Extraction of Cynaroside from Honeysuckle by Nonionic Surfactant-mediated Aqueous Extraction with Cloud Point Enrichment and Purification by Metal Complexation

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Abstract

Honeysuckle is rich in cynaroside (Cy), but its extraction and purification are difficult due to its poor solubility in water. Therefore, nonionic surfactants were used to extract Cy from honeysuckle plants, enabling the formation of micelles that solubilized Cy in aqueous solution. The effects of temperature, extraction time, surfactant concentration, and the solid-liquid ratio on the extraction rate of Cy were investigated to determine the optimal extraction conditions. The cloud point effect of the optimal nonionic surfactant was exploited to separate the extracting solution into two distinct phases, which resulted in the concentration of Cy into the up phase. The phase containing the extracted Cy was then purified by metal complexation after phase separation using $CaCl_2$ followed by EDTA, resulting in a purity of Cy of 93.7%. Surfactants have high biological safety, making the extraction process safer and more environmentally friendly. As a result, the process developed in this study enabled the efficient and environmentally friendly extraction of Cy from plants, which has potential value in the production of various medicines and health products containing natural botanicals, such as Cy.

Introduction

For many years, plant extracts have been utilized as raw materials in the development of health products and nutraceuticals (Wilson et al. 2022). Modern extraction methods, such as microwave radiation, ultrasonication, as well as the use of supercritical fluids and enzymes, have demonstrated significant advantages over conventional extraction methods to augment the extraction efficiency and selectivity of bioactive components (Ke et al. 2023). However, these new and improved extraction methods consume more energy and create significant waste, posing serious environmental concerns (Shentu et al. 2023; Tran Thai Ha et al. 2023). With increasingly prominent global environmental issues, the development of sustainable technologies to facilitate the extraction and/or production of natural products from plants has received widespread attention.

Surfactants are amphiphilic compounds that reduce the interfacial tension between similar phases and are used in variety of applications as emulsifiers, detergents, and wetting agents, to name a few (Kergomard et al. 2021; Nagtode et al. 2023). Surfactants are typically prepared on an industrial scale by chemical synthesis from fossil fuels and organic matter. When the concentration of a surfactant reaches or exceeds the critical micelle concentration (CMC), the individual surfactant molecules undergo spontaneous aggregation, resulting in the formation of micelles (Smith et al. 2022). In these micelles, the polar heads of the surfactant molecules are oriented toward the aqueous phase, while the hydrophobic chains are oriented towards the hydrophobic interior of the micelle, the polarity of which is favorable for poorly water-soluble molecules (Berardino et al. 2023). Therefore, micelles are critical tools for increasing the apparent solubility of poorly water-soluble compounds in water.

Flavonoids are a class of naturally occurring phytochemicals biosynthesized that are produced in the wood, leaves, flowers, fruits, seeds, and roots of plants (Deng et al. 2023; Lydia Pui Ying et al. 2023). Flavonoids comprise a 15-carbon skeleton, in which two benzene rings are coupled together through a

three-carbon chain (Malla et al. 2022). These compounds are frequently found in combination with sugars, forming glycosides. Over the years, flavonoids have been extensively investigated for potential applications in medicines (Zhang and Yan 2023) and pharmaceuticals (Johan et al. 2023), as well as natural health foods and beverages (Oei et al. 2023), because they have demonstrated anti-inflammatory, antiviral, antioxidant, and anticancer properties and are nontoxic (Ling et al. 2023; Peng et al. 2023). They are also utilized in agricultural production and animal husbandry (Schnarr et al. 2022; Lin et al. 2022).

Cynaroside (Cy) is the 7-*O*-glucoside of luteolin, a flavone (subclass of flavonoids) naturally occurring in several plant species, including *Lonicera caerulea* L. (honeysuckle) (Bouyahya et al. 2023). Several studies have reported that Cy has numerous pharmacological properties, such as antibacterial, antifungal, antileishmanial, antioxidant, hepatoprotective, antidiabetic, anti-inflammatory, and anticancer properties (Bouyahya et al. 2023; He et al. 2023; Liu et al. 2023). Although Cy has good biological activity, its application is limited due to its low extraction efficiency and high cost.

In this study, Cy was extracted from honeysuckle plants using a surfactant-mediated extraction with cloud point enrichment. First, we determined which nonionic surfactant enabled the highest extraction efficiency of Cy. We then determined the optimal extraction conditions of the optimal nonionic surfactant by investigating the effects of temperature, extraction time, surfactant concentration, and the solid-liquid ratio on the extraction rate of Cy. The cloud point properties of the optimal nonionic surfactant enabled the selective concentration of Cy after phase separation. Finally, we determined the optimal metal chelating agent for achieving the most efficient purification of Cy. The results reported herein highlight the development of a novel, sustainable, and highly efficient method for extracting bioactive components from plants.

Material and Methods

Materials

Honeysuckle (the content of Cy in honeysuckle was found to be 0.711 ± 0.023 mg/g) was collected from Linyi, Shandong Province, China, stoved, and then ground into a powder. Cy (99%) was obtained from Chengdu Zhibiao Pure Biotechnology Co., Ltd. (Sichuan, China). Tween-60 (T-60), tween-80 (T-80), span-80 (S-80), decacylglycerol monolaurate (POL-10), and tween-40 (T-40) were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Alkyl polyglucoside-0810 (APG-0810), nonylphenol ethoxylate-10 (NP-10), soap nut saponin (SOS 80%), polyethylene glycol 400 (PEG-400), sodium lauryl ether sulfate (SLES), cocamidopropyl betaine (CAB), dodecyl dimethyl betaine (BS-12), and Saponin (SAP, 98%) were obtained from Chengdu Dianchun Technology Co., Ltd. (Sichuan, China). Acetonitrile (99.9%) and phosphoric acid (85%) were obtained from Beijing Bailingwei Technology Co., Ltd. (Beijing, China).

Extraction of Cy

The ground honeysuckle powder (5.0 g) was added to beakers containing aqueous solutions of different surfactants. Then, the beakers were placed in a water bath, and the solutions were stirred to perform the extraction. Single factor experiments were conducted to optimize the extraction rate of Cy based on temperature (20, 30, 40, 50, 60, 70℃), time (30, 60, 90, 120, 150, 180 min), surfactant concentration (2, 4, 6, 8, 10, 12%), and solid-liquid ratio (1:5, 1:10, 1:15, 1:20, 1:25, 1:30). After the extraction was complete, the suspensions were cooled to room temperature and centrifuged at 8000 rpm for 10 minutes. The supernatant was diluted with methanol and filtered through a 0.45 μm filter for hyper-performance liquid chromatography (HPLC) analysis.

HPLC Analysis and Quantification

An LC-20A HPLC (Shimadzu, Japan) equipped with a C18 reversed-phase column (Diamonsil, 5 μm, 250 mm × 4.6 mm; Dikma Technologies) was employed for the quantitative analysis of Cy. Acetonitrile and water with 0.2% phosphoric acid were mixed in a 20:80 (v/v) ratio and used as the mobile phase, and the flow rate was maintained at 1.0 mL/min. The detection wavelength used for analysis was 350 nm. The injection volume of each sample was 10 μL, and the column temperature was maintained at 30℃ (Balu Alagar Venmathi et al. 2023). To quantitate Cy after each extraction, a standard curve of Cy was generated by plotting the area under the curve against the corresponding concentration of a series of Cy standard solutions over the concentration range of 0.015625–1.0 mg/mL. The standard curve and HPLC chromatogram are shown in Fig. 1. The linear regression equation of the standard curve was (y = 31824574x – 21766 (R^2 = 0.999).

Comparison of Extraction Methods

First, 5.0 g of the ground honeysuckle powder was added to a mixture of ultrapure water and anhydrous ethanol in a solid-liquid ratio of 1:20, and the mixture was subjected to Soxhlet extraction for 8 hours. After cooling to room temperature, the mixture was centrifuged at 8000 rpm for 10 minutes, and the supernatant was diluted with methanol to determine the amount of Cy by HPLC. Under the same solid-liquid ratio, honeysuckle powder was extracted using ultrasonication and microwave irradiation, with anhydrous ethanol as the solvent, for 30 minutes. The methods for determining the Cy content by HPLC can be found in the "**HPLC analysis and quantification**" section.

Concentration of Cy at the Nonionic Surfactant Cloud Point

First, NaCl (80 mg) was added to the 800 mL Cy extraction solution, and the mixture was stirred at low speed at 60℃ for 1 hour, after which the mixture was left to stand until separation occurred (Yuanyuan et al. 2022). The upper layer was analyzed by HPLC to determine the Cy content and calculate the yield. The upper layer solution was diluted with 1/5 of its volume of chloroform, and the solution was vigorously stirred at room temperature at 5000 r/min, after which the mixture was centrifuged. The precipitate was washed with chloroform and then centrifuged at a speed of 8000 r/min for 10 minutes 3 times, combined the centrifuged mixture, rotate and evaporate to recover chloroform, and the precipitate was dried by nitrogen at room temperature to obtain a yellow-brown solid powder (crude product). The

dried solid was accurately weighed it and then analyzed by HPLC to determine the Cy content according to the methods in the "**HPLC analysis and quantification**" section.

Purification of Cy by Metal Complexation

To determine which metal salt would enable the most efficient complexation of Cy to isolate from the honeysuckle extract, a 5 mL ethanol solution of Cy standard (1 mg/mL) containing 0.1 mol/L of various metal salts (Ca^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+}, Fe^{2+}, Zn^{2+}) were prepared (Marie et al. 2024; Roberto et al. 2024). After fully shaking for 15 minutes, the solutions were supplemented with 1 mol/L of NaOH dropwise to adjust the pH to 9.5, after which they were stirred thoroughly and left to stand at 4℃ for 4 hours. Then, the mixtures were centrifuged at a speed of 8000 r/min for 10 minutes, and the sediment was collected respectively. The collected sediments were dispersed into 10 mL 30% aqueous ethanol solution until the precipitate was completely dissolved, and then the same quality as the each sediment of EDTA were added. Each mixture was shaken in a 60℃ water bath until a new precipitate is formed, which is a complex of EDTA and metal ions. After the reaction is completed, the mixture was subjected to 0.2 μM filter membrane filtration and concentrate the filtrate under reduced pressure until dry, and obtain the precipitate as Cy pure product. Weigh it. Dissolve the prepared pure Cy in DMSO to a concentration of 1 mg/mL, and determine the purity of Cy by HPLC.

Results and Discussion

Extraction Optimization

Designing extraction methods necessitates the evaluation of extraction solvents that are both environmentally friendly and cost-effective. New extraction solvents should possess enhanced solubility of the target compounds, while also offering cost reduction, improved economic efficiency, and eco-friendliness. In this study, we screened 13 naturally derived surfactants to compare their extraction rates of Cy from honeysuckle and determine the saturation solubility of Cy in micelles formed from each of the 13 different surfactants. As shown in Fig. 2, the saturation solubility of Cy in T-60, APG-0810, and SLES was relatively high, which were 0.24 mg/mL, 0.18 mg/mL and 0.19 mg/mL respectively. When T-60, T-80 and BS-12 were used for extraction, the extraction rate of Cy was higher, which were 49.03%, 33.59% and 30.40% respectively. Based on the saturation solubility results, T-60 was ultimately selected as the extraction micelle solvent. T-60 demonstrates a significant advantage in terms of economic affordability compared to other solvents. It is widely recognized that upon reaching the CMC, free surfactant molecules tend to aggregate and form micelles. Therefore, all subsequent experiments were conducted using T-60 as the extraction surfactant.

The effects of surfactant concentration, solid-liquid ratio, extraction time, and extraction temperature on the extraction rate of Cy are shown in Fig. 3. T-60 is a surfactant that aggregates in aqueous solution to form micelles with both spherical and cylindrical morphologies. T-60 enables the solubilization of both heavily lipophilic and hydrophilic substances during the extraction process. As shown in Fig. 3A, the Cy extraction rate increased as the concentration of T-60 increased from 2–10%. However, when the

concentration surpassed 10%, the extraction rate of Cy decreased slightly. Therefore, x was determined to be the optimal concentration of T-60.

When determining the optimal solid-liquid ratio, it is advantageous to determine which solvent is capable of extracting the maximum amount of compound using the minimum volume of solvent, so as to ultimately reduce the production costs of the extraction system on an industrial scale. The results depicted in Fig. 3B illustrate that increasing the volume of the solvent, thereby decreasing the ratio, led to an increase in the extraction rate of Cy. The quantity of solvent was insufficient to fully immerse the substance when the solid:liquid ratio was less than 1:25. Futhermore, there was no statistically significant difference between the extraction efficiency of Cy between the 1:25 and 1:30 ratios. Therefore, 1:25 was selected as the optimal solid-liquid ratio for subsequent experiments. As shown in Fig. 3C, the extraction rate of Cy increased from 30 minutes to 60 minutes but remained stable as the extraction time increased beyond 60 minutes. Therefore, 60 minutes was determined to be the optimal extraction time. Lastly, Fig. 3D compares the extraction rate of Cy at different temperatures using T-60. As the temperature increased from 20°C to 50°C, the extraction rate of Cy gradually increased, reaching its highest value at 50℃ and flattening thereafter. Therefore, 50℃ was considered the optimal extraction temperature.

Comparison of Extraction Methods

As shown in Table 1, the Soxhlet extraction method, which uses water as the solvent, is the most traditional extraction method with low extraction efficiency. After replacing the solvent with ethanol, the extraction efficiency significantly increases. However, using organic solvents for extraction has a significant impact on the environment. Ultrasonic extraction and microwave extraction are currently widely used new extraction processes, which significantly increase the extraction rate but also increase energy consumption (Shahram et al. 2024, Lizárraga-Chaidez et al. 2024). Lastly, extraction of endogenous biomolecules using nonionic surfactants solubilizes poorly water-soluble target compounds through hydrophobic interactions to form micelles. This particular method is trivial, safe, and sustainable, and it typically results in high extraction efficiencies (Leite et al. 2017; Podlesny et al. 2023).

Table 1
Comparison of the different extraction methods on the extraction yield of Cy

NO.	Extraction method	Extraction medium	Time (min)	The yield of Cy (mg/g)
a	Soxhlet extraction	Ultrapure water	120	0.149
b	Soxhlet extraction	Anhydrous ethanol	120	0.483
c	Ultrasonication	Ultrapure water	30	0.455
d	Microwave irradiation	Ultrapure water	30	0.496
e	Micelle extraction	T-60 (10%) aqueous solution in ultrapure water	60	0.525

Concentration of Cy at the T-60 Cloud Point

The T-60 in the extraction solution has cloud point properties. As shown in Fig. 4A, the solubility of T-60 in aqueous solution decreases with increasing temperature. When the temperature reaches a certain value, the solution becomes turbid and reaches the turbidity point. After adding inorganic salts to the extraction solution, electrostatic interactions between ions and between ions and dipoles result in water molecules surrounding ions, reducing the amount of free water, making it easier for surfactants to precipitate from the solution, and lowering the cloud point temperature. After further placement or centrifugation, separate the liquid phase to concentrate certain components in one phase of the extraction solution. Therefore, as shown in Fig. 4B, we took 800 mL of honeysuckle extract (preparation method as described above), added 80 mg of NaCl, heated the water bath to 60°C, and the solution became turbid. After standing at room temperature, stratification occurred, causing Cy to accumulate in the upper layer. After separation by a separating funnel, the upper layer was taken and dissolved in 5 times the volume of trichloromethane. As Cy is insoluble in trichloromethane, it was precipitated out. After collecting the sediment and drying it with nitrogen gas, the crude product of cy is obtained, as shown in Fig. 4C.

Purification of Cy by Metal Complexation Method

Ca^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} ions have high valence charges and are prone to complexation reactions with flavonoids (Li et al. 2019; Binbin et al. 2023). Therefore, these six ions were selected to determine which would enable the most efficient complexation of Cy for purification from the crude Cy product (Fig. 5A). The ethanol solution of Cy in the presence of the metal ions was centrifuged, after which the supernatant was analyzed by HPLC to determine the Cy content and its transfer rate. As shown in Fig. 5B, Ca^{2+}, Cu^{2+}, and Al^{3+} formed metal-ligand complexes with Cy relatively better than the other three ions. Among them, Ca^{2+} had the highest transfer rate and is non-toxic, so $CaCl_2$ was ultimately chosen as the complexing reagent. EDTA is a strong metal complexing agent that has a significantly higher binding affinity for divalent metal ions than flavonoids. After adding EDTA to the mixture containing the Cy-Ca^{2+} complexes, the EDTA competitively chelated the Ca^{2+} from the Cy-Ca^{2+} complexes, thereby affording the pure Cy (Cy-sample) in 93.7% yield (Fig. 5C).

Conclusion

In summary, a nonionic surfactant-mediated aqueous extraction method was optimized to enable the efficient and sustainable extraction of Cy from honeysuckle. The optimum extraction condition: the ratio of T-60 is 10%, the solid:liquid ratio is 1:25, the extraction time is 60 min and the extraction temperature is 50℃. The yields of Cy is 0.525 mg/g, which is 3.52 times that of Soxhlet extraction method (extraction solvent is water), 1.09 times that of Soxhlet extraction method (extraction solvent is ethanol), 1.15 times that of ultrasonic extraction method, and 1.06 times that of microwave extraction method.. The cloud point properties of the nonionic surfactants enabled the preconcentration of flavonoid glycosides.

Finally, the high purification of Cy was achieved by metal complexation, according to HPLC detection, its purity is 93.7%. This method not only avoided the risks and environmental hazards brought by traditional solvents but also significantly reduced costs and achieved effective the extraction of Cy using the environmentally friendly T-60 surfactant. We anticipate that this method will serve as a promising alternative to traditional organic solvent extraction and separation procedures and can be employed for large-scale extraction applications.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Credit authorship contribution statement

Wen Zhu: Methodology, Investigation, Conceptualization, Writing-original draft, Writing-review & editing.

Cuiman Tang: Methodology, Investigation.

Bin Wang: Methodology, Investigation.

Yue Lv: Writing - review & editing.

Jialin Liu: Writing - review & editing.

Chaofan Sun: Writing - review & editing.

Yuangang Zu: Conceptualization, Visualization.

Xiuhua Zhao: Supervision, Funding acquisition, Conceptualization, Resources.

Data Availability

Data produced and/or examined in the course of this study can be obtained from the authors upon request.

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Figures

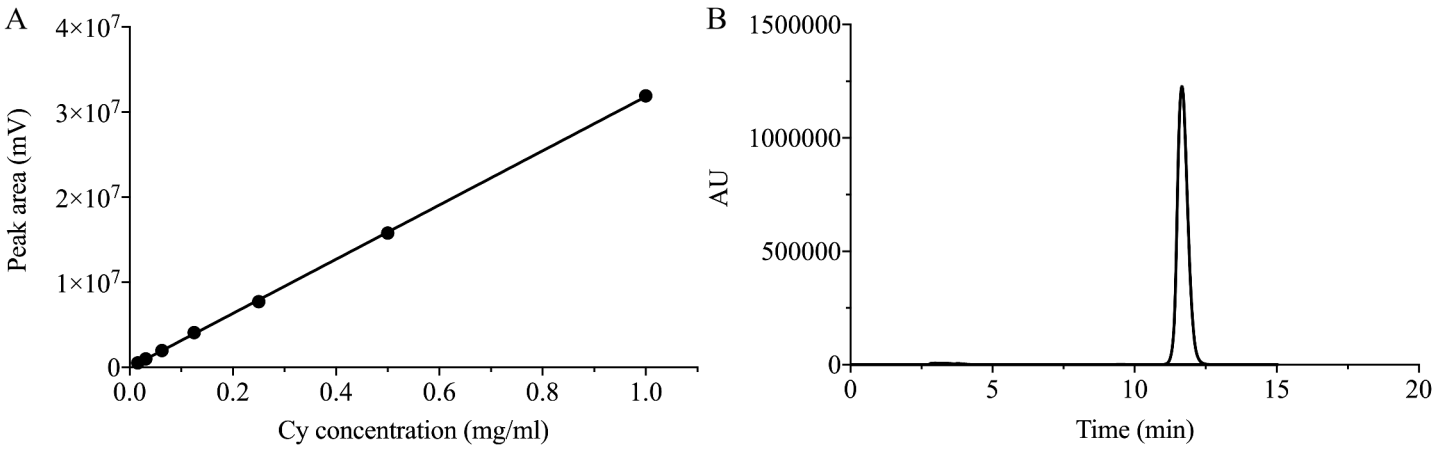

Figure 1

A standard curve of Cy and **B** HPLC spectrum of Cy

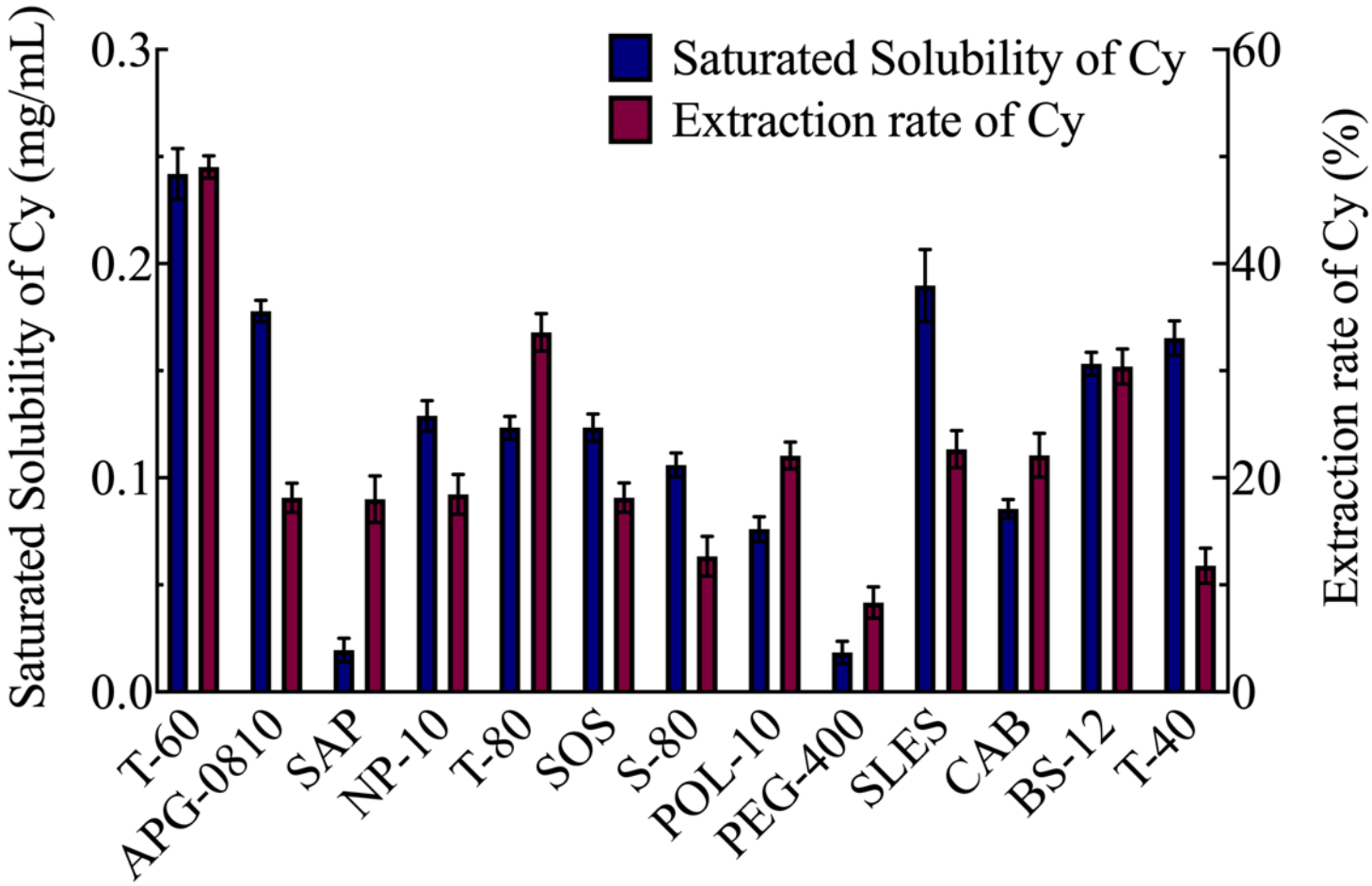

Figure 2

The saturation solubility of Cy in each of the 13 surfactants investigated and the extraction rate of Cy from honeysuckle using the different surfactants

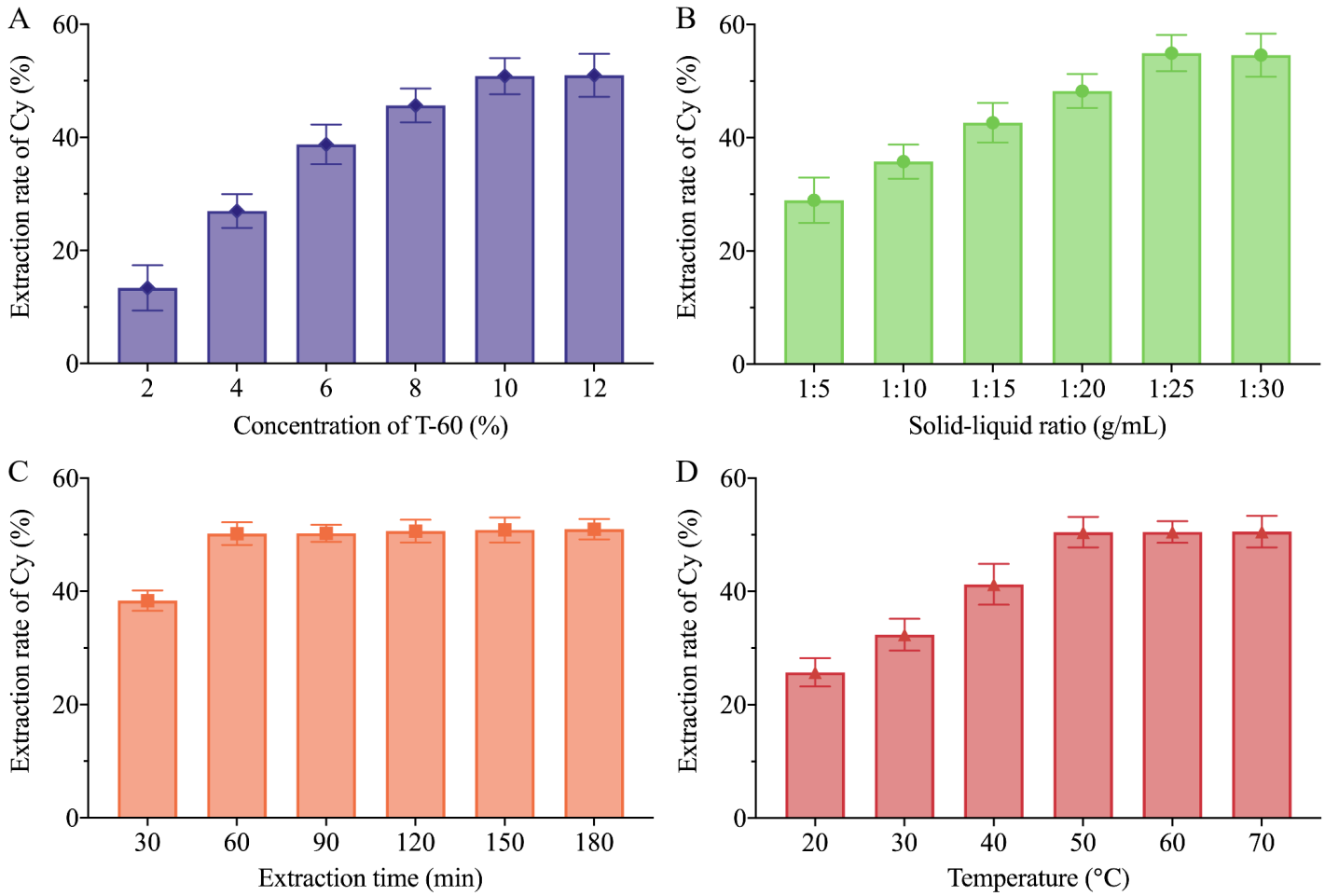

Figure 3

The effects of concentration of T-60 (**A**), solid-liquid ratio (**B**), extraction time (**C**), and temperature (**D**) on the extraction yields of Cy

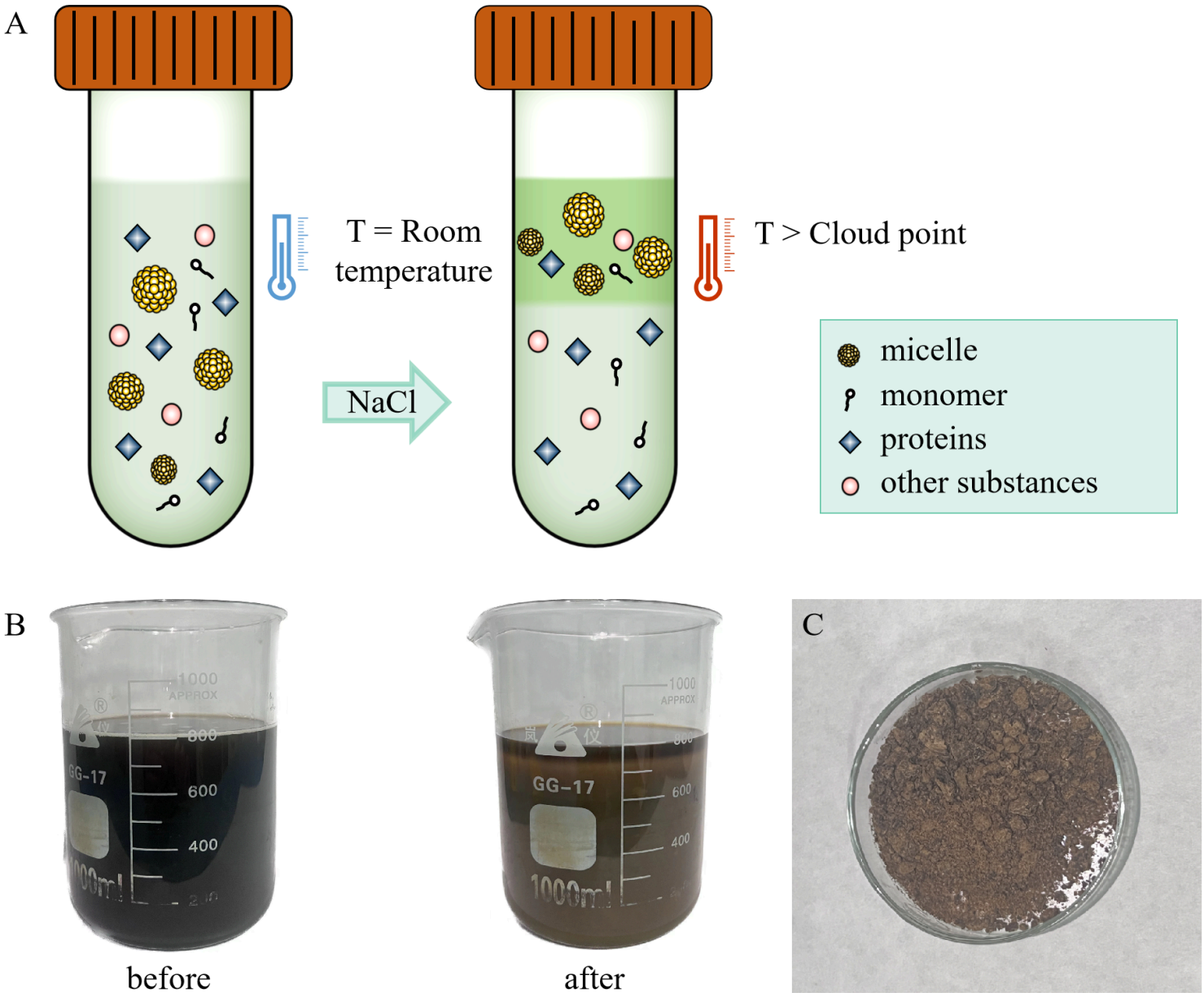

Figure 4

A Schematic diagram of the enrichment of Cy in T-60 using the cloud point method; **B** Concentration of Cy by cloud point method; **C** photograph of the crude Cy after precipitation by the cloud point method.

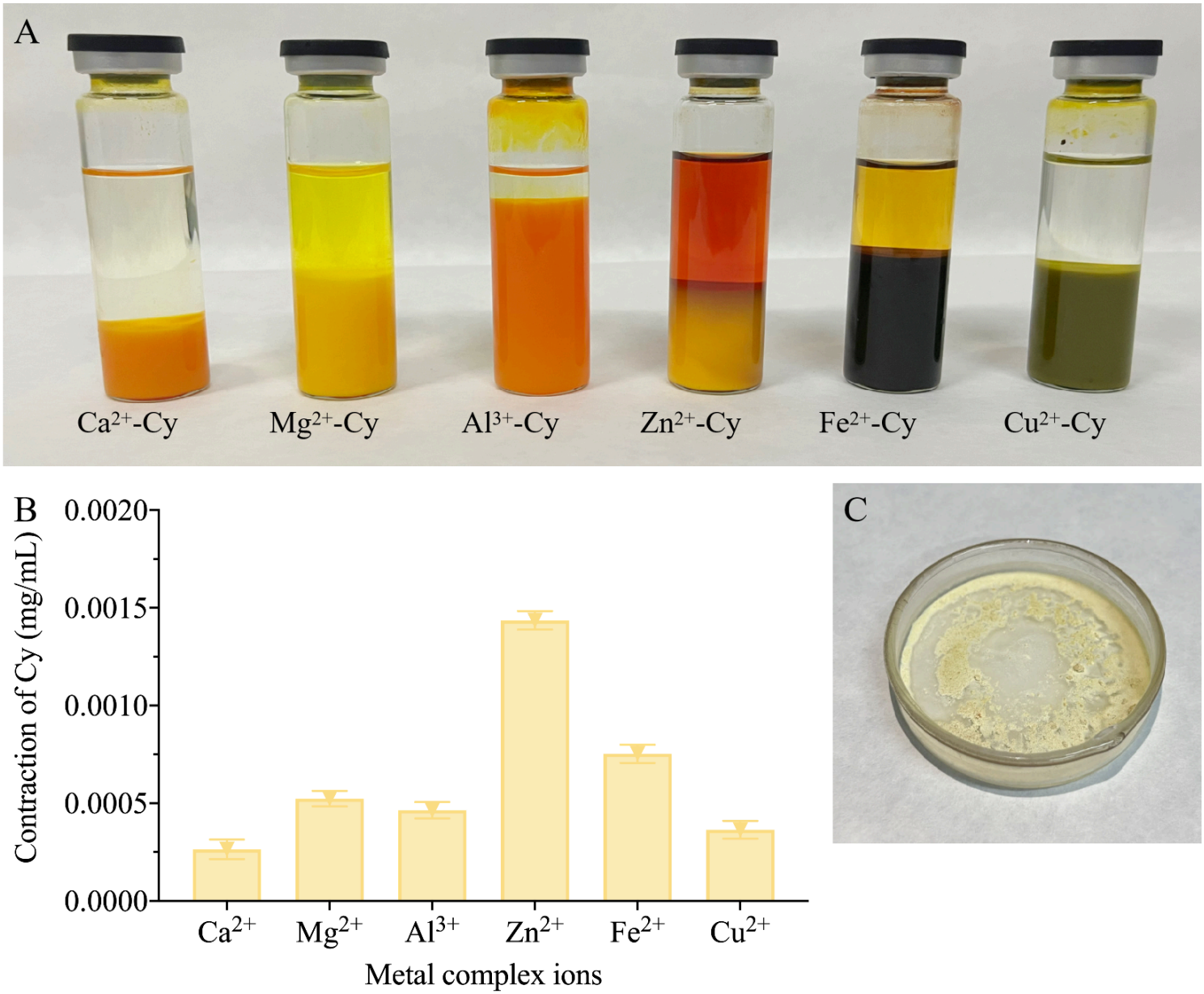

Figure 5

A Ca^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+}, Fe^{2+} and Zn^{2+} combine with Cy to form metal complexes; **B** contention of free Cy after complexation; **C** photograph of the pure Cy (Cy-sample) after purification by metal complexation method.