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Ultrasonic-assisted Micellar Extraction and Cloud-point Pre-concentration of Major Saikosaponins in *Radix Bupleuri* using High Performance Liquid Chromatography with Evaporative Light Scattering Detection

Joon Hyuk Suh,[a] Dong-Hyug Yang,[a] and Sang Beom Han[*]

Department of Pharmaceutical Analysis, College of Pharmacy, Chung-Ang University, Seoul 156-756, South Korea
[*]E-mail: hansb@cau.ac.kr
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A new ultrasonic-assisted micellar extraction and cloud-point pre-concentration method was developed for the determination of major saikosaponins, namely saikosaponins -A, -C and -D, in *Radix Bupleuri* by high performance liquid chromatography with evaporative light scattering detection (HPLC-ELSD). The non-ionic surfactant Genapol X-080 (oligoethylene glycol monoalkyl ether) was chosen as the extraction additive and parameters affecting the extraction efficiency were optimized. The highest yield was obtained with 10% (w/v) Genapol X-080, a liquid/solid ratio of 200:1 (mL/g) and ultrasonic-assisted extraction for 40 min. In addition, the optimum cloud-point pre-concentration was reached with 10% sodium sulfate and equilibration at 60 °C for 30 min. Separation was achieved on an Ascentis Express C18 column (100 × 4.6 mm i.d., 2.7 μm) using a binary mobile phase composed of 0.1% acetic acid and acetonitrile. Saikosaponins were detected by ELSD, which was operated at a 50 °C drift tube temperature and 3.0 bar nebulizer gas (N_2) pressure. The water-based solvent modified with Genapol X-080 showed better extraction efficiency compared to that of the conventional solvent methanol. Recovery of saikosaponins ranged from 93.1 to 101.9%. An environmentally-friendly extraction method was successfully applied to extract and enrich major saikosaponins in *Radix Bupleuri*.

Key Words : Saikosaponin, Cloud-point extraction, Genapol X-080, *Radix Bupleuri*, HPLC-ELSD

Introduction

Radix Bupleuri, the dried roots of *Bupleurum falcatum* L. (Umbelliferae), is a well-known traditional herbal medicine widely used in China, Japan and Korea for the treatment of the common cold, influenza, inflammation, and liver diseases.[1,2] The phytochemical constituents associated with the pharmacological and biological activities of *Radix Bupleuri* include saikosaponins, essential oils, lignans and polysaccharides. Among them, saikosaponins -A, -C, and -D (SSa, SSc, and SSd, respectively; chemical structures are shown in Figure 1) are generally accepted to be the representative bioactive components, exhibiting a variety of pharmacological activities including *anti*-inammatory, immunomodulatory, *anti*-cancer, *anti*-hepatitis, and antiviral effects.[3] Accordingly, the quality and efficacy of *Radix Bupleuri* are highly dependent on the contents of the three saikosaponins. Because differences in the compositions of medicinal plants may affect the efficacy of herbal medicine, it is important to quantify the bioactive constituents in *Radix Bupleuri* to ensure its quality. Various analytical methods including HPLC-UV,[4] HPLC-ELSD,[5,6] HPLC-MS/MS,[7] and capillary zone electrophoresis[8] have been developed to measure saikosaponin content in *Radix Bupleuri*.

Sample preparation, always the first critical step in the determination of bioactive or marker compounds in herbal extracts, requires an integrated approach solution that com-

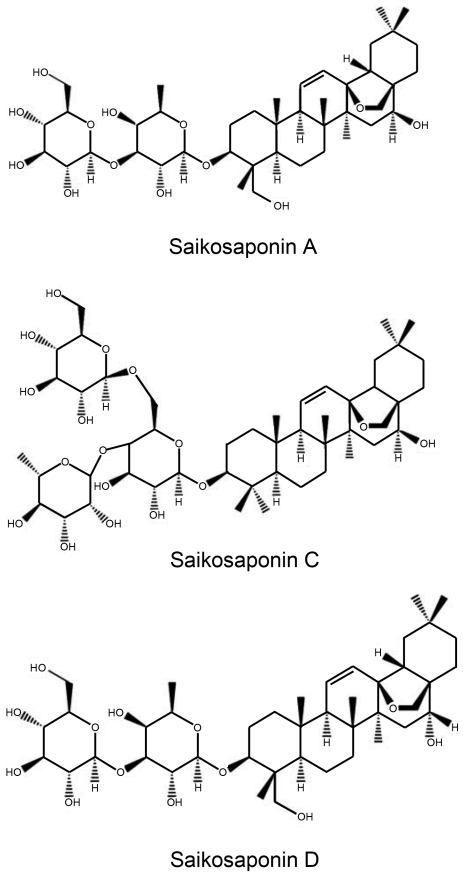

Figure 1. Chemical structures of saikosaponins -A, -C, and -D.

[a]These authors contributed equally to this work.

bines extraction and chromatographic techniques.[9] Extraction methods such as maceration, percolation, heat-reflux extraction, sonication, and Soxhlet extraction are commonly used to prepare herbal materials.[10] However, such methods may be time-consuming, require the use of organic solvents, and may have low extraction efficiencies. An ideal method of extraction would be simple, rapid, and reproducible, with high extraction efficiency, and requires little or no organic solvent.

Recently cloud-point extraction (CPE) has emerged as an environmentally-friendly extraction method, similar to liquid-liquid extraction. The CPE methodology requires only a small amount of non-volatile surfactants and offers the merits of safety, low cost, easy disposal of surfactants, limited environmental pollution effects, and low toxicity.[11-13] Furthermore, the unique behaviors of surfactants at cloud-point can purify and yield highly enriched samples through a simple procedure. The CPE method has been applied in many studies for the extraction of various analytes, including metal ions, vitamins, estrogen, and proteins from biological and environmental samples.[14-17] In contrast, only a few reports have described the extraction of chemical ingredients from herbal medicines using CPE.[13,18,19] To the best of our knowledge, there has been no report on the extraction of saikosaponins from *Radix Bupleuri via* CPE.

We developed a two-steps CPE method to extract major saikosaponins (SSa, SSc, and SSd) from *Radix Bupleuri* in an aqueous solution containing non-ionic surfactant Genapol X-080. At first step, we extracted saikosaponins from the solid matrix and solubilized them into the aqueous surfactant solution, i.e., micelle-mediated extraction. The second step eliminated chemical interferences and pre-concentrated the saikosaponins based on a phase separation mechanism, i.e., cloud-point pre-concentration. We optimized the experimental conditions, including the concentration of the surfactant solution, liquid/solid ratio, extraction time, electrolyte concentration, equilibration time, and temperature. The developed method was applied to determine the level of SSa, SSc, and SSd in *Radix Bupleuri* samples using HPLC-ELSD.

Experimental

Samples and Materials. Dried roots of *Bupleurum falcatum* L. were purchased from a local market (Kyungdong herbal market) in Seoul, South Korea. Pure SSa, SSc, and SSd standards were obtained from Wako Pure Chemical (Osaka, Japan). Genapol X-080 (oligoethylene glycol monoalkyl ether), Triton X-45, Triton X-100, and Triton X-114 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium sulfate was purchased from Kanto Chemical (Tokyo, Japan), and acetic acid (≥ 99.7%) was obtained from Fluka (Buchs, Switzerland). Acetonitrile, methanol, and water (Fisher Scientific, Fair Lawn, NJ, USA) were HPLC grade, and all other reagents used in this study were analytical grade. Nylon membrane filters (0.21 μm) were obtained from Whatman (Maidstone, England).

Micelle-mediated Extraction. *Radix Bupleuri* samples were finely ground using a laboratory mill and sieved through a mesh (No. 70, < 380 μm). One hundred milligrams of powdered sample was accurately weighed and mixed with 20 mL of Genapol X-080 aqueous solution (0.5-20%, v/v) in a 50 mL plastic tube. The tube was placed in an ultrasonic bath (Daihan Scientific, Seoul, South Korea) to extract the saikosaponins from the sample. After ultrasonic extraction at 25 °C for 10-60 min, the resultant extract solution was centrifuged at 4000 rpm for 10 min, and the supernatant was filtered through a 0.21 μm nylon membrane filter. An aliquot of 20 μL was injected into the HPLC-ELSD system.

Cloud-point Pre-concentration. In order to pre-concentrate the aqueous surfactant solution *via* phase separation, 100 mg of *Radix Bupleuri* sample was extracted with 20 mL of 10% Genapol X-080 aqueous solution in an ultrasonic bath at 25 °C for 40 min. After centrifugation, the supernatant was transferred into a 50 mL plastic tube. Various amounts of sodium sulfate (1-6 g) were added to the tube and mixed vigorously for 3 min using a vortex mixer. The resultant cloudy sample solution was incubated in a water bath at 10-70 °C for 10-50 min. Temperature-induced phase separation occurred and was then accelerated *via* centrifugation for 10 min at 4000 rpm. After the aqueous phase was removed with a syringe, the sticky surfactant-rich phase was obtained. One milliliter of the surfactant-rich phase was diluted with an equal amount of methanol to reduce its viscosity and filtered through a 0.21 μm nylon membrane filter prior to HPLC-ELSD analysis.

Extraction Efficiency Comparison. Extraction efficiency was defined as the proportion (mg/g) of the amount of extractable saikosaponins to the amount of *Radix Bupleuri* powdered sample. To compare the extraction efficiencies of 10% Genapol X-080 and conventional solvents (methanol, 70% methanol, and water), uniform extraction conditions were applied (100 mg of sample, 20 mL of solvent, 25 °C of extraction temperature, and 40 min of extraction time) for the quantification of three major saikosaponins (SSa, SSc, and SSd) from *Radix Bupleuri* using the HPLC-ELSD method described below.

HPLC-ELSD Analysis. The determination of saikosaponins was performed using a slight modification of a previously described method.[20] HPLC-ELSD analysis was performed on a PerkinElmer series 200 pump (Waltham, MA, USA), a Waters 717 Plus autosampler (Milford, MA, USA), and a PerkinElmer series 200 column oven equipped with a SEDERE Sedex 75 evaporative light scattering detector (Cedex, France). The injection volume was 20 μL. The three saikosaponins were separated on a Supelco Ascentis® Express C18 column (100 × 4.6 mm i.d., 2.7 μm particle size, Bellefonte, PA, USA) with a Phenomenex Security Guard C18 column (4.0 × 3.0 mm i.d., Torrance, CA, USA) at a flow rate of 1.0 mL/min. The column and autosampler temperatures were maintained at 27.5 °C and 4 °C, respectively. The binary gradient system consisted of 0.1% acetic acid-acetonitrile (90:10, v/v) (solvent A) and 0.1% acetic acid-acetonitrile (10:90, v/v) (solvent B). The

linear gradient program started with 80% solvent A and 20% solvent B for 5 min, decreased to 50% solvent A and 50% solvent B over 35 min, decreased again to 10% solvent A and 90% solvent B for 35-45 min, and then returned to 80% solvent A and 20% solvent B for column equilibration. ELSD was adjusted to a drift tube temperature of 50 °C, and the nebulizer gas (N_2) pressure was set to 3.0 bar. All eluents were filtered through 0.21 μm nylon membrane filters. The acquisition and processing of data were analyzed using Empower software (version 5.00.00.00, Waters).

Results and Discussion

The use of a micelle-mediated extraction and CPE process for the separation and pre-concentration of sample preparation offers distinct advantages: (a) the surfactants are less toxic; (b) the experimental operation is simple; (c) there is little or no loss of analytes due to the absence of an evaporation step; (d) the surfactant-rich phase is compatible with the HPLC mobile phase; and (e) the extraction factor can be optimized according to the type and concentration of surfactant as well as other experimental conditions.[17] It is already known that SSa, SSc, and SSd contain an unstable allyl oxide linkage and are readily converted into diene saponins *via* mild acid treatment.[5,7] Consequently, the addition of a pH modifier for micelle-mediated extraction and CPE process was not examined in this study.

Optimization of Micelle-mediated Extraction Conditions.

Selection of the Surfactant: In our initial study, several types of commercially available non-ionic surfactant, including Triton X-45, Triton X-100, Triton X-114, and Genapol X-080, were evaluated for use as the prospective medium. Genapol X-080 was chosen as the CPE surfactant because it showed the highest extraction efficiency among them. Genapol X-080, a poly-oxyethylene glycol monoether-type surfactant with eight oxyethylene units and tridecyl alkyl moieties, has a critical micellar concentration of 0.028% (w/v) and a cloud-point temperature of 42 °C in pure water.[21] To optimize the micelle-mediated extractions of SSa, SSc, and SSd from *Radix Bupleuri*, parameters affecting the performance of the CPE, including the Genapol X-080 concentration (%, w/v), liquid/solid ratio, and ultrasonic-assisted extraction time, were investigated under different conditions.

Effect of Surfactant Concentration: A surfactant concentration above the critical micellar concentration is required to achieve the cloud-point of the system.[11] Figure 2(a) shows the extraction percentages of the three saikosaponins from *Radix Bupleuri* with an increase in surfactant concentration ranging from 0.5 to 20% (v/v). The properties of the aqueous non-ionic Genapol X-080 solution for extracting saikosaponins may be related to the solubility-enhancement effect of the surfactant micelles.[12] The extraction efficiencies of SSa, SSc, and SSd were higher with a surfactant concentration between 2.5% and 10%, and no remarkable enhancement in extraction efficiency was obtained above 10% due to increased viscosity. In addition, the extract was too sticky for subsequent handling at surfactant percentages greater than 15%. Based on the extraction efficiency and manipulability, 10% Genapol X-080 was selected for further studies.

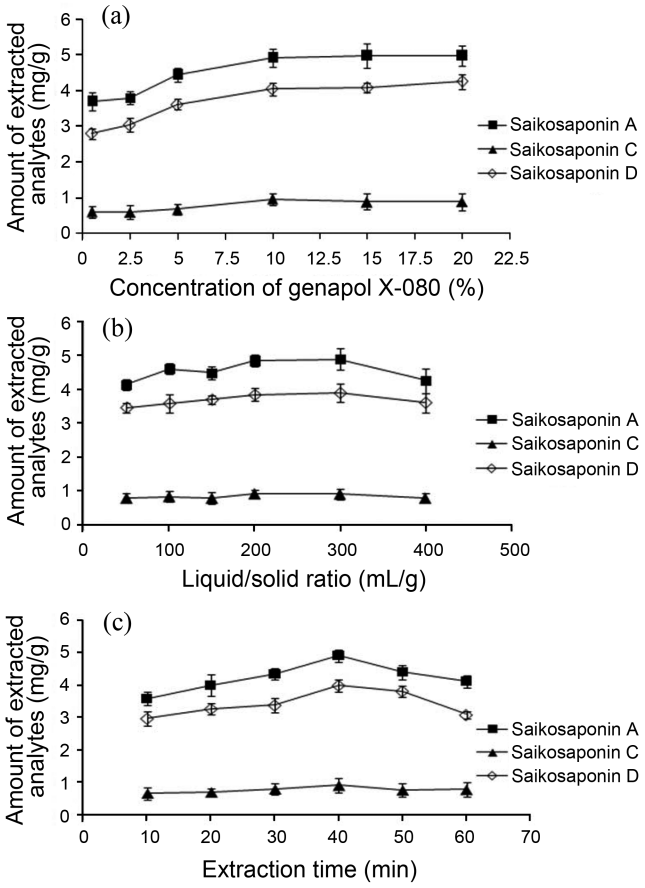

Figure 2. Micelle-mediated extraction. The effects of (a) Genapol X-080 (%, w/v) concentration, (b) liquid/solid ratio, and (c) ultrasonic-assisted extraction time on the extraction efficiencies of saikosaponins -A, -C, and -D (n=3).

Effect of the Liquid/Solid Ratio: The liquid/solid ratio (solvent/matrix), the ratio of the volume of solvent to the amount of solid material, influences the extraction yield. The relationship between liquid/solid ratio and the extracted amounts of SSa, SSc, and SSd is presented in Figure 2(b). The saikosaponin extraction efficiencies were highest when the ratio was 300:1 (mL/g). At the same time, both sensitivity and reproducibility were poor at that ratio because of the relatively small amounts of analytes. There was no significant difference in extraction efficiency between the ratios of 300:1 and 200:1; therefore, a liquid/solid ratio of 200:1 was employed in subsequent steps.

Ultrasonic-assisted Extraction Time: The effect of ultrasonic-assisted extraction time on the extractions of SSa, SSc, and SSd was investigated by varying the sonication time between 10 to 60 min (Figure 2(c)). The highest extraction efficiency was obtained with 40 min of sonication time. When sonication time was longer than 40 min, the contents of extracted saikosaponins, especially SSa, declined, pre-

sumably due to the degradation of saikosaponins by the excess heat generated during sonication. Consequently, 40 min was chosen for the extraction of saikosaponins.

Optimization of Cloud-point Pre-concentration. After the first micelle-mediated extraction step was optimized to extract saikosaponins from a solid herbal matrix into an aqueous surfactant solution, phase separation based on the cloud-point phenomenon of the surfactant was performed. Analytes were pre-concentrated in a small volume of surfactant-rich phase by raising the temperature and adding electrolytes to obtain phase separation. To optimize the cloud-point pre-concentration, we investigated several parameters, including the amount of sodium sulfate, equilibration temperature, and equilibration time according to the recoveries of SSa, SSc, and SSd. The recovery was calculated as the ratio of the analyte contents after cloud-point pre-concentration to the analyte contents before cloud-point pre-concentration.

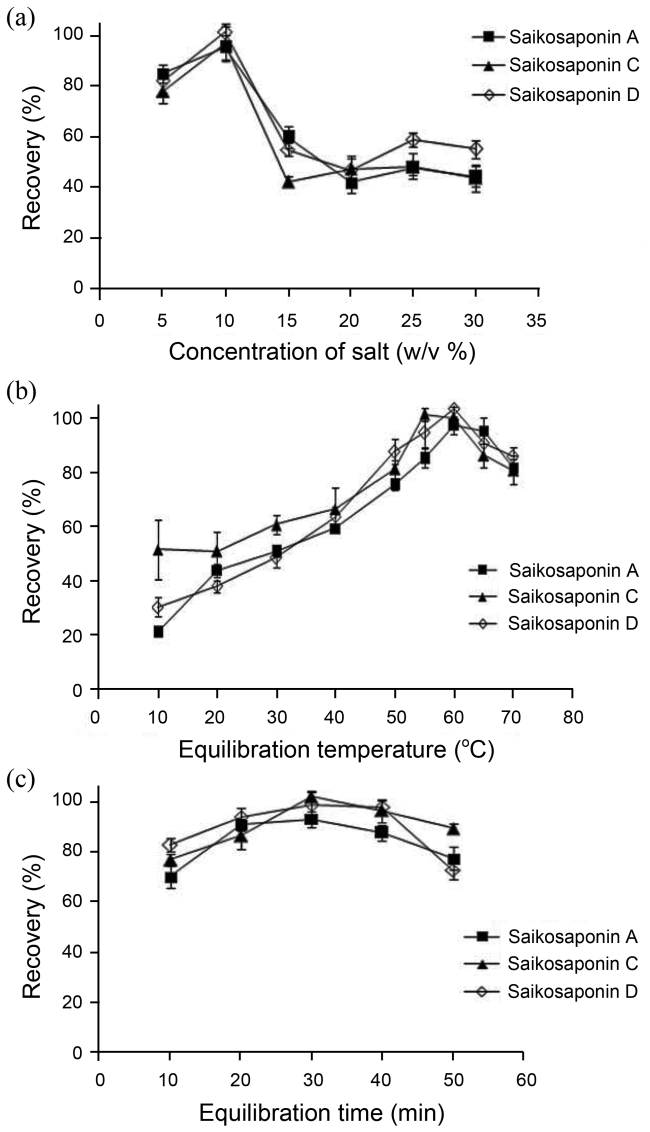

Figure 3. Cloud-point pre-concentration. The influences of (a) the concentration of sodium sulfate, (b) equilibration temperature, and (c) equilibration time on the recoveries of saikosaponins -A, -C, and -D (n=3).

Effect of Sodium Sulfate Concentration: The addition of an electrolyte to the surfactant solution can influence the extraction/pre-concentration process by altering the density of the aqueous phase and inducing phase separation, as well as changing the cloud-point temperature of the non-ionic surfactant.[17] Inert electrolytes such as sodium chloride, sodium sulfate, urea, potassium chloride, and sodium azide have been used in the CPE method.[22] We chose sodium sulfate because it resulted in more obvious phase separation. The extraction recovery was calculated by varying the concentration of sodium sulfate between 5% and 30% (w/v). As shown in Figure 3(a), the sodium sulfate concentration at 10% produced the maximum recovery. Accordingly, sodium sulfate at 10% was used in the subsequent studies.

Effect of Equilibration Temperature: The equilibration temperature is a crucial factor in the pre-concentration step because phase separation depends upon the cloud-point temperature of the surfactant. We measured the recoveries of SSa, SSc, and SSd from 10 to 70 °C in order to optimize the equilibration temperature. It is known that the optimal equilibration temperature of CPE occurs when the temperature is 15-20 °C higher than the cloud-point temperature of the surfactant.[23] Therefore the expected equilibration temperature for Genapol X-080 (cloud-point temperature; 42 °C) was between 57 °C and 62 °C. Overall extraction recovery increased when the temperature increased from 10 to 60 °C (Figure 3(b)). Above 60 °C, the recovery declined. Accordingly, the equilibration temperature was set at 60 °C, which was within the expected temperature range.

Effect of Equilibration Time: Equilibration time is related to the time needed for interactions between the analytes and micelles.[24] The optimal equilibration time provides a favorable environment in which the analytes move into the micelles. The effect of equilibration time on the recoveries of SSa, SSc, and SSd was investigated by changing the equilibration time from 10 to 50 min, maintaining the salt concentration at 10% (w/v) and the equilibration temperature at 60 °C. Because the equilibration time of 30 min showed the highest recoveries of SSa, SSc, and SSd, it was selected as the optimum equilibration time (Figure 3(c)).

Comparison of Extraction Efficiencies between Genapol X-080 and Conventional Solvents. Figure 4 shows the extraction percentages for the three saikosaponins. Under optimal conditions with 10% Genapol X-080, the quantities of extracted SSa, SSc, and SSd were 4.1 mg/g, 0.7 mg/g, and 3.0 mg/g, respectively. With 70% methanol, the SSa, SSc, and SSd quantities were 3.3 mg/g, 0.6 mg/g, and 2.6 mg/g, respectively. The overall extraction efficiency in 70% methanol was better than that in pure methanol. When pure water was used as an extraction solvent, the recoveries were very low. Genapol X-080 had the highest extraction efficiency, possibly due to the more complete diffusion of surfactant solution into the particles of the plant matrix and the solubility-enhancing effect of the surfactant micelles.[17]

Evaluation of Pre-concentration Factor. In this study, the pre-concentration factor (F_c) was defined as the phase

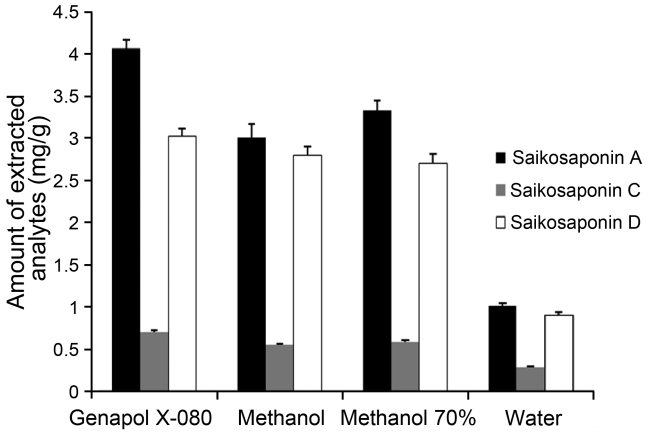

Figure 4. Comparison of extraction efficiencies between Genapol X-080 and other conventional extraction solvents (n=3).

Table 1. Pre-concentration factors at different concentrations of Genapol X-080

Genapol X-080 (%, w/v)	V_o [a] (mL)	V_s [b] (mL)	F_c [c] (V_o/V_s)
0.5	20.0	0.6	33.3
2.5	20.0	1.0	20.0
5	20.0	1.6	12.5
10	20.0	2.6	7.7
15	20.0	5.2	3.8
20	20.0	7.0	2.9

[a]volume of the original sample solution. [b]volume of the obtained surfactant-rich phase. [c]pre-concentration factor

ratio of the volume (V_o) of the original sample solution (20 mL of 10% Genapol X-080 aqueous solution) to the volume (V_s) of the obtained surfactant-rich phase which was a upper phase of two-phase system and had a relatively small volume. The surfactant concentration has a predominant impact on the volume of the surfactant-rich phase, and F_c increases as the surfactant concentration decreases.[25] Accordingly, we evaluated F_c by varying the concentration of Genapol X-080 (0.5-20%, v/v)(Table 1). With 10% Genapol X-080, 2.6 mL of the surfactant-rich phase was obtained and the F_c was 7.7. When the concentration of Genapol X-080 was less than 10%, the F_c was greater than 10, but the surfactant-rich phase volume was less than 2.0 mL, which was too small to handle. The chosen surfactant for use in the proposed CPE method was 10% Genapol X-080.

HPLC-ELSD Profiles. HPLC-ELSD analysis was performed to identify SSa, SSc, and SSd in *Radix Bupleuri* samples using the proposed micelle-mediated extraction and cloud-point pre-concentration. Figures 5(a) and 5(b) present chromatograms of both the aqueous Genapol X-080 solution and the extract of *Radix Bupleuri* before cloud-point pre-concentration. In contrast, Figure 5(c) shows a chromatogram of pre-concentrated saikosaponins present in the pre-concentrated surfactant-rich phase, clearly demonstrating the pre-concentration effect of the CPE process. The retention times of SSa, SSc, and SSd were 13.1, 19.7, and 27.0 min, respectively. All analytes were completely separated

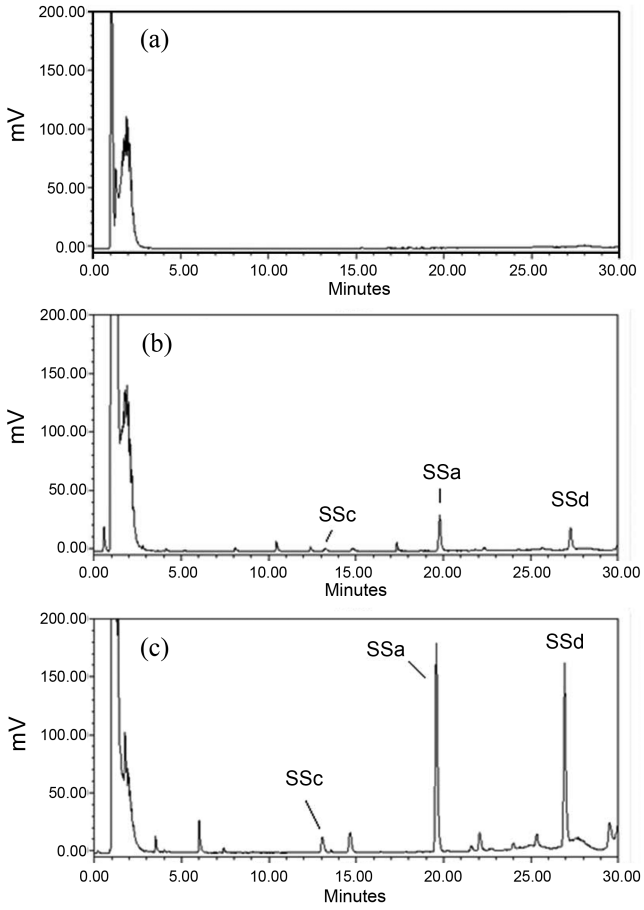

Figure 5. HPLC-ELSD chromatograms of (a) Genapol X-080 (10%, v/v), (b) the extract of *Radix Bupleuri* before cloud-point pre-concentration, and (c) the extract of *Radix Bupleuri* after cloud-point pre-concentration.

from the sample matrices, and the Genapol X-080 did not disturb the detection of the target analytes. The average extraction recoveries of SSa, SSc, and SSd for the proposed method were 93.1%, 101.9%, and 98.8%, respectively (n=3). Our proposed technique is a simple, rapid, effective, and environmentally-friendly extraction method for separation of bioactive constituents in *Radix Bupleuri*. Furthermore, this method also provides the possibility of large-scale extraction and purification of active ingredients from herbal materials since it has already been reported that non-ionic surfactant can be easily removed *via* hydrophobic adsorption with polystyrene resins.[26,27]

Conclusions

To the best of our knowledge, this is the first report on the micelle-mediated extraction and cloud-point pre-concentration of SSa, SSc, and SSd from *Radix Bupleuri*. Without the use of any volatile or potentially toxic organic solvents, CPE provides a simple process for distinctive extraction and pre-concentration of analytes. In our study, satisfactory results for the extraction and pre-concentration of saikosaponins from *Radix Bupleuri* samples were obtained using

10% Genapol X-080. Ultrasonic-assisted extraction combined with a CPE process may be a promising eco-friendly alternative for the solubilization, extraction, and pre-concentration of bioactive components in herbal medicines.

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