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Investigation on ultrasound assisted extraction of saikosaponins from *Radix Bupleuri*

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Abstract

Ultrasound assisted extraction (UAE) of saikosaponins from *Radix Bupleuri* (*Bupleurum Chinense DC*) was studied. The effects of various factors such as time (t), temperature (T), ultrasound power (I), particle size (S), solvent to solid ratio (R) and solvent concentration (C) on the yield of target components were investigated. The experimental results indicated that the extraction by UAE is six times faster than those by conventional thermal extraction. It is also found that fast extraction rate was obtained in the first 30 min of sonication. Prolonged sonication did not give a proportional increase in the yield. For the effect of ultrasound power, the maximum yield was obtained at the power level of around 21 W. Scanning electron microscope (SEM) images of the plant cells after UAE treatment were obtained to provide visual evidence of the sonication effect. The effects of particle size, solvent to solid ratio and solvent concentration on the yield are also discussed. The preliminary optimum conditions for UAE of saikosaponins were found at time of 30 min, temperature of 80 °C, power of 21 W, particle size <0.3 mm, solvent to solid ratio of 25 ml/g and solvent concentration of 50%.

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Keywords: Extraction; Ultrasound; Saikosaponins; *Radix Bupleuri*

1. Introduction

In 2002, an apparently new infectious disease termed as the severe acute respiratory syndrome (SARS) emerged and rapidly spread to different areas around the world. It caused over 8000 probable cases and more than 700 deaths in less than 8 months since its outbreak [1]. In the battle against SARS, *Radix Bupleuri* [2] appeared on the list of treatment therapy, and caught attention as it helped people fight this new infectious disease.

As an old member in the family of Chinese herbs, bupleurum root has been used for more than 2000 years. It is primarily used to treat cold fevers, chills and fevers along with congestion and stuffiness in the chest and hypochondria. The extracts of bupleurum contain many active components such as volatile oil, polysaccharide, flavonoid and saikosaponins, which exhibit a broad range of biological functions [3–5] and serve a wide variety of harmonizing activities. The main medical uses of saikosaponins are anti-inflammatory, anti-hepatitis

and anti-tumor. Saikosaponins were obtained through traditional extraction methods, which were extremely time consuming and material wasting. The majority of previous studies focused on the types of saikosaponins and the analysis of their structures [6,7]. In recent years, many published reports have shown that the use of ultrasound leads to an increase in the yield of various phytochemicals [8–10]. When ultrasound is applied, a large amount of bubbles are generated in a liquid medium. When agitated by intense ultrasound, they oscillate violently, undergo initial explosive growth and subsequently collapse [11]. Because there is limited “space” for them to expand, most of the bubbles collapse asymmetrically in the vessels, and the inside of bubble can reach thousands of bar in pressure and Kelvin in temperature. Extraction can be significantly improved with the aid of an ultrasound wave.

The main objective of this work was to evaluate the effects of certain factors on ultrasound assisted extraction (UAE) of saikosaponins from the bupleurum root. Saikosaponin-a, c and d were selected as the marks in this study. The results from UAE were then compared with that found from conventional solvent extraction (CSE). Microscopic images by scanning electron microscope (SEM) were also obtained to provide visual evidence of the existence of ultrasonic effects.

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2. Material and methods

2.1. Materials and reagents

The roots of raw *Radix Bupleuri* with a moisture content of 10.3% were purchased from a regional market (Guangzhou, China). The herb was pulverized into powder form by a grinder (DF-15, DADE, China). The herb powder was then sieved with stainless steel sieves to classify the particle size, ranging as <0.3, 0.3–0.45 and 0.45–0.9 mm. The classified herb powder was packaged in polyethylene bags and stored at room temperature for later use. All solvents used, namely ethanol (MERCK, Germany), methanol and acetonitrile (both for HPLC use; from TEDIA, USA) were analytical grades. The standard saikosaponin-a, c (Nacalai Tesque, Japan) and d (WAKO, Japan) were bought from Japan.

For SEM, the roots of raw *Radix Bupleuri* were cut into 2 mm × 2 mm squares; treated by CSE or UAE, then frozen and dehydrated in a freeze dryer (Alpha 1-4 LD, Christ, Germany) for 2 days before imaging.

2.2. Apparatus

A 600 W, 20 kHz ultrasonic homogenizer (model CPX600, Cole-Parmer, USA) equipped with a probe transducer and a flat tip of 12.7 mm was employed in the UAE experiments. The amplitude control in the equipment allowed the setting of the ultrasound output power at a desired level ranging from 0 to 100% of the nominal power.

An evaporator (RE-52A, SHYR, China) was used for dehydrating the extracts and a centrifuge (5415D, Eppendorf, Germany) for a rapid separation of the unresolved residues from the solution.

A scanning electron microscope (model Leica 440 Stereoscan, Leica, UK) was applied for generating the SEM images.

2.3. Extraction method

In this study, all experiments were carried out in triplicate for each set of conditions, and the results reported were the average of the three trials.

2.3.1. Conventional solvent extraction

In the experiment, the herb matrix was placed in a 100 ml glass container with a cooling/heating jacket (Fig. 1) with 75 ml of 70% ethanol aqueous solution (v/v), stirring with a magnetic stirrer at a speed of 420 rpm. The temperature was controlled within ±1 °C by varying the temperature and flow rate of the cooling/heating water. The treated mixture was cooled down, filtered and quantified in a 100 ml volumetric flask. Then 10 ml solution was taken from the flask and evaporated at reduced pressure. The residue was dissolved with 1 ml methanol, centrifuged and filtered through 0.45 μm filter paper. 10 μl of the final solution was injected into HPLC to analyze the content of target compounds.

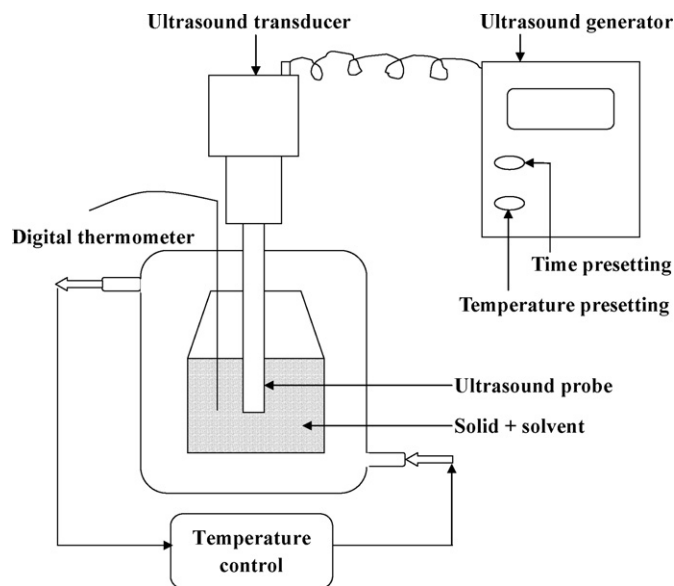


Fig. 1. Experiment set-up of ultrasound assisted extraction (UAE).

2.3.2. Ultrasound assisted extraction

In UAE method, a standard ultrasound probe with a flat tip of 12.7 mm in diameter was inserted into the mixture directly. The distance from the tip to the bottom of the vessel was preset. The samples were extracted with continuous ultrasound waves at frequencies of 20 kHz with different levels of power output. During the extraction period, temperature was controlled at a desired level within ±1 °C. The post-treatment of the extracts was the same as that mentioned in CSE method.

In UAE, the energy entering the extraction medium was found to be a linear function of the output power of the ultrasonic horn within the range used in the experiments. The ultrasound power expressed in this study is the actual power entering the system converted by calorimetrically from the output power.

2.3.3. Extraction yield

The percentage yield of saikosaponins was calculated using the following equation:

$$\text{Yield (\%)} = \frac{W_i}{W_t} \times 100\% \quad (1)$$

where W_i is the weight of saikosaponins (including saikosaponin-a, c and d) obtained from one extraction trial and W_t is the total weight of saikosaponins (including saikosaponin-a, c and d) obtained from repeated CSEs, in which the solid matrix was extracted several times until no more saikosaponins was detected by HPLC.

2.4. HPLC analysis

A HPLC system consisted of a quaternary pump with a degasser (G1311A, Hewlett-Packard, USA), a variable wavelength detector (G1314A, Hewlett-Packard) and a Hypersil ODS C₁₈ reverse phase column (200 mm × 4.6 mm × 5 μm; Alltech, IL, USA) was used for analysis.

Table 1
Parameters of the calibration equations for the three saikosaponins

Name of compound	Parameter		
	<i>a</i>	<i>b</i>	<i>R</i> ²
Saikosaponin-a	535.97	153.37	0.9976
Saikosaponin-c	510.24	17.78	0.9989
Saikosaponin-d	383.56	66.60	0.9993

The HPLC conditions were set based on Lin's method [12] with some modification to give satisfactory resolution for the three mentioned saikosaponin marks.

Elution was carried out with acetonitrile–water solution of 30:70 (v/v) at 0.8 ml/min in the first 10 min. The water percentage was then decreased at a speed of 0.8 ml/min in the following 8 min, reaching an acetonitrile–water ratio of 40:60. The ratio was changed to 45:55 in the next 10 min, then kept for 7 min, and raised again to 30:70. The analytical runs could be completed in 40 min. The three saikosaponins were completely separated from the extract mixture. The retention times for saikosaponin-a, c and d were about 18, 23 and 32 min, respectively. This method was sensitive and accurate with good reproducibility.

Calibration equations for all three saikosaponins were obtained for the range from 0.2 to 2 µg with six intervals. All equations are linear and can be expressed in the following formula:

$$Y = aX + b \quad (2)$$

where *X* is the amount of standard used in calibration experiments and *Y* is the related peak area in HPLC chromatographs. The values of the slope (*a*), the intercept (*b*) and the correlation coefficient (*R*²) are listed in Table 1. As shown in Table 1, *R*² is very close to 1, which means that *Y* is linearly correlated with *X* and the equations are adequate for quantitative analysis.

2.5. SEM imaging

The treated samples in CSE or UAE were placed into a preparation chamber, which was attached to a scanning electron microscope. The samples were first sputter coated with a thin layer of conductive gold at a thickness 30–100 nm. The shape and the surface characteristics of the samples were magnified and digitally recorded.

3. Results and discussion

3.1. Comparison of UAE with CSE

For the same conditions such as temperature, particle size, solvent to solid ratio and solvent concentration, the results from UAE were compared with those obtained from CSE. As shown in Fig. 2, both UAE and CSE had a similar extraction yield at prolong time, indicating that the application of ultrasound did not alter the terminal extraction yield. Moreover, the extracted amount of saikosaponins by UAE for 30 min was competitive with that by CSE for 180 min. At 30 min, the extracted amount

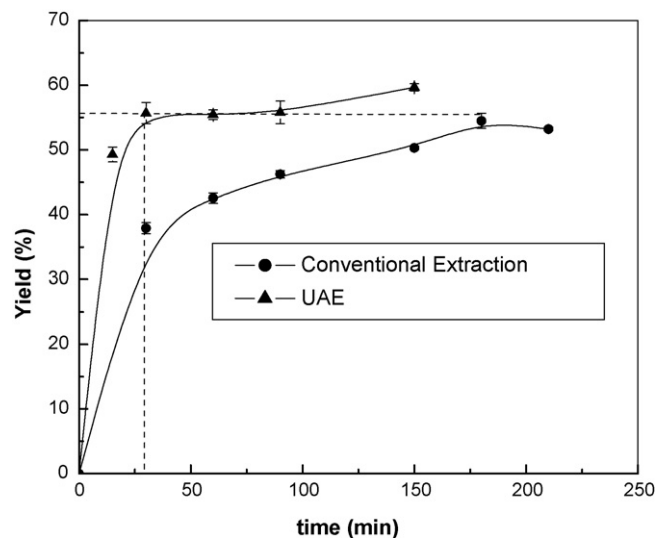


Fig. 2. Comparison of CSE and UAE in the yield of total saikosaponins.

of saikosaponins by UAE was 47% larger than that by the conventional method. In UAE, induced by ultrasound waves, the expansion and collapse of cavities near the cell wall as well as the turbulent vibration on the solvent–solid interfaces led to a cell disruption and speeded up both the release and diffusion of the target components into the extraction medium, thus extraction was significantly improved.

The results demonstrated that UAE gave a larger yield at the early stage of extraction compared with CSE, and the extraction time could be greatly shortened by the application of ultrasound.

The SEM images showed in Fig. 3 provide the evidence on the disruption of the plant cells after UAE treatment, indicating the cavitation did take place during UAE, which disrupting the cell and facilitating the release of saikosaponins inside the cells to the exterior solvent. Fig. 3a shows the cells of the *Radix Bupleuri* roots tissues were kept intact after CSE; while after ultrasound treatment, as shown in Fig. 3b, hollow openings were generated, indicating the cell walls of the plant tissues were broken and all the cell constituent components were washing away by rinsing effect.

3.2. Effects of operation parameters of UAE on the yield

The operation parameters to be studied were time (*t*), temperature (*T*), power (*I*), particle size (*S*), solvent to solid ratio (*R*) and solvent concentration (*C*), respectively.

3.2.1. Sonication time

UAE was carried out for different periods of time from 15 to 150 min with other conditions fixed at *T* = 60 °C, *I* = 21 W, *S* = 0.3–0.45 mm, *R* = 25 ml/g and *C* = 70% (ethanol to water, v/v).

The results shown in Fig. 4 clearly indicated that the extraction yield increased rapidly in the first 30 min of sonication. The raise was then leveled when sonication was prolonged. Such a trend was similar for all three saikosaponins, and thus the total saikosaponins as well. Ultrasound facilitated the release of

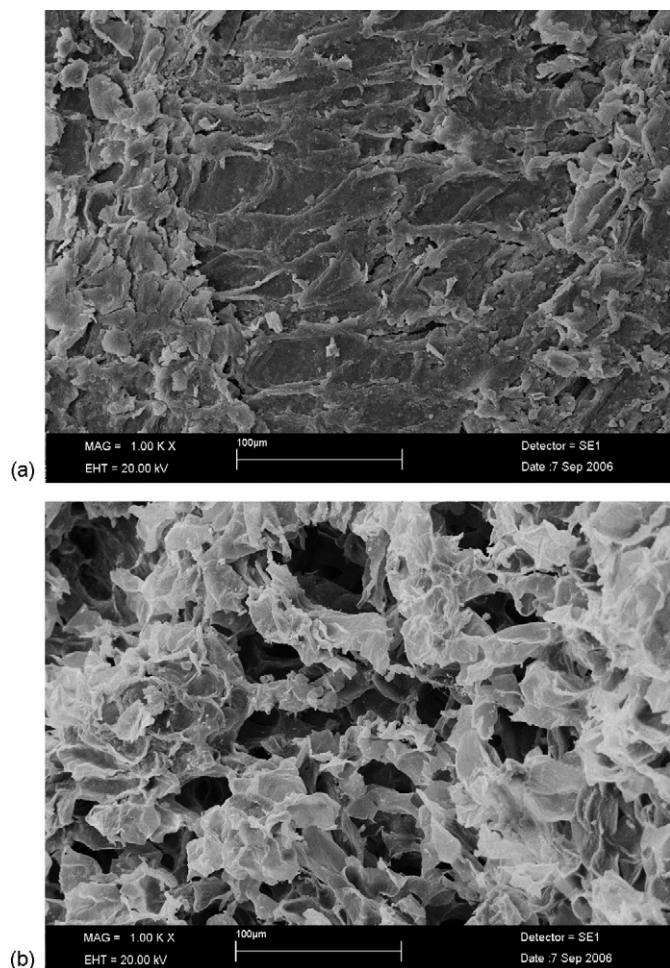


Fig. 3. SEM images of *Radix Bupleuri* root tissues after the extraction: (a) treated by CSE and (b) treated by UAE.

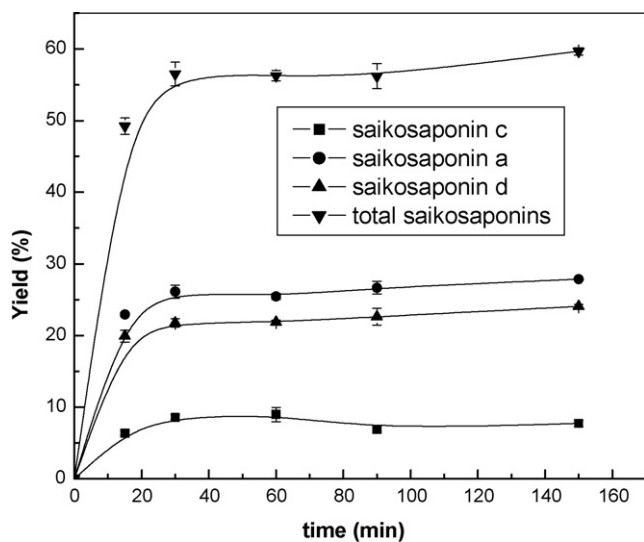


Fig. 4. Effect of sonication time on the yield of saikosaponins (other conditions were fixed at $T = 60^{\circ}\text{C}$, $I = 21\text{ W}$, $S = 0.45\text{--}0.9\text{ mm}$, $R = 25\text{ ml/g}$, $C = 70\%$).

saikosaponins inside the plant cells to the exterior solvent and gave a large yield at the early stage of extraction. The rinsing effect may release most of the saikosaponins in the broken cells at the first 30 min. Furthermore, as the diffusion front moved towards the interior of the tissues, the diffusion area reduced, diffusion distance increased and the diffusion rate would decrease accordingly. Therefore, there was no obvious observed yield change in the prolonged time periods. Based on these results, the optimum sonication time was set for 30 min for later experiments.

3.2.2. Temperature

The effect of temperature on UAE was explored with sonication time at 30 min and other conditions fixed as mentioned previously. Previous researches had shown that as temperature approached the boiling point of the solution, ineffective sonication occurred as a result of the decrease in surface tension and increase in vapor pressure with microbubbles, which in turn caused the damping of the ultrasonic wave. To fully investigate the effect of ultrasound on extraction, the temperature of the extraction mixture was kept below its boiling point. As shown in Fig. 5, the higher the temperature, the larger the extraction yield obtained at a given time. It was well proved that the extraction rate of most compounds increased with temperature. Thus, the diffusion of active components was intensified when temperature increased, so was the extraction yield at a fixed time period. Thus, 80°C was chosen as the optimum temperature.

3.2.3. Power

As shown in Fig. 6, the yield of saikosaponins-a increased to the maximum at a power output of 21 W, while that of saikosaponin-d reached the peak at 26 W. Saikosaponin-c remained in a relative low level and increased with the power applied. Because saikosaponin-a was the dominant component in the herb, the total saikosaponins showed a trend similar to it. The difference between the yield with and without ultrasound

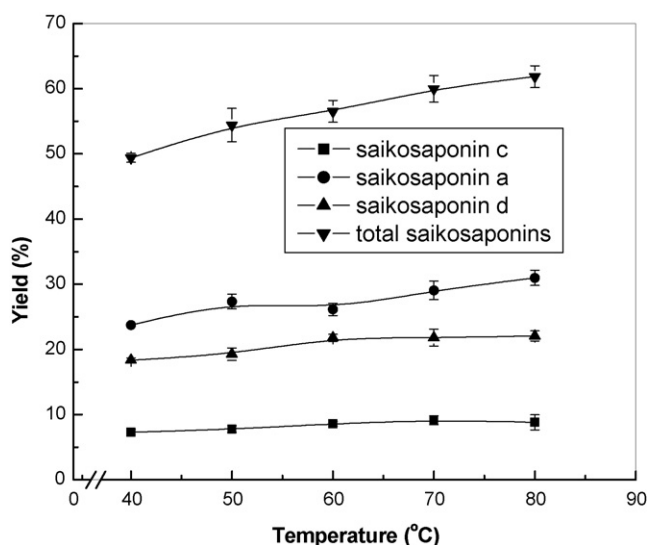


Fig. 5. Effect of temperature on the yield of saikosaponins (other conditions were fixed at $t = 30\text{ min}$, $I = 21\text{ W}$, $S = 0.45\text{--}0.9\text{ mm}$, $R = 25\text{ ml/g}$, $C = 70\%$).

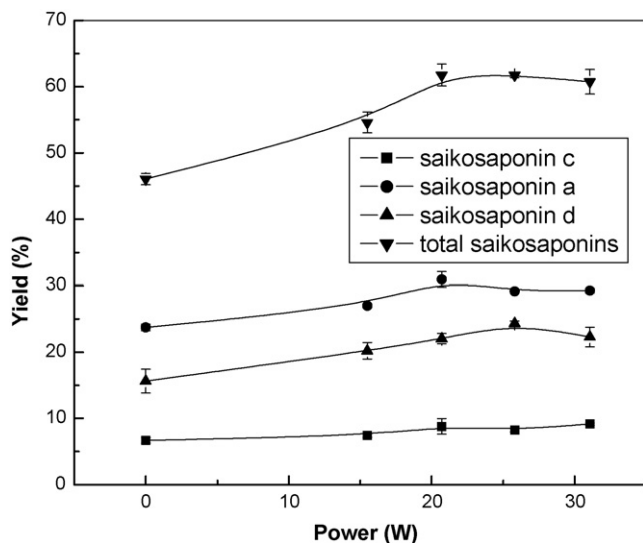


Fig. 6. Effect of power on the yield of saikosaponins (other conditions were fixed at $t = 30$ min, $T = 80$ °C, $S = 0.45$ – 0.9 mm, $R = 25$ ml/g, $C = 70\%$).

power was also presented in this figure. For the given conditions, there was an approximate 33% increase in the yield of total saikosaponins at a power of 21 W compared with that at 0 W. The power output of the ultrasound generator was directly related to the intensity of the ultrasound in the medium. Thus, an increase in the power output would bring about an increase in the extraction yield. However, a power too high would cause an increase in the bubble numbers in the solvent during cavitation, which might reduce the efficiency of the ultrasound energy transmitted into the medium [13]. After 21 W, the curve for power effect on the yield of extraction became plain. Therefore, sonication power of 21 W was seen to be suitable for the extraction.

3.2.4. Particle size

The particle size used in this work was in three ranges: <0.3 , 0.3 – 0.45 and 0.45 – 0.9 mm. As expected, when the particle size decreased, the extraction rate for all saikosaponins increased (Fig. 7). However, saikosaponins-a and c showed a trend that a further decrease in size would not result in a further increase in the extraction yield. For a fixed weight of material, the surface area increased as the particle size decreased. For the particle in size range of 0.45 – 0.9 mm, diffusion may play a significant role so that the yield at a given time would be expected to increase with the reduction of size, as the surface area increasing. However, when the particle size was small enough, most of the cells were ruptured by the application of ultrasound, and diffusion would not be a significant step in the extraction of such small particles, so that a further decrease in the size would not result in a corresponding increase in the extraction rate. This hypothesis was supported by our experimental results for the size change from 0.3 – 0.45 to <0.3 mm, and Mason's report [14]. Consequently the particle size range of 0.3 – 0.45 mm was chosen.

3.2.5. Solvent to solid ratio

In this work, the solvent to solid ratio was investigated in the range of 15 – 50 ml/g. As shown in Fig. 8, the extraction yield

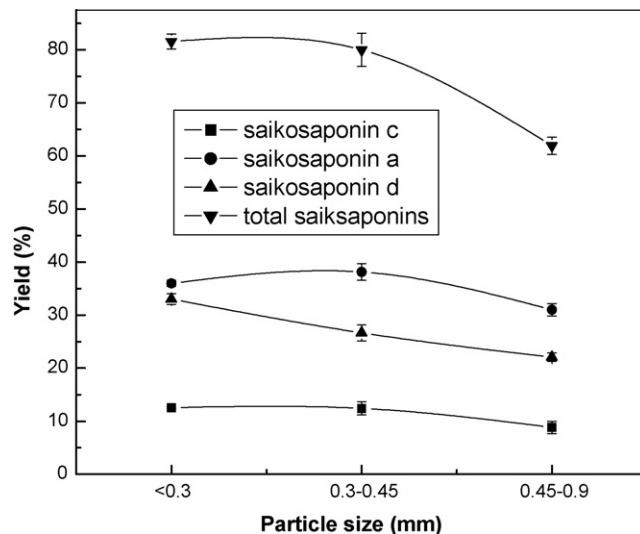


Fig. 7. Effect of particle size on the yield of saikosaponins (other conditions were fixed at $t = 30$ min, $T = 80$ °C, $I = 21$ W, $R = 25$ ml/g, $C = 70\%$).

was promoted when the solvent to solid ratio was increased up to 25 ml/g, and then weakened as this ratio was further increased.

Theoretically, for a fixed amount of solid matrix, the more quantity of solvent used, the more dilute effect in the solvent side. This gave a larger concentration difference between the interior of the plant cells and the external solvent, thus a faster extraction rate could be obtained. But if the solution was very dilute, an extra solvent increase would not lead to a sufficient increase in the concentration difference, the increase in extraction yield would be limited. Therefore, there was no apparent increase in the yield when the solvent to solid ratio increased from 25 to 50 ml/g.

For commercial application, a solvent to solid ratio of 25 ml/g should be optimum to avoid waste of solvent and bulky handling in the subsequent processes.

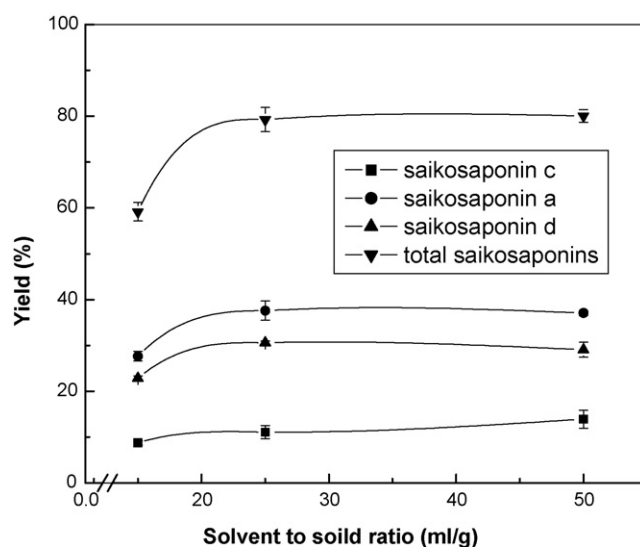


Fig. 8. Effect of solvent/solid ratio on the yield of saikosaponins (other conditions were fixed at $t = 30$ min, $T = 80$ °C, $I = 21$ W, $S = 0.3$ – 0.45 mm, $C = 70\%$).

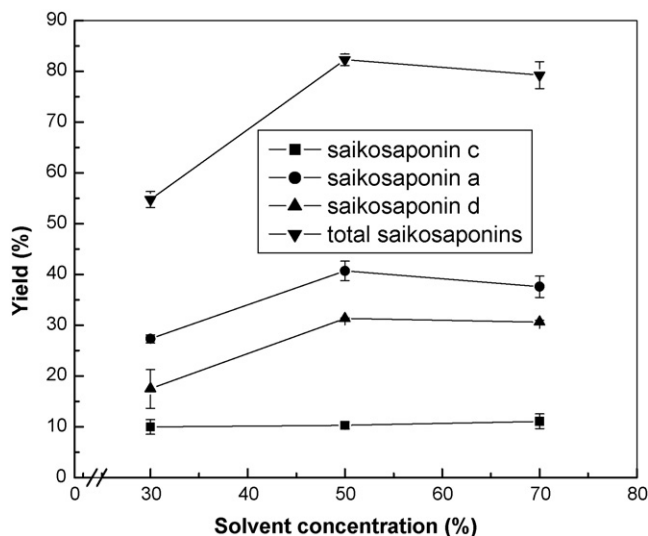


Fig. 9. Effect of solvent concentration on the yield of saikosaponins (other conditions were fixed at $t = 30$ min, $T = 80$ °C, $I = 21$ W, $S = 0.3$ – 0.45 mm, $R = 25$ ml/g).

3.2.6. Solvent concentration

There was limited study for the effect of solvent concentration on the extraction of saikosaponins. Thus, in UAE of saikosaponins, solvent concentration at different levels was investigated. As shown in Fig. 9, there were maximum yields of saikosaponins-a and d at 50% (v/v) solvent concentration. Further increase in the ethanol concentration cannot enhance the yield of the target compounds accordingly. Accordingly, the maximum yield for total saikosaponins was obtained at the same condition. Therefore, a 50% concentration was selected for high extraction yield.

4. Conclusions

Systematic study on UAE of saikosaponins was carried out with a focus on the effects of parameters such as time, temperature, power, particle size, solvent to solid ratio and solvent concentration. The ultrasound applied in extraction clearly promoted the release of the constituent components within the plant cells and the mass transfers between the solid matrix and solvent, and enhanced the yield of extraction at a fixed time. SEM images provided visual evidence that the cell walls were sufficiently ruptured by ultrasound with the power intensity used. Compared with CSE, the terminal yield of extraction could be achieved six times faster with the application of ultrasound. The suitable conditions for UAE were found to be $t = 30$ min, $T = 80$ °C, $I = 21$ W, $S = 0.3$ – 0.45 mm, $R = 25$ ml/g and $C = 50\%$ (EtOH/H₂O, v/v).

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