Enhanced Extraction of Tanshinones from Salvia miltiorrhiza Using Natural Surfactant-Based Cloud Point Extraction

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Article

Enhanced Extraction of Tanshinones from *Salvia miltiorrhiza* Using Natural Surfactant-Based Cloud Point Extraction

Ye Rim Shin [1], Byeong Ryeol Ryu [1], Min Ji Kang [1], Min Jun Kim [1] and Jung Dae Lim [1,2,*]

[1] Department of Bio-Health Convergence, Kangwon National University, Chuncheon 24341, Korea
[2] Institute of Cannabis Research, Colorado State University-Pueblo, 2200 Bonforte Blvd, Pueblo, CO, 81001-4901, USA
[3] Department of Bio-Functional Material, Kangwon National University, Samcheok 25949, Korea
* Correspondence: ijdae@kangwon.ac.kr; Tel.: +82-33-540-3323

Abstract: *Salvia miltiorrhiza* (SM) contains the tanshinones, a compound with various pharmacological effects, and has been extensively studied as a pharmaceutical material. However, conventional methods for extracting tanshinones face challenges such as environmental hazards and high cost. In this study, we aimed to effectively extract tanshinones from SM using cloud point extraction (CPE) with lecithin, a natural surfactant. By optimizing various extraction conditions including the solid-to-liquid ratio, lecithin concentration, NaCl concentration, pH, and equilibrium temperature, the optimal extraction efficiency was achieved using 20 mL of solvent per 1 g of sample, 3% lecithin (w/v), 2% NaCl (w/v), pH 6, and room temperature (25 ± 2°C). The CPE method, which minimizes the use of organic solvent and is eco-friendly demonstrated improvements in extraction efficiency, with a 4.55% increased for dihydrotanshinone I, 8.32% for cryptotanshinone, 15.77% for tanshinone I, and 6.81% for tanshinone IIA compared to the conventional water extraction method. These results suggest that CPE is promising, environmentally friendly, and efficient approach for extracting hydrophobic components from pharmacologically active materials such as SM, with potential applications across various fields of natural product extraction.

Keywords: *Saliva miltiorrhiza*; cloud point extraction (CPE); lecithin; tanshinone

1. Introduction

Salvia miltiorrhiza (SM) is a genus of the *Labiatae* family, has been traditionally used in China, Korea, and Japan in Asian countries [1]. SM contains over 100 active compounds which are classified two main categories: water-soluble phenolic acid components and hydrophobic tanshinones [2]. The tanshinone group primarily consists of lipophilic phenanthrene-quinone and its derivatives [3], including dihydrotanshinone I (DHTS), cryptotanshinone (CT), tanshinone I (Tan I), and tanshinone IIA (Tan IIA). These secondary metabolites accumulate mainly in the roots and exhibit various pharmacological effects such as antibacterial, antioxidant, and antitumor properties, making them promising candidates for the treatment of diseases like cardiovascular and cerebrovascular diseases disorders [4,5]. Notably, due to their natural origin, tanshinones have been extensively incorporated into various formulations such as tablets, injections, and ointments in combination with modern medicine, providing new therapeutic options and significant industrial value as high-value natural product materials [6]. However, despite the high value, the extraction yield of tanshinones remains low, and ongoing research to improve their efficient utilization [7,8].

The conventional approach to extracting tanshinones involves the use of organic solvents such as ethanol and methanol. However, the application of these solvents in industries like food and pharmaceuticals is limited due to their toxicity and high flammability. Additionally, even when solvents are used, extraction steps are required to remove them at end of the process, which increases

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production cost. Moreover, residual solvent may inadvertently remain, raising concerns about product quality and safety [9].

The International Council for Harmonisation (ICH) guidelines set limits for residual solvent in organic solvents specifying a maximum of 30 mg/L for methanol and 50 mg/day for ethanol. While the Food and Agriculture Organization (FAO) does not regulate ethanol, it does recommend a limit 10 mg/kg for methanol in most foods [10,11].

Alternative extraction methods include reflux extraction with polar solvents such as $CHCl_3$ and ethyl acetate [12], soaking, percolation, and ultrasonic extraction [13], continuous ultrasound-assisted extraction with high intensity ultrasonic probe (CUAE-HIUP) [14], supercritical fluid extraction (SFE) [15], pressurized-liquid extraction [16], infrared-assisted extraction [17], and ionic liquid-based ultra-high pressure extraction [18]. However, the need to develop new, simple, and safe extraction methods remains critical, as existing methods often require expensive equipment or involve complex processes that can increase production costs.

Cloud point extraction (CPE) is a technique in which a surfactant is heated to a temperature to reach its 'cloud point' where the solution becomes cloudy, leading to the formation of micelles. These micelles separate into a surfactant-rich layer and an aqueous layer, trapping the analytes within the surfactant layer [19]. The micelles have a hydrophobic core that captures hydrophobic compounds, while the hydrophilic outer layer stabilizes the micelles in the aqueous layer [20]. CPE is simple, cost-effective, and environmentally friendly method that minimized or eliminates the use of organic solvent [19,21]. It has been applied to extract organic compounds from food or heavy metals from water, and bioactive substances from plants [22–24]. Nonionic surfactants such as Triton X-100, Triton X-114, and Tween 80 are commonly used in CPE. Previous studies have been used, synthetic surfactants like Genapol X-080 or Triton X-100 to extract tanshinones from SM [25,26]. While these surfactants are approved as edible by the U.S. Food and Drug Administration (FDA), they are not naturally derived. In contrast, lecithin, a natural surfactant obtained from sources, like sunflower, soybean, and egg, is widely used in the food, pharmaceutical, and cosmetic industries due to its non-toxic and biocompatible nature. Lecithin is also designated as GRAS (Generally Recognized as Safe) by FDA [27]. Additionally, it is classified as a food additive in the European Union (EU) under the code E322 and can be used in any quantity [27,28]

Given the growing demand for tanshinone derived from *Salvia miltiorrhiza* (SM), there is an urgent need for efficient extraction methods that maximize yield and preserve bioactivity. This study aims to optimize cloud point extraction (CPE) using lecithin, a natural surfactant, as an alternative to organic solvents for extracting tanshinones, the hydrophobic compounds from SM. The extraction efficiency of the optimized CPE method will be evaluated by comparing it with extracts obtained using conventional solvent-based methods through component analysis.

2. Materials and Methods

2.1. Chemicals

Solid lecithin derived from soybean, along with standards for HPLC analysis, dihydrotanshinone I (DHTS), cryptotanshinone (CT), tanshinone I (Tan I), and tanshinone IIA (Tan IIA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaCl, citric acid anhydrous, and methanol were obtained from DAEJUNG (Siheung, Korea). Filter paper (No. 6) was sourced from Whatman International Ltd. (Maidstone, England), and syringe filters (13JP020AN, 13HP020AN, 0.2 ㎛) were purchased from Advantec Mfs. Inc. (Dublin, CA, USA). HPLC-grade acetonitrile and water were supplied by DUKSAN (Ansan, Korea), and acetic acid glacier was purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Sample Pretreatment

The SM samples were provided by the Korean variety 'Dasan', cultivated and harvested in April 2021 by the SM Specialty Division of the Rural Development Administration in Eumsung, Korea. The

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samples were dried at 55 °C and ground into fine powder. The powder was then stored at 4 °C until used in the experiments.

Samples (1 g each) were mixed with water containing 5% (w/v) lecithin, and subjected to ultrasonic-assisted extraction (UAE) at room temperature for 40 minutes. The supernatant was then separated by centrifugation at 3500 rpm for 15 minutes. The supernatant was collected, and as a control, the SM water extract was subjected to the same conditions as the optimized CPE, excluding the CPE process.

2.3. Cloud Point Extraction (CPE) Procedure

The CPE process was optimized by systematically adjusting the solid-to-liquid ratio, surfactant concentration, NaCl concentration, pH, and equilibrium temperature. The CPE method was adopted from Alibade et al. [27] and Bi et al. [26]. After the supernatant was separated, 5% (w/v) NaCl was added, and the pH was adjusted to 3 using 1 M citric acid. The mixture was equilibrated in a water bath at 40 °C for 30 minutes, then centrifuged at 3500 rpm for 15 minutes to separate the surfactant layer from water layer. Both layers were freeze-dried, with the water layer diluted with distilled water and the surfactant layer diluted with methanol to a concentration of 10 mg/mL for analysis. The final selection was based on the content of tanshinones (DHTS, CT, Tan I, and Tan IIA), which showed the highest levels in the surfactant layer and the lowest levels in the water layer.

2.4. HPLC Analysis

The constituents in the extract were analyzed using a modified method based on Chen et al. [29] targeting four components (DHTS, CT, Tan I, Tan IIA). Standards for each tanshinone were prepared at concentrations of 2 ug/mL, 4 ug/mL, 8 ug/mL, 16 ug/mL, and 32 ug/mL. Calibration curves were generated to calculate regression equation and R^2 values to determine the sample's component content. All samples were analyzed at 10000 ug/mL. High-performance liquid chromatography (HPLC) analysis was conducted using a Shimadzu LC-20AT HPLC system equipped with a YMC-Pack ODS-AM column (250 mm× 4.6 mm I.D., 5 um) maintained at 30 °C. The flow rate was set at 1.0 mL/min, with an injection volume of 10 uL and detection was performed at 280 nm. The mobile phases consisted of 0.8% (v/v) acetic acid in water (A) and 0.8% (v/v) acetic acid in acetonitrile (B) were eluted in a gradient manner as follows: 2-46% B from 0 to 40 min, 46-66% B from 40 to 60 min, 66-48% B from 60 to 70 min, 48-90% B from 70 to 71 min, and 90-90% B from 71 to 80 min.

2.5. Transmission Electron Microscopy (TEM) Analysis

Transmission electron microscopy (TEM; JEM-2100F, JEOL, Akishima, Japan) was used to visually analyze the morphology and size of micelles, as well as the transmittance of the samples based on surfactant concentration. All lyophilized surfactant layer samples were dispersed in distilled water and then dried on filter paper (1002-090, 90 mm, Whatman, Maidstone, UK). Measurements were conducted at an accelerating voltage of 200 kV.

2.6. Statistical Evaluation

Each experiment was performed in triplicate, and the mean value and standard error were calculated. Statistical analysis was conducted using GraphPad Prism 8.0.1 (San Diego, CA, USA) to assess significance at the 1% level of the T-test ($p < 0.01$).

3. Results and Discussion

3.1. Calibration Curves and Linearity

The standard curves and coefficients of determination for each component are shown in Table 1. The results confirm the method's suitability for both quantitative and qualitative analysis, validating its linearity and accuracy.

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Table 1. Linear equations and correlation coefficients of each tanshinone.

Compounds	Linear equation	Correlation coefficient
Dihydrotanshinone I (DHTS)	Y=32230x + 6450.7	0.9999
Cryptotanshinone (CT)	Y =17924x – 1518.5	0.9999
Tanshinone I (Tan I)	Y =34243x + 6435.1	0.9998
Tanshinone IIA (Tan IIA)	Y =42974x + 13509	0.9996

3.2. Effect of Solid-to-Liquid Ratio

The solid-to-liquid ratio is a crucial factor in sample extraction as it influences the concentration gradient between the extraction solvent and the sample surface, thereby affecting the extraction kinetics [30]. Figure 1 illustrates the impact of varying the extraction solvent ratio from 20 to 80 mL per gram of sample.

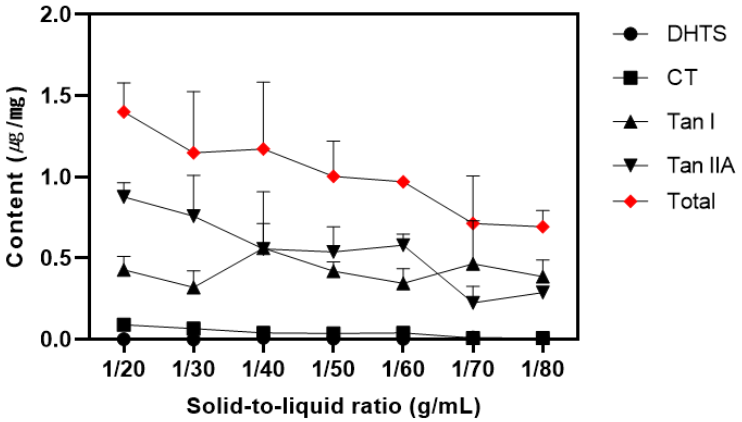

Figure 1. Effect of solid-to-liquid ratio on the tanshinone content in surfactant layer.

For the surfactant layer, CT and Tan IIA showed a gradual decrease in component content as the amounts of extraction solvent increased. Although DHTS displayed statistically significant differences, the practical impact was negligible considering the margin of error. Tan I exhibited high mean values at 40, 50, and 60 mL, but due to the large standard deviation, none of the ratios showed statistically significant differences. Considering the overall content, the extraction ratio of 1 g/20 mL was found to yield the highest content (Figure 1).

In the water layer, the content of CT, Tan I, and Tan IIA generally increased as the extraction solvent ratio increased (Table S1). This was attributed to excessive extraction solvent use, which hindered proper layer separation. While a higher solvent ratio can enhance extraction efficiency, an excessive amount actually reduces efficiency and prolongs the concentration time [31]. A study by Leite et al. [32] demonstrated that reducing the proportion of non-ionic surfactant solution in the chlorophyll extraction from spinach leaves increased the amount of chlorophyll extracted. Similarly, Shi et al. [25] found that an extraction ratio of 1 g/20 mL was optimal for extracting tanshinones from SM using micelles and measuring them by HPLC. Therefore, based on the above studies, 1 g/20 mL was concluded to be the optimal extraction ratio.

3.3. Effect of Surfactant Concentration

Surfactant concentration is a key factor in optimizing the CPE process and, together with the solid-to-liquid ratio, plays a significant role in determining extraction efficiency. To effectively capture hydrophobic components, sufficient micelle formation above the critical micelle concentration (CMC) is required [19]. In this study, lecithin concentration was tested within the range of 2% - 10% (w/v) (Figure 2).

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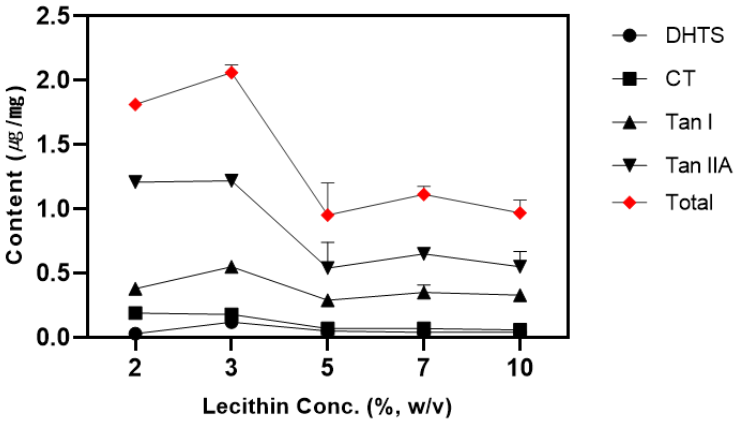

Figure 2. Effect of surfactant concentration on the tanshinone content in surfactant layer. Conc. denotes concentration.

In the surfactant layer, there was a statistically significant difference between 2% and 3% for CT and Tan IIA, while the 3% concentration showed the highest content in DHTS and Tan I. The total tanshinone content increased with lecithin concentrations up to 3%, but showed a decreasing trend thereafter. This is likely due to the effect of surfactant concentration on micelle size, interfacial area, surfactant layer formation, extraction step dilution, and preconcentration factor. High surfactant concentrations can reduce extraction efficiency by decreasing micelle size and causing surfactant saturation in the solution, while low concentrations can result in decreased solubility due to insufficient surfactant layer formation and extraction [33,34]. As surfactant concentration decreases, the ratio of the preconcentrated aqueous solution volume to the surfactant layer volume increases, leading to a higher preconcentration factor [35]. In the water layer, the content of all components increased with rising lecithin concentration, indicating unstable layer separation at higher surfactant concentrations (Table S2).

Interestingly, the DHTS content in the water layer was relatively high at 2% lecithin concentration, likely due to the lower hydrophobicity of DHTS. Hydrophobicity is usually expressed by the log *P* value, based on the octanol/water partition coefficient, with negative values indicating hydrophilicity and positive values indicating hydrophobicity [36]. Due to the relatively low log *P* value (3.904) of DHTS, it likely does not preferentially bind to micelles and is more distributed in the water layer than other tanshione components (Table 1). According to Fischer et al. [37], hydrophobic components with a high log *P* value are fully soluble in micelles, while hydrophilic components are either evenly distributed between the micelle and aqueous phase or are more present in the aqueous phase. At low lecithin concentrations, micelle formation may be insufficient, resulting in decreased DHTS adsorption into micelles and increased content in the water layer. Based on these results, the optimal lecithin concentration for maximizing extraction efficiency of all components was determined to be 3% (w/v).

Table 2. Lists of log *P* values of tanshinones.

Compounds	Log *P*	Reference
Dihydrotanshinone I	3.904	[38]
Cryptotanshinone	4.931	
Tanshinone I	4.443	
Tanshinone IIA	5.471	

3.4. Morphology of Micelle

The morphology and particle size changes of micelles with the varying lecithin concentration were analyzed using transmission electron microscopy (TEM) and compared to the control sample

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without lecithin (lecithin 3%) (Figure 3). The smallest spherical micelles were observed at 2% lecithin concentration with the largest size at 3% lecithin, followed by a gradual decrease in size and increase in particle number with increasing surfactant concentration (Figure 3a–e). At 10% lecithin, a mixture of spherical and elongated micelles was observed (Figure 3e).

According to Pisárčik et al. [39], an increase in cationic surfactant concentration lead to a decrease in micelles size due to stronger charge repulsion. Although lecithin is neutral as a zwitterionic surfactant, under experimental conditions at pH 3, lecithin became positively charged, leading to a decrease in micelle size due to charge repulsion [40]. This finding is consistent with Pisárčik et al. [39].

High concentrations of surfactants can cause spherical micelles to transform into worm-like micelles [41]. Various shapes of micelles were observed at 10% lecithin, considered a transitional concentration where micelle morphology changes at high concentrations.

Comparing micelles with and without SM samples, it was found that micelles in the control group without the sample were relatively arranged compared to micelles with the sample (Figure 3b,f). This is likely due to the presence of the hydrophobic component, tanshinone, which influenced micelle binding density. The largest micelles capable of effectively entrapping the component were formed at 3% lecithin, corresponding to the most stable concentration for CPE. Correlation with HPLC results also supports this finding.

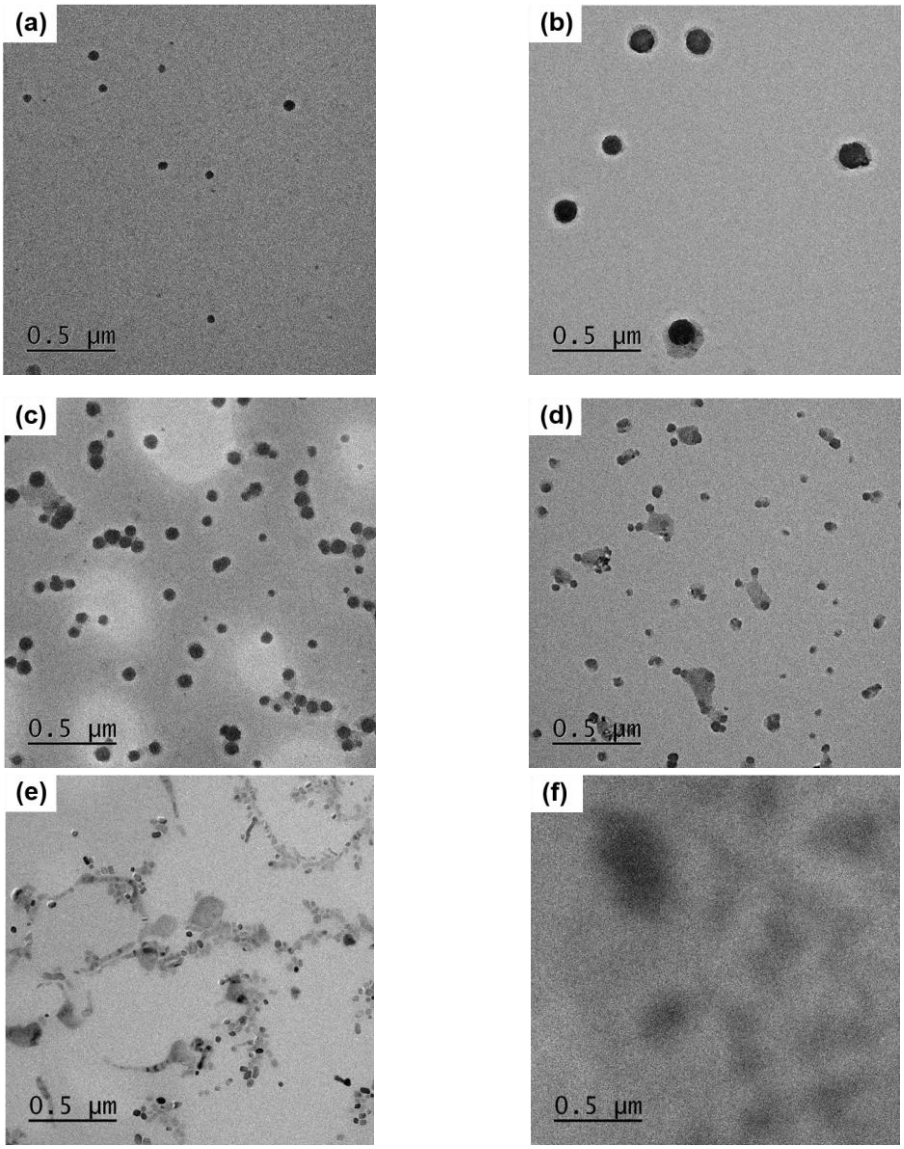

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Figure 3. Transmission electron microscopy (TEM) images of micelle formed by lecithin concentration. (a) 2% lecithin; (b) 3% lecithin; (c) 5% lecithin; (d) 7% lecithin; (e) 10% lecithin; (f) 3% lecithin control.

3.5. Effect of NaCl Concentration

Generally, phase separation in CPE is achieved by heating to the cloud point temperature (CPT). However, adding salts such as NaCl and Na_2SO_4 can induce phase separation at lower temperatures to enhance extraction efficiency [42]. We chose NaCl for its cost-effectiveness and availability and tested concentrations 2%, 3%, 5%, 7%, 10% (w/v). The results are shown in Figure 4.

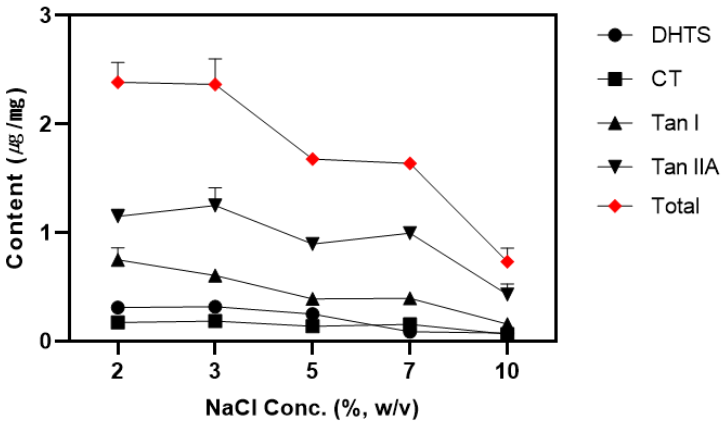

Figure 4. Effect of NaCl concentration on tanshinone content in surfactant layer. Conc. denotes concentration.

The tanshinone content in the surfactant layer was relatively consistent at 2% and 3% NaCl but showed a significant decrease starting at 5%. Tan IIA exhibited the highest content at 3% NaCl, but there was no statistically significant difference between 2% and 3% when considering the total tanshinones content. The other tanshinones showed similar behavior. NaCl induce "salting-out", where the addition of NaCl increase the attraction between water molecules and salt ions, weakening the interaction between the micelle's hydrophilic head and water molecules, promoting tanshinone migration into the micelle [43,44]. However, when the NaCl concentration exceeds 5%, the tanshinone content tends to decrease because the increased charge on the micelle surface hinders micelle formation [45]. The content of tanshinones that could not be extracted into micelles and remained in the water layer increased slightly with rising NaCl concentration, but the content was low across the entire range due to stabilization by the surfactant concentration (Table S3). Considering the economic and environmental factors, the optimal NaCl concentration for tanshinones extraction was determined to be 2%.

3.6. Effect of pH Value

The pH was adjusted by adding 1 M citric acid in the range of pH 2 to 6 with pH 6 serving as the baseline without citric acid. The extraction efficiency was evaluated and shown in Figure 4 (pH 6 was without citric acid).

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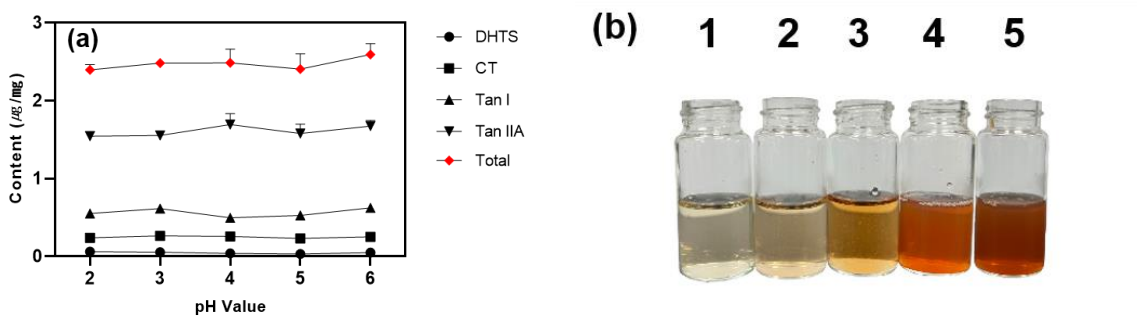

Figure 5. (**a**) Effect of pH on tanshinone content in surfactant layer. Conc. denotes concentration; (**b**) Water layer with surfactant layer removed (1: pH 2; 2: pH 3; 3: pH 4; 4: pH 5; 5: pH 6).

DHTS and CT maintained similar contents at all pH values, with Tan I showing the highest content at pH 3 and Tan IIA at pH 4, though no statistically significant differences were observed. CPE efficiency is generally pH-dependent, influencing complex formation [46]. For neutral or non-ionic compounds, the impact of pH change is minimal [47], a finding corroborated by Bi et al. [26], who isolated CT and Tan I from SM using Triton X-100.

Interestingly, as the pH increased, the color of the water layer darkened to brown, and layer separation became unstable. HPLC analysis confirmed that more tanshinones remained in the water layer at higher pH levels (Table S4). Tanshinone compounds are known to be sensitive to cations [48], and lecithin, neutral in aqueous solution, can become positively or negatively charged depending on pH [40]. At low pH, lecithin is protonated and forms strong bonds with tanshinone, facilitating layer separation. At higher pH, lecithin is deprotonated and becomes neutral or negatively charged, weakening its interaction with tanshinones leading to less separation and more tanshinones remaining in the water layer, darkening its color.

The analysis revealed that adjusting the pH improved the speed and clarity of the layer separation but did not significant impact the tanshinone content in the extract layer. This suggests that effective tanshinone extraction can be achieved without pH adjustment, and the influence of pH on the overall CPE process was deemed insignificant, leading to the elimination of the pH adjustment step.

3.7. Effect of Equilibrium Temperature

Lecithin is known for forming micelles at lower equilibrium temperatures than other surfactants, with the cloud point temperature (CPT) further reduced by adding salt [42]. Leveraging these characteristics of lecithin, tanshinones were extracted at temperatures ranging from 25°C (room temperature) to 50 °C, with phase separation observed (Figure 6).

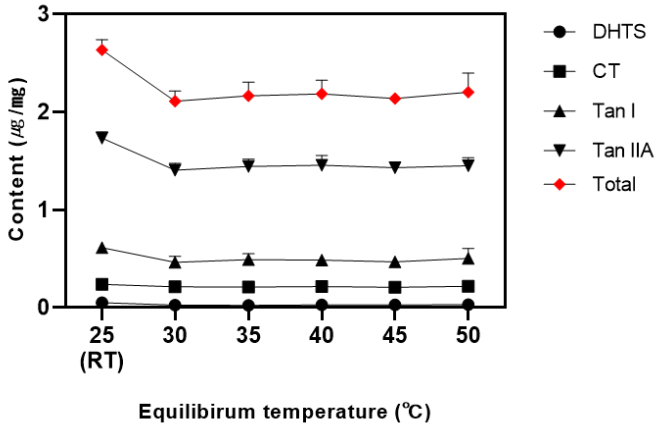

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Figure 6. Effect of equilibrium temperature on tanshinone content in surfactant layer. RT denotes room temperature.

The result showed that the sample reacted at room temperature had the highest content of all tanshinones, with no significant difference at other temperature ranges. The content of tanshinones in the aqueous phase was found to be invariant with temperature (Table S5). These results likely related to lecithin's low cloud point temperature (CPT). Consistent with previous findings [19] that certain surfactants exhibit layer separation even at room temperature when salts are added, this study suggests that adding NaCl to lecithin allows effective extraction at lower temperatures. Although low temperature result in slower layer separation, centrifugation achieved the same effective separation as higher temperature. Therefore, 25°C (room temperature) was determined to be the appropriate temperature for the equilibrium reaction.

3.8. Comparison with Other SM Extract

The optimized *Salvia miltiorrhiza* (SM) CPE (SM-CPE) conditions were compared with SM water extract (SMW) regarding component content and extraction efficiency. SMW is a common herbal medicine extraction method, was used as the control without CPE. For the comparison of extraction efficiency, the tanshinone content of SM extracted with ethanol (EtOH) supplemented with 5% acetic acid (A.A) (5% A.A+EtOH) was considered the total content. Generally, organic solvents are the easiest and most efficient way to extract tanshinone, and Zhu et al. [49] reported that adding 5% A.A. to the organic solvent is more effective for tanshinone extraction. Due to solvent limitations in food, EtOH was chosen instead of methanol. The relative extraction efficiency was calculated by comparing the tanshinone content of each extract with the SM 5% A.A+EtOH. The calculation was performed using Equation (1):

$$\text{Extraction efficiency}(\%) = \frac{Cet}{Ttc} \times 100 \quad (1)$$

where *Cet* is the respective tanshinone content in each extract, and *Ttc* is the respective tanshinone content in the SM 5% A.A+EtOH.

Table 2. Extraction efficiency of each SM extracted method.

Sample	Extraction efficiency(%)				
	DHTS	CT	Tan I	Tan IIA	Total
SM-CPE	8.47±1.34	9.57±1.16	17.15±2.08	7.11±0.57	42.30±0.62
SMW	3.92±0.38	1.25±0.19	1.38±0.21	0.30±0.02	6.85±0.14
T-test ($p<0.01$)	*	**	**	**	**

Comparing the two extracts, SMW had significantly lower content than SM-CPE (Figure 7a–d). For Tan IIA, a representative tanshinone, SM-CPE contained 1.74±0.03 ug/mg, while SMW contained 0.07±0.00 ug/mg, which is about 23.5 times higher. DHTS was about 2.16 times higher, CT was 7.64 times higher, and Tan I was about 12.4 times higher (Figure 7d). The extraction efficiencies for each tanshinone by different extraction methods were calculated based on 100% content of SM 5% A.A+EtOH and are shown in Table 2. For all tanshinones, SM-CPE showed higher extraction efficiency than SMW, with difference ranging from 15.77% to 4.55%. Considering the total tanshinone content, SM-CPE recovered about 42.3% of tanshinones compared to 5% A.A+EtOH extract, demonstrating that the optimized CPE conditions were more effective for extracting tanshinones from SM than the conventional water extraction method.

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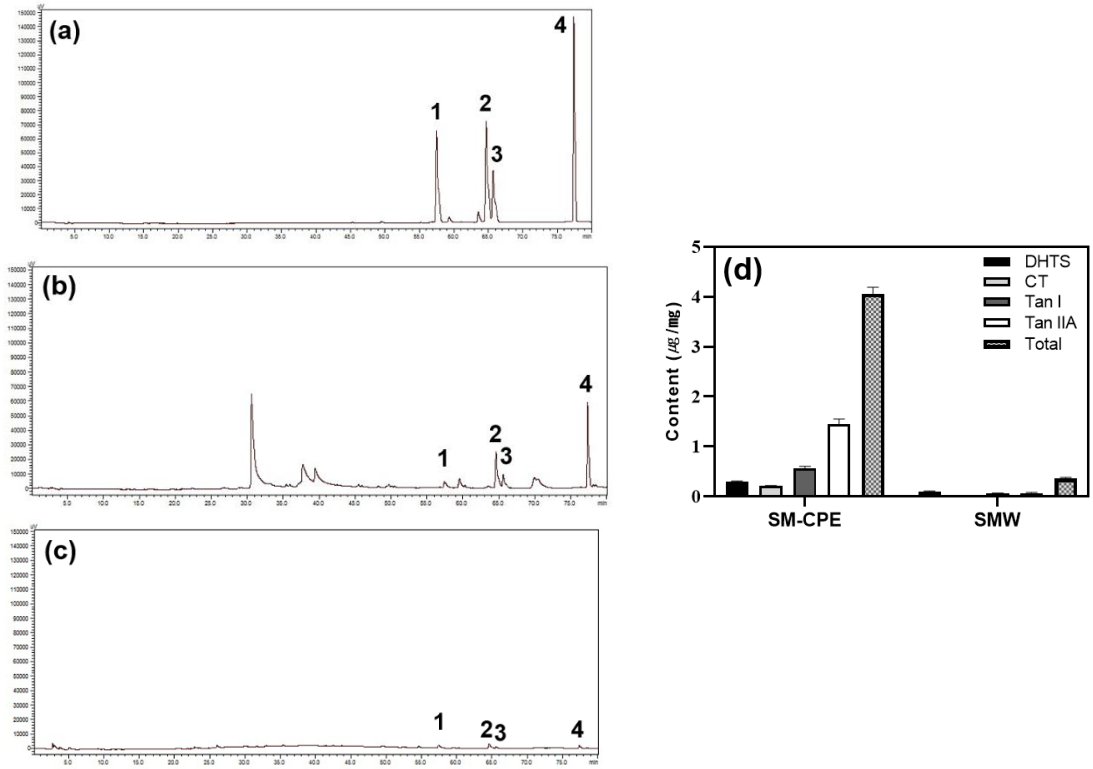

Figure 7. (a) HPLC chromatogram of 4 tanshinones standard mixture (50 ug/mL, 1 dihydrotanshinone I (DHTS); 2 cryptotanshinone (CT); 3 tanshinone I (Tan I); 4 tanshinone IIA (Tan IIA); (b) HPLC chromatogram of SM-CPE; (c) HPLC chromatogram of SMW; (d) Contents of tanshinones in SM sample extracted with different solvent.

4. Conclusions

In this study, we applied cloud point extraction (CPE) technology to *Salvia Miltiorrhiza* (SM) to establish optimal extraction conditions for tanshinones, a hydrophobic component, and compared it with conventional water extraction methods. The results showed that 20 mL of solvent, 3% lecithin (w/v), 2% NaCl (w/v), pH 6 (no pH adjustment required), and room temperature (25°C) per 1g of sample yielded the best extraction efficiency. Notably, high extraction efficiency can be achieved without pH adjustment or high temperature reaction, simplifying the process. Additionally, micelle formation promoted by lecithin expected to improve the bioavailability of tansinone. Although the total content is lower than that obtained by organic solvent extraction, this study introduces a new approach to effectively extract tansinones, a hydrophobic component, from aqueous extraction systems. This method is eco-friendly and safe by eliminating the use of organic solvents, making it a promising technology for future natural product extraction.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Effect of solid-to-liquid ratio on the content of tanshinone content in water layer; Table S2: Effect of surfactant concentration on the tanshinone content in water layer, Table S3: Effect of NaCl concentration on the tanshinone content in water layer, Table S4: Effect of pH on the tanshinone content in water layer, Table S5: Effect of equilibrium temperature on the tanshinone content in water layer.

Author Contributions: Conceptualization, Y.R.S.; methodology, Y.R.S. and J.D.L.; formal analysis, M.J.K.; investigation, Y.R.S. and M.J.K.; data curation, B.R.R.; writing—original draft preparation, Y.R.S.; writing—review and editing, Y.R.S. and J.D.L.; supervision, J.D.L.; project administration, B.R.R. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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