27±0.68[bE]	0.57±0.00[aA]	88.29±0.85[bBC]
	α-T500	299.3±9.6[aD]	-29.35±0.71[bE]	0.57±0.00[aA]	88.26±0.05[bBC]
15	α-T0	323.3±11.0[bBC]	-25.35±0.36[aA]	0.44±0.16[bB]	87.15±0.12[bD]
	α-T100	270.7±6.1[cE]	-29.46±0.44[cE]	0.57±0.00[aA]	88.42±0.69[aBC]
	α-T200	314.3±10.4[bC]	-27.70±0.60[bBC]	0.57±0.00[aA]	88.82±0.46[aBC]
	α-T500	331.2±4.04[aB]	-27.75±0.78[bBC]	0.57±0.00[aA]	87.83±0.12[abCD]
30	α-T0	341.6±6.46[aA]	-25.03±0.74[aA]	0.57±0.00[aA]	79.62±1.14[aEF]
	α-T100	271.6±7.7[cE]	-28.47±0.59[cC]	0.57±0.00[aA]	79.19±0.12[aF]
	α-T200	322.2±7.64[bBC]	-27.24±0.47[bB]	0.57±0.00[aA]	80.37±0.20[aE]
	α-T500	342.0±7.29[aA]	-27.30±0.25[bB]	0.57±0.00[aA]	79.15±0.24[aF]

Each value is expressed as mean±standard deviation.

Means with different letters (a, b et al) within respective storage day are significantly different at $p < 0.05$.

Means with different letters (A, B et al) in all storage days are significantly different at $p < 0.05$.

3.2. Effect of α-tocopherol on the particle size distribution of HO/W emulsion

Initial particle size distribution of the HO/W emulsions added with 0, 100, 200 and 500 ppm α-tocopherol are presented in Fig. 4(A). The particle size distributions of all emulsions had two separate small and main particle diameter. The range of mean initial diameter at small particle diameter was 35 to 57 nm and that at main particle diameter was 116 to 384 nm. As α-tocopherol concentration increased, the main particle diameters were increased and particle size distribution were broad except for α-T100 emulsion. These might be caused by the viscosity of oil phase which was increased as increasing α-tocopherol concentration (Walstra 1993). After 30 days of storage, the particle diameters of α-T0 and α-T100 emulsions increased (Fig. 4(B)). However, the increase rate of main particle diameter decreased with the increase of α-tocopherol concentration at 34% in α-T200 emulsion and 6% in α-T500 emulsion. In those emulsions, particle growth by Ostwald ripening was slowly happened because of low solubility of tocopherol (Ziani et al., 2012). The high concentration of α-tocopherol in dispersed phase protected particles from the Ostwald ripening. These results indicated that increasing of α-tocopherol concentration in horse oil prevented the change of particle size, moreover enhanced the emulsion stability during storage.
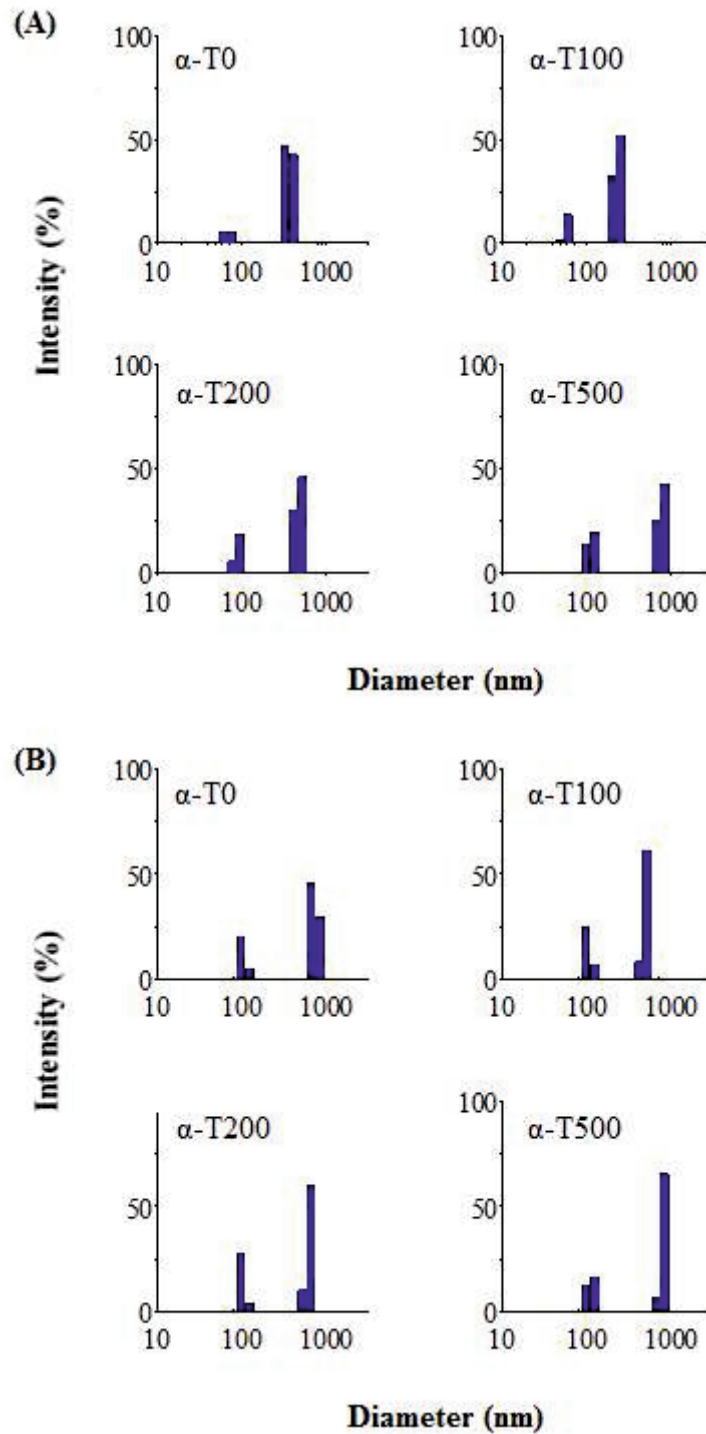
Fig 4. Particle size distribution of the HO/W emulsion added with α
−tocopherol at 0, 100, 200, and 500 ppm stored at 40℃ for 0 day (A)
and 30 day (B).

3.3. Oxidative stability of HO/W emulsions and bulk horse oil added with α-tocopherol

The changes in peroxide values of HO/W emulsions added with α-tocopherol at 0, 100, 200, and 500 ppm stored at 40°C for 30 days in the dark were shown in Fig. 5 (A). Initial hydroperoxide concentrations of the HO/W emulsion added with α-tocopherol at 0, 100, 200, and 500 ppm were in the range of 2.88 to 2.96 mmol/kg oil, which were not significantly different (p>0.05). As the storage day increased, hydroperoxide concentrations were increased. Especially, after 30 days, the hydroperoxide concentrations of the HO/W emulsion added with α-tocopherol at 100 and 200 ppm were 8.81 and 8.86 mmol/kg oil, respectively . When compared to them, the hydroperoxide concentration in α-T0 and α-T500 emulsions were high. These results showed that α-tocopherol at 100 and 200 ppm acted as antioxidant but at 500 ppm acted as prooxidantin the HO/W emulsion system.

The lipid oxidation rate and stability of the O/W emulsion system are different compared to the bulk oil system (Waraho et al., 2011). In this study, the changes in hydroperoxides of the bulk horse oil containing α-tocopherol were shown in Fig. 5 (B). The hydroperoxide concentration of the bulk oil without α-tocopherol (α-T0) increased from 2.12 to 5.48 mmol/kg oil after 10 days of storage. As the storage days increased, α-tocopherol inhibited the formation of hydroperoxide. Among three emulsions with α-tocopherol, the concentration at 100 and 200 ppm provided more protection than at 500 ppm. α-Tocopherol at 100 and 200 ppm was acted as an antioxidant similarly to the bulk oil system and O/W emulsion system; however, the antioxidant activity at high concentration of α-tocopherol decreased and finally α-tocopherol at 500 ppm acted as prooxidant in the HO/W emulsion.

The changes in TBARS of HO/W emulsions added with α-tocopherol at 0, 100, 200, and 500 ppm stored at 40°C for 30 days in the dark were shown in

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Fig. 5 (A). A different concentration in the range of 2.07 to 2.46 of initial TBARS value with α-tocopherol at 0, 100, 200, and 500 ppm was detected. The secondary oxidation products concentration gradually increased during storage at 40°C, being the increase faster in α-T0 and α-T500 emulsions than in α-T100 and α-T200 emulsions. After 30 days, α-T200 emulsion was slightly lower in TBARS value (4.04 mmol/kg oil) compared to α-T100 (4.35 mmol/kg oil). Sharif et al., (2017) reported the added dual antioxidants, ß-carotene and α-tocopherol, in flaxseed oil-in-water emulsions produced more malonaldehyde at higher concentration of α-tocopherol, which was the similar result with this study. These results showed that the HO/W emulsion added with α-tocopherol at 100 and 200 ppm helped to reduce the secondary oxidation products.

The changes in TBARS value of the bulk horse oil containing α-tocopherol were shown in Fig. 6 (B). The initial secondary oxidation products concentration of the bulk syatem were in the range of 0.83 to 0.88 mmol/kg oil, which were not significantly different (p>0.05). Bulk horse oil without α-tocopherol (α-T0) rapidly increased from 0.86 to 3.89 mmol/kg oil after 10 days of storage and gradually increased after storage at 30 days. Chen et al., (2011) reported that oxidation stability in bulk oil has been shown to lose effectiveness as α-tocopherol concentration increases due to promote to autoxidation linoleic acid. However, in this study TBARS value of bulk horse oil containing α-tocopherol did not change significantly during 30 days of oxidation regardless of tocopherol concentration. These results showed that α-tocopherol at 500 ppm acted as prooxidant in O/W emulsion system unlike bulk oil system.
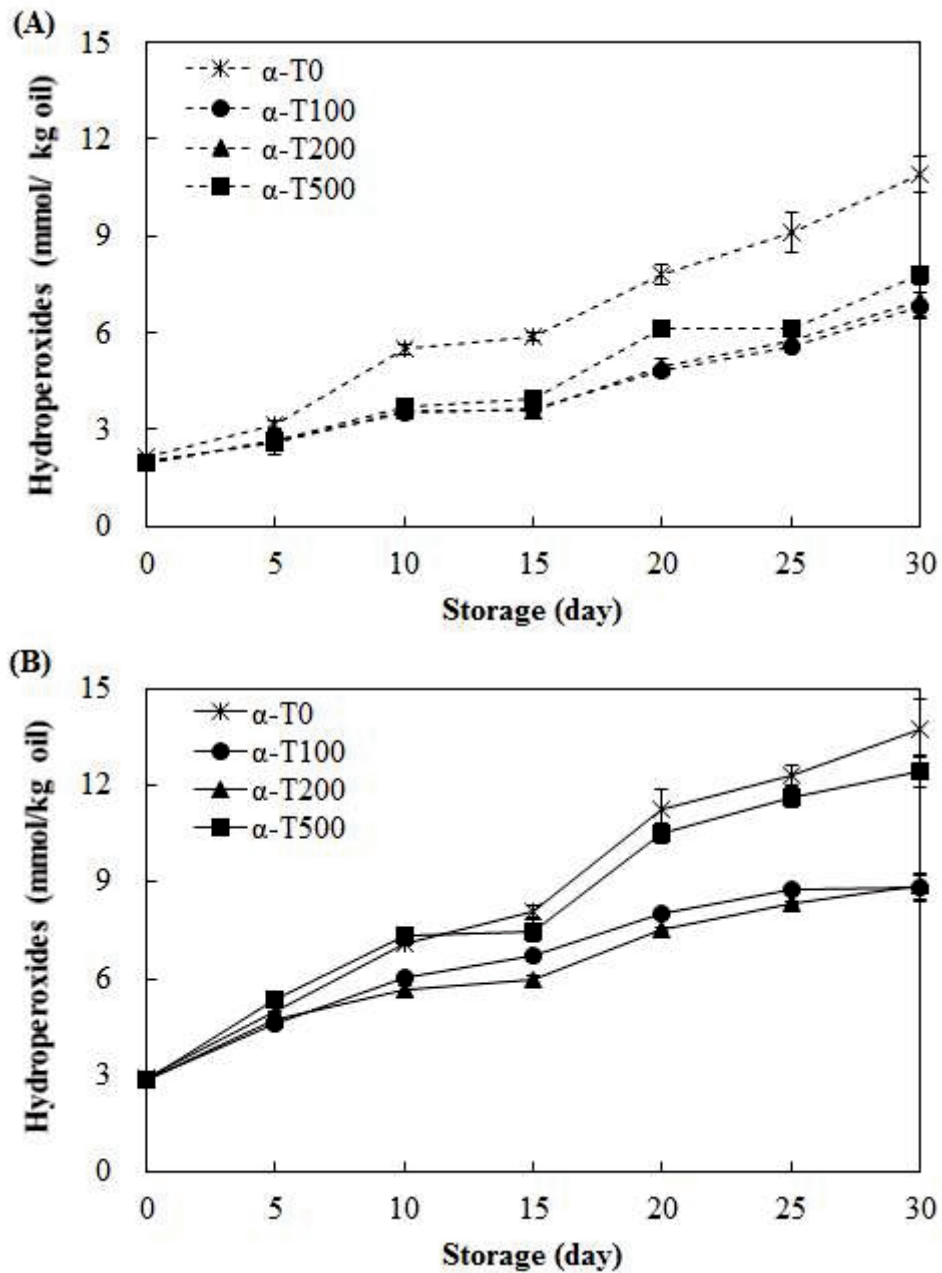
Fig 5. Changes in peroxide value of HO/W emulsion (A) and bulk horse oil (B) added with α-tocopherol at 0, 100, 200, and 500 ppm stored at 40℃ during storage of 30 day.
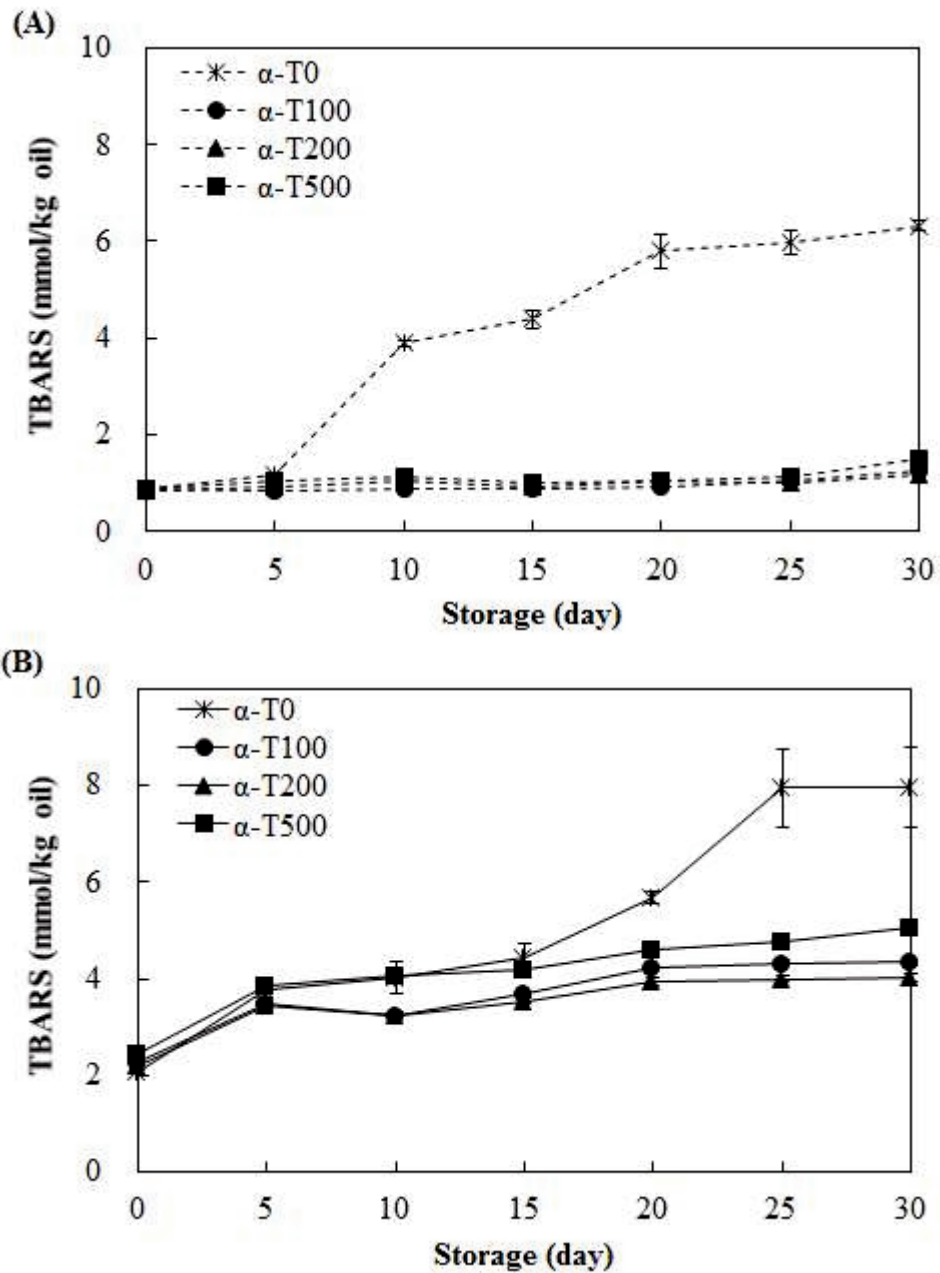
Fig 6. Changes in TBARS value of HO/W emulsion (A) and bulk horse oil (B) added with α-tocopherol at 0, 100, 200, and 500 ppm stored at 40°C during storage of 30 day.

4. Conclusion

The emulsion stability and oxidative stability of HO/W emulsions added with α-tocopherol at 0, 100, 200, and 500 ppm were investigated. The presence of α-tocopherol at the appropriate concentration made the HO/W emulsion maintain stable particle sizes. In oil-in-water emulsion system, α-tocopherol at 100 and 200 ppm showed the similar tendency to inhibit the formation of hydroperoxide and malonaldehyde while in oils α-tocopherol at 500 ppm improved the oxidative stability during storage at 40°C. These results showed that the concentration of α-tocopherol impacted the oxidative stability of HO/W emulsions suggesting that α-tocopherol could provide a stable and efficient emulsion system.

국문요약

본 연구에서는 70℃에서 감압 추출한 마유를 이용하여 hydrophilic-lipophilic balance (HLB) system에 따라 최적 비율 조건의 수중유적형 에멀션을 조제하였다. 또한 최적 HLB가 및 비율의 에멀션에 α-tocopherol을 농도 별로 첨가한 뒤 에멀션의 특성 및 산화 안정성을 측정하여 화장품 산업에서 활용될 수 있는 자료를 제시하고자 하였다. 내상이 30%인 수중유적형 마유에멀션에서 두 가지 비이온 계면활성제인 Span 60 과 Tween 60을 혼합하여 HLB 10-14 유화제조합을 사용하여 실험을 진행하였으며, 그 농도는 10, 15, 20%를 사용하였다. 조제된 에멀션에서 10%의 계면활성제 농도는 저장 기간 모두 분리가 일어나 적합하지 않은 농도임을 규명하였다. HLB 11에서 12사이의 유화제 조합에서 제타 전위의 절댓값이 가장 높음을 확인할 수 있었으며, 농도 15%의 HLB 12로 제조된 에멀션(E8) 의 입자들은 모두 500 nm 이하의 입자크기를 얻어 나노 에멀션의 양상을 나타냈다. 또한 E8 및 E9 제형물은 15일동안 40℃ 저장 중 평균입자의 크기 및 EE(%)가 유의적으로 변하지 않았으나 피부의 안정성을 고려하여 낮은 계면활성제 농도인 E8 제형을 최적비율의 수중유적형 마유에멀션으로 선택하여 후속 연구를 진행하였다. 이 제형은 후속 연구에서 α-T0을 대조구로 명명하였으며, α-tocopherol 농도 100, 200, 500 ppm 첨가량에 따라 α-T100, α-T200, α-T500으로 명명하였다. α-T100 은 대조구에 비하여 평균 입자의 크기는 감소하였으나, α-T200 및 α-T500은 증가하는 경향을 보였다. α-tocopherol 첨가구는 대조구에 비하여 제타전위의 절댓값이 증가하였으나 다분산상에 가까워졌다. α-T200 및 α-T500은 Ostwald ripening에 의한 현상은 다른 제형에 비하여 나타나지 않음을 확인하였다. α-Tocopherol 농도에 따른 산화 방지 활성은 100 및 200 ppm은 수중유적형 에멀션 및 bulk oil에서 모두 뛰어난 활성을 나타냈지만 500 ppm에서의 산화 방지 활성은 저하됨을 확인하였으며, 특히 bulk oil에 비하여 수중유적형 에멀션 성상에서 비교적 산화 방지 활성이 급격히 감소됨을 확인하였다. 본 연구를 토대로 내상이 30%인 마유에서의 HLB 값과 계면활성제 및 토코페롤의 농도를 선택할 수 있으며 화장품 산업에서 마유를 함유한 수중유적형 에멀션이 보다

안정적이고 효율적인 시스템을 제공할 수 있을 것으로 기대된다.

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감사의 글

대학교 2학년부터 실험실 생활을 하고 석사생활을 거치며 5년간의 식품생화학실험실 생활을 잘 마칠 수 있게되어 감사의 글을 올리게 되었습니다. 실험실 및 대학원 생활은 마무리 단계까지 많은 배움과 경험을 얻을수 있는 기회였습니다. 이러한 마무리를 잘 할수있게 옆에서 힘이되고 가르침을 주신 분들게 감사의 마음을 전하고자 합니다.

먼저 간단한 실험데이터 정리도 할 줄도 모르고, 사고뭉치 였던 저를 석사학위 논문까지 작성하도록 교육해주신 지도교수님이신 김현정교수님에게 먼저 감사를 드립니다. 그리고 바쁘신 와중에도 논문심사와 조언을 주신 고영환교수님께 감사드립니다. 에멀젼에 대한 지식이 없는 제게 많은 가르침과, 실험을 할 수 있도록 도움을 주신 천지연 교수님께 감사드립니다. 또한 학부생부터 대학원까지 많은 가르침을 주신 임상빈교수님, 박은진교수님께도 진심으로 감사드립니다.

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