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STABILITY ENHANCEMENT OF L-GLUTATHIONE BY ENTRAPMENT IN WATER-IN-OIL MICROEMULSION

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Abstract: L-glutathione (GSH) is one of popular skin-lightening active ingredients since it can reduce the formation of eumelanin or brown-to-black pigment in the skin, resulting in pale color. However, its high hydrophilicity and low stability are hindrances for topical delivery. Thus, development in form of effective formulations such as microemulsion (ME) which can enhance stability and skin penetration of GSH is necessary. This study aimed to increase stability of GSH by incorporation in a water-in-oil (w/o) ME which designated as GSH-ME. GSH-ME was composed of 50% w/w 1:1 Tween80:Span80 as surfactant mixture, 40% w/w palm oil as oil phase, 9.8% w/w 1:1 water:propylene glycol as aqueous phase and 0.2% w/w GSH as the active ingredient. The stability of GSH-ME was evaluated comparing with that of 0.2% w/w GSH aqueous solution after kept in ice box (0±2°C) and at ambient temperature (30±2°C). The chemical analysis was carried out by high performance liquid chromatography (HPLC). The results showed that GSH in aqueous solution was quickly degraded to glutathione disulfide (GSSG) by autoxidation. The changing was found to depend on storage temperature. Degradation of GSH solution when kept at ambient temperature was faster than that when kept in ice box. GSH-ME could retard the degradation rate of GSH to GSSG in comparison with GSH aqueous solution since the oxidation sensitive moiety of GSH was possibly protected in the internal aqueous phase. However, the extended duration of stability of GSH-ME was still not enough for development of a commercial cosmetic product.

Keywords: L-glutathione, autoxidation, microemulsion, skin-lightening, stability

บทคัดย่อ: แอล-กลูตาไธโอน (GSH) เป็นสารไวท์เทนนิ่งชนิดหนึ่งที่ได้รับความนิยมเนื่องจากสารนี้สามารถลดการสร้าง eumelanin หรือเม็ดสีน้ำตาลถึงดำในผิวหนัง ส่งผลให้สีผิวแลดูอ่อนลง อย่างไรก็ความชอบน้ำสูงและความคงตัวต่ำของสารนี้เป็นอุปสรรคต่อการนำส่งเฉพาะที่ ดังนั้นการพัฒนาให้อยู่ในรูปแบบที่มีประสิทธิภาพ เช่น ไมโคร-อิมัลชัน (ME) ซึ่งสามารถเพิ่มความคงตัวและการซึมผ่านผิวหนังของ GSH จึงเป็นสิ่งจำเป็น การศึกษานี้มีวัตถุประสงค์เพื่อเพิ่มความคงตัวของ GSH โดยบรรจุในไมโครอิมัลชันประเภทน้ำในน้ำมัน และเรียกชื่อตำรับว่า GSH-ME ซึ่งประกอบด้วย 50% w/w 1: 1 Tween80: Span80 เป็นของผสมของสารลดแรงตึงผิว, 40% w/w น้ำมันปาล์มเป็นวัฏภาคน้ำมัน, 9.8% w/w 1: 1 น้ำ:โพรพิลีนไกลคอลเป็นวัฏภาคน้ำ และ 0.2% w/w GSH เป็นสารสำคัญ ความคงตัวของ GSH-ME ได้รับการประเมินเทียบกับสารละลาย 0.2% w/w GSH ในน้ำ หลังการเก็บในกล่องน้ำแข็ง (0±2°C) และที่อุณหภูมิห้อง (30±2°C) การวิเคราะห์ทางเคมีใช้วิธีโครมาโทกราฟีของเหลวสมรรถนะสูง (HPLC) ผลการศึกษาแสดงว่า GSH ในรูปสารละลายในน้ำสลายตัวเป็นกลูตาไธโอนไดซัลไฟด์ (GSSG) โดยออโทออกซิเดชันอย่างรวดเร็ว การเปลี่ยนแปลงนี้ขึ้นอยู่กับอุณหภูมิในการเก็บรักษา การสลายตัวของ GSH ในรูปสารละลายในน้ำเมื่อเก็บไว้ที่อุณหภูมิห้องเร็วกว่าเมื่อเก็บในกล่องน้ำแข็ง GSH-ME สามารถลดอัตราการสลายตัวของ GSH เป็น GSSG เมื่อเทียบกับสารละลายในน้ำ เนื่องจากเป็นไปได้ที่โครงสร้างส่วนที่ไวต่อออกซิเดชันของ GSH ถูกปกป้องในวัฏภาคน้ำซึ่งเป็นวัฏภาคภายใน อย่างไรก็ตามระยะเวลาของความคงตัวที่เพิ่มขึ้นของ GSH-ME ยังคงไม่เพียงพอต่อการพัฒนาเป็นผลิตภัณฑ์เครื่องสำอางเชิงพาณิชย์

คำสำคัญ: แอล-กลูตาไธโอน, ออโทออกซิเดชัน, ไมโครอิมัลชัน, ไวท์เทนนิ่ง, ความคงตัว

INTRODUCTION

Nowadays, skin-lightening products are very popular. They appear to be the largest and continually growing segment in cosmetic market, especially in Asia. It is expected that the global market of these products will reach $19.8 billion by 2018 (McDougall, 2013) and $23 billion by 2020 (SpecialChem, 2015). L-glutathione (GSH) is one of popular skin-lightening active ingredients. It is a tripeptide composing of three amino acids, i.e., glutamate,

cysteine and glycine. Thiols (cysteine and glutathione) can react with L-dopaquinone and subsequently lead to the switch from eumelanogenesis to pheomelanogenesis, resulting in pale skin color (Villarama and Maibach, 2005). Thus, GSH is in demand for cosmetic market as a skin-lightening agent. However, it is freely water soluble and easily degraded to form glutathione disulfide (GSSG) by autoxidation (Deshmukh et al., 2009; Mahlapuu et al., 2006), resulting in impediments for topical delivery. Moreover, skin penetration of GSH is obstructed by barrier function of stratum corneum while its target site is stratum basale. Therefore, formulation development in form of microemulsion (ME) is of interest. ME is a carrier system composed of two immiscible phases (i.e., water and oil) and stabilized with the interfacial film of a surfactant or a mixture of surfactant and cosurfactant. Advantages of ME include spontaneous formation, good appearance and thermodynamic stability. Moreover, ME can increase stability of the loaded active ingredients and has potential for skin penetration enhancement (Boonme, 2007; Boonme, 2009; Boonme et al., 2009; Lopes, 2014). Water-in-oil (w/o) MEs have been found to provide lower transdermal fluxes of benzophenone-3, a sunscreen, than oil-in-water (o/w) ones, resulting in suitability for topical delivery of a cosmetic active ingredient (Songkro et al., 2014). The w/o MEs have been also reported to protect GSH from enzymatic degradation and to increase permeability across the intestinal epithelium via oral delivery (Wen et al., 2013). This study aimed to compare stability of GSH in ME with that in aqueous solution. Sample extraction was also investigated for finding the most suitable diluent in analytical procedure.

MATERIALS AND METHODS

Materials

GSH and GSSG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Palm oil was purchased from Oleen Co., Ltd. (Samut Sakhon, Thailand). Polyoxyethylene (20) sorbitan monooleate (Tween80), sorbitan monooleate (Span80) and propylene glycol (PG) were purchased from P.C. Drug Center (Bangkok, Thailand). Potassium dihydrogen phosphate was purchased from Ajax Finechem (Taren Point, NSW, Australia). Sodium 1-heptanesulfonate was purchased from Alfa Aesar (Heysham, UK). Absolute ethanol, acetonitrile, methanol and ortho-phosphoric acid were purchased from RCI Labscan (Bangkok, Thailand). Ultrapure water was produced in-house by a Millipore Direct-Q® 5 system (EMD Millipore, Billerica, MA, USA). All chemicals were used as received without any modifications.

Sample preparation

A w/o ME was selected from the ME region in pseudoternary phase diagram from our previous study (Wuttikul and Boonme, 2016). GSH-ME was prepared by incorporation of 0.2% w/w GSH in the chosen ME. Hence, GSH-ME was composed of 50% w/w 1:1 Tween80:Span80 as surfactant mixture, 40% w/w palm oil as oil phase, 9.8% w/w 1:1 water:PG as aqueous phase and 0.2% w/w GSH as the active ingredient. It was prepared by simply mixing. GSH aqueous solution was prepared by dissolving 0.2% w/w GSH in water.

Microemulsion characterization

Type of ME was confirmed by staining and conductivity measurement methods. For staining method, the difference of diffusion between a water soluble dye and an oil soluble dye which were dropped into the ME were observed. For conductivity measurement, a pen conductivity meter (ST10C-A, OHAUS®, China) was used. Viscosity was measured by Brookfield rheometer model DV-III ultra (Brookfield Engineering Laboratories, USA) with SC4-31 spindle at speed of 100 rpm. All experiments were performed in triplicate at 25±1°C.

Stability study

In previous reports (Appala et al., 2016; Sutariya et al., 2012), solutions of GSH in water and other solvents were stored at 4°C before used. Therefore, effect of storage temperature on stability of GSH aqueous solution was investigated in this study. GSH aqueous solution was kept in ice box (0±2°C) and at ambient temperature (30±2°C). Subsequently, it was analyzed for amounts of remained GSH and formed GSSG at 0, 1, 2, 4, 6, 8 and 12 hr after storage. GSH-ME was analyzed after stored at ambient temperature for 7 days and 3 months. The areas under the peak of GSH were obtained by high performance liquid chromatography (HPLC). Afterward, the GSH decreasing was calculated by comparing the areas under the peak at the studied time with those at the initial time.

Preparation of standard solutions

The standard solutions were freshly prepared and used within 1 day. GSH, GSSG and GSH+GSSG standard solutions were prepared by dissolving 0.5 mg GSH, 1.0 mg GSSG and 0.5 mg GSH as well as 1.0 mg GSSG in water to make the solutions of 10 ml, respectively. Raggi et al. (1998) suggested that GSH standard solutions should be prepared daily to guarantee reliable results since GSH is low stable.

Chemical analysis

The chemical analysis for determining amounts of GSH (the active ingredient) and GSSG (the degraded compound) was performed by HPLC (Agilent HPLC series 1100, Basel, Switzerland). The method was adapted from USP37-NF32 (2014). A reversed phase column (Luna® C18, 150x4.6 mm, 5 µm particle size, Phenomenex Inc., California, USA) was used as a stationary column and controlled at 25 ºC. The mobile phase composed of phosphate buffer pH 3.0 and methanol at the ratio of 90:10 by volume and the flow rate was 0.65 ml/min. Phosphate buffer pH 3.0 was prepared from potassium dihydrogen phosphate, sodium 1-heptanesulfonate and ortho-phosphoric acid. Injection volume was 20 µl. Wavelength detector was set at 214 nm. The ChemStation software version Rev. B.02.01 was used for data analysis. All sample extracts were filtered with 0.45 µm nylon filter before used and protected from light. The results were recorded in the form of areas under the peak. Effects of diluents in extraction of GSH from samples were also investigated. Various diluents, i.e., absolute ethanol, acetonitrile, methanol and water, were studied for suitability in sample extraction with no interferes in the obtained chromatograms. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Microemulsion characteristics

Formulation of GSH-ME was selected from point H of ME region in previous studied pseudoternary phase diagram (Wuttikul and Boonme, 2016) as exhibited in Figure 1. This point was chosen according to three following reasons. It contained low concentration of surfactant mixture, leading to reduce risk of skin irritation (Boonme, 2009; Lopes, 2014). It was not too near border of ME region, resulting in low risk of microstructure changing after incorporation with the active ingredient (Kaewbanjong et. al., 2017; Yuan et. al., 2006). The preliminary results showed that GSH could be incorporated in this ME without physical changing when compared with other eight MEs at points A-G and I. The obtained GSH-ME was clear yellowish liquid. Oil soluble dye could diffuse in GSH-ME faster than water soluble dye. The conductivity value of GSH-ME was 0.67±0.06 µS/cm. The results of faster diffusion of oil soluble dye than water soluble dye in the sample and low conductivity of the sample confirmed for w/o type. Viscosity value of GSH-ME was 299.34±0.26 cPs while that of the

blank counterpart was 321.43±0.22 cPs. It could be noted that addition of 2% w/w GSH into the selected ME slightly decreased viscosity. The reason was unclear; however, the incorporation of GSH into the ME might affect interaction between the internal droplets (Podlogar et. al., 2005).

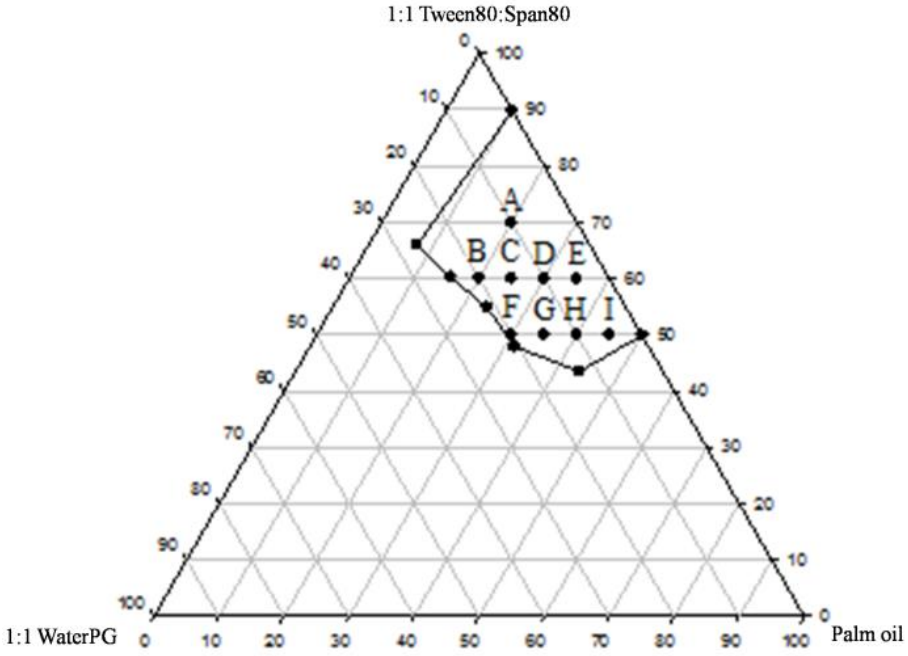

Figure 1. ME region of the studied system (modified from Wuttikul and Boonme, 2016)

Chromatographic conditions

Figure 2 exhibits that the HPLC method in this study could separate the peaks of the active ingredient (GSH) and its degraded compound (GSSG) when water was used as diluent at different retention times of around 5 and 9 min, respectively. However, it was found that degradation of GSH occurred very quickly since peak of GSSG was also observed when freshly prepared GSH standard solution was analyzed at ambient temperature as seen in Figure 2(B). In addition, sample extraction was studied to obtain the most suitable diluent in analytical procedure. Figure 3 shows that organic solvents, i.e., absolute ethanol, acetonitrile and methanol, were inapplicable diluents to extract and dilute GSH from the sample (aqueous solution) since interferences were found. Therefore, water was suitable to be used as diluent since no interferes were observed as presented in Figure 2 which was according to solubility property of GSH. It was also found that extraction of GSH-ME with water provided no interferes from blank ME as illustrated in Figure 4.

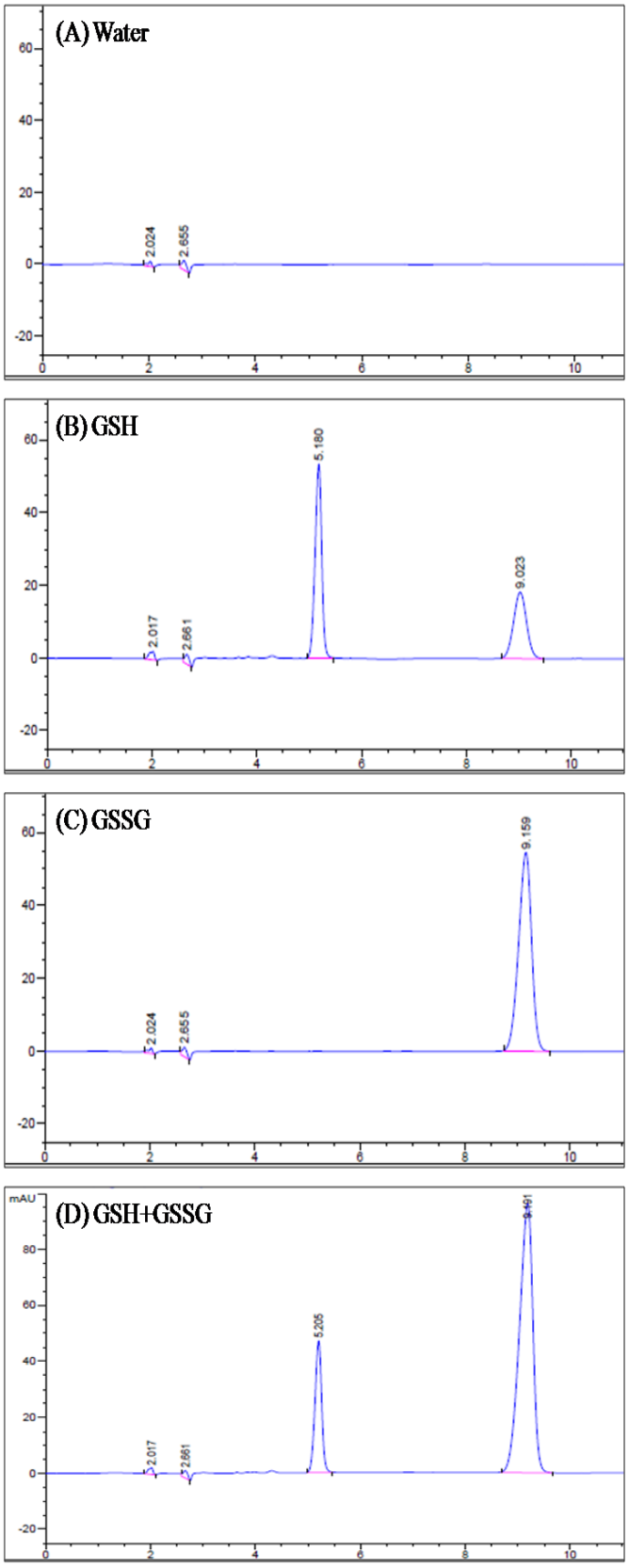

Figure 2. Chromatograms of (A) water and aqueous solutions of (B) GSH, (C) GSSG and (D) GSH+GSSG

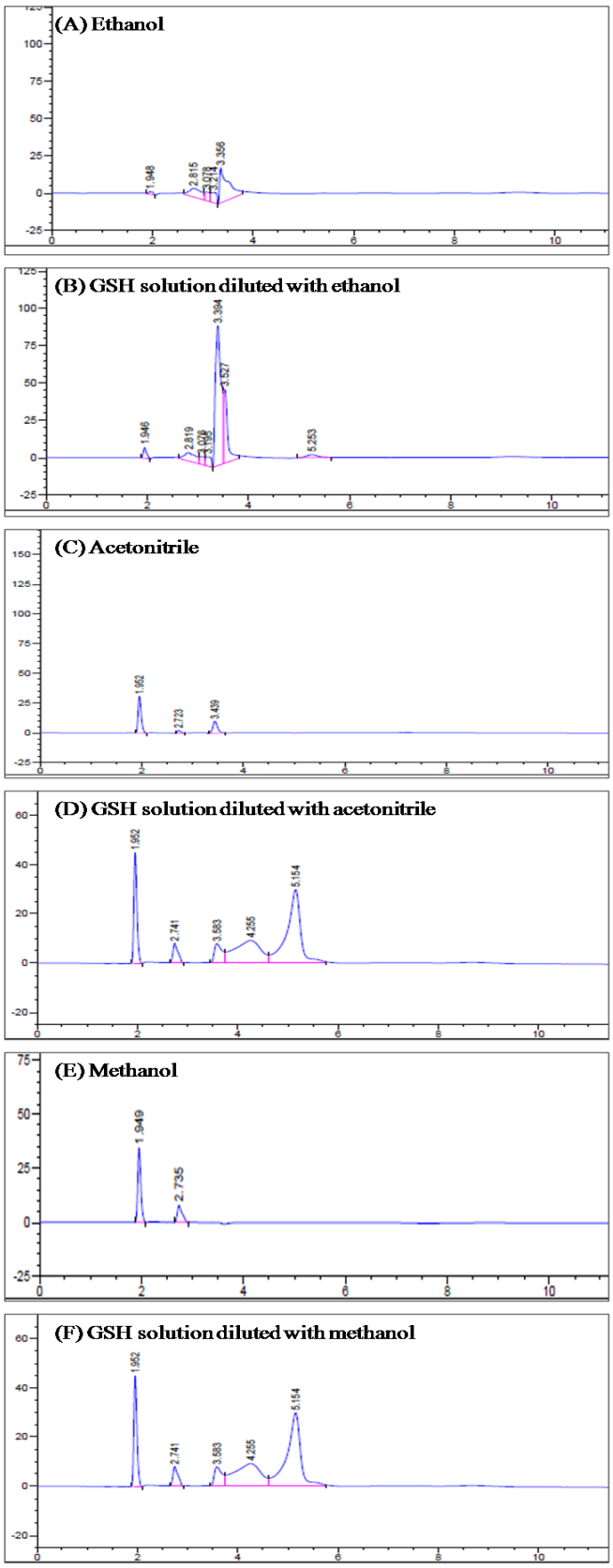

Figure 3. Chromatograms of studied organic solvents and GSH solution diluted with these solvents

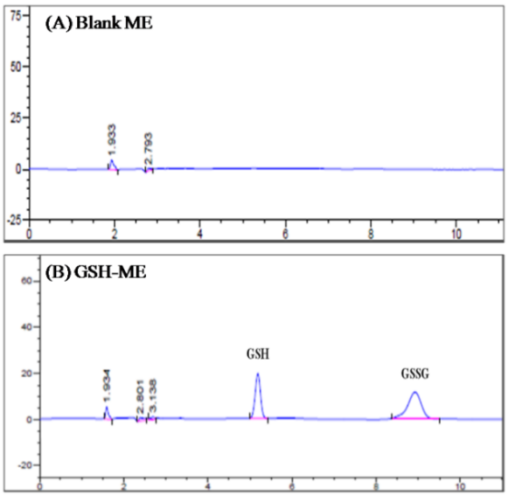

Figure 4. Chromatograms of (A) blank ME and (B) GSH-ME

Stability

It was seen in Table 1 that the areas under the peak of GSH averagely decreased to 96.27% and 78.91% when compared to those at the initial after kept in ice box and at ambient temperature for 12 hr, respectively. In the meantime, the areas under the peak of GSSG gradually increased. GSH degraded to GSSG faster when kept at ambient temperature than when kept in ice box. The degradation was caused by autoxidation (Deshmukh et al., 2009; Mahlapuu et al., 2006) and adversely affected by high temperature (Lin et al., 2006).

Table 1. Areas under the peaks (mAU) of GSH and GSSG after kept GSH aqueous solution in ice box and at ambient temperature (n = 3)

Time (hr)	In ice box		At ambient temperature	
	GSH	GSSG	GSH	GSSG
0	851.9±2.25	0.0±0.00	809.9±5.49	64.2±6.52
1	849.2±2.49	12.5±11.06	791.5±6.95	87.0±8.40
2	843.8±0.72	23.7±0.96	772.0±5.66	109.5±7.95
4	840.5±0.83	29.7±1.75	737.4±5.76	150.1±5.75
6	837.1±2.89	34.9±2.63	706.0±4.94	179.1±3.21
8	828.9±1.77	44.5±1.20	682.5±3.64	208.0±7.95
12	820.1±1.08	59.3±0.87	639.1±3.70	266.3±2.34

Incorporation GSH in ME might decrease the autoxidation rate of GSH to GSSG since peak of GSH was still found after stored GSH-ME in clear bottles without light protection at ambient temperature for 7 days as shown in Figure 4(B). It might be explained that the oxidation sensitive moiety of the hydrophilic active ingredient was entrapped in the internal aqueous phase, resulting in protection effects (Spiclin et al., 2001). However, peak of GSH disappeared after further kept GSH-ME for 3 months (data not shown). Although GSSG can be converted to GSH by enzymatic mechanism of GSSG reductase (Lu, 2009), the data indicated that the current GSH-ME was not desirable for development of a commercial cosmetic product since its long-term stability was not adequate.

CONCLUSION

GSH was formulated in form of w/o ME composed of 50% w/w 1:1 Tween80:Span80, 40% w/w palm oil, 9.8% w/w 1:1 water:PG and 0.2% w/w GSH. It could be qualitatively analyzed by HPLC using water as the diluent for sample extraction. GSH was changed to

GSSG by autoxidation depending on storage temperature. GSH-ME could retard the degradation rate of GSH to GSSG when compared with GSH aqueous solution. However, the extended duration of stability of GSH-ME was still too short to be used in development of a commercial cosmetic product. This problem may be coped with changing ME components to form rigid film around internal droplets or adding antioxidant into the formulation to delay the degradation reaction. Changing to use GSSG as the main material in the formulation is also challenging since GSSG can be converted to GSH by enzymatic mechanism of GSSG reductase.

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