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Optimization of Ultrasonic-Assisted Aqueous Two-Phase Extraction of Flavonoids from Hawthorn Leaves Using Response Surface Methodology

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Abstract. In this paper, using hawthorn leaves as raw materials, the effect of extracting total flavonoids from hawthorn leaves by aqueous two-phase system (ATPS) combined with ultrasonic assisted was studied. Through single factor test and response surface method Box-Behnken design (response surface method, BBD), according to the regression equation, the optimal process conditions were obtained as follows: the ratio of solid to liquid 1:37, ultrasonic time 40 min, ultrasonic power 360 w, ultrasonic temperature 65℃, and the yield of flavonoids was 2.869±0.0004%. This study shows that ultrasound combined with aqueous two-phase system (ATPS) is an effective method to extract total flavonoids from hawthorn leaves.

Keywords. Hawthorn leaves, flavonoids, aqueous two-phase system, ultrasound, response surface methodology.

1. Introduction

Hawthorn is generally grown in China as a medicinal and edible fruit [1]. Hawthorn leaf is a traditional medicinal material in China with biological and pharmacological properties, among which flavonoids play a key role in the pharmacological properties [2]. Some researchers have shown that hawthorn leaves are much higher in flavonoids than hawthorn berries and has special effects in regulating blood sugar and blood lipids, decreasing blood pressure, anti-atherosclerosis, and anti-oxidation [3, 4].

At present, traditional solvent extraction method is mainly used to extract flavonoids from plants, such as ethanol extraction. Although this method is simple, it has problems of long extraction time and low efficiency and is not conducive to the further purification of compounds [5]. Enzyme-assisted method has also been reported in recent years, but the conditions of this method are difficult to control [6]. Another method is microwave-assisted extraction, which can save the extraction time, but this method requires special equipment, thus raising costs in a disguised way [7]. Therefore, new extraction methods are urgently needed. As a new method of extracts, ATPS have been used to extract natural compounds [8]. In order to improve efficiency, ultrasonic assisted ATPS was used to extract flavonoids. According to previous literature, ultrasonic-assisted extraction has similar advantages to ATPS extraction, with short extraction time, less extraction solvent, and high extraction rate of flavonoids. Therefore, ultrasonic-assisted ATPS (UA-ATPS) extraction of flavonoids is feasible [9]. In this study, based on the single factor experiment, the UA-ATPS process conditions were optimized by response surface methodology.

2. Materials and Methods

2.1. Plant Material
Hawthorn leaves were provided by Laiwu Wanbang Food Co., Ltd. After the hawthorn leaves are dried by hot air, they are ground into powder with an electric grinder, sieved through a 70-mesh sieve, and stored in a sealed bag.

2.2. Determination of Flavonoids
The methods reported by Ma were used to determine flavonoids from hawthorn leave with some modifications [10].

2.3. UA-ATPS Extraction from Hawthorn Leaves
1.0000g leaves powder was added into an Erlenmeyer flask and mixed with ATPS (PEG 11.5% and $(NH_4)_2SO_4$ 14%). UA-ATPS extraction was conducted in a numerical controlled ultrasonic cleaner (KQ-600DB, Kunshan). After extraction, centrifuge at 3800 rpm for 20 min. The formula for calculating the yield of flavonoids is:

$$\text{Yield of flavonoids (\%)} = \frac{100C_tV_t}{M}$$

In the formulae Ct and Vt, the measured apical phase concentration (mg/ml), volume (ml) and M are the weight (g) of the leaf powder.

2.4. Optimization of UA-ATPS Extraction
Since the single factor test, the response surface test was designed by using the statistical analysis system. A Box-Behnken design (BBD) dominated by 4 factorial points, 3 layers, and 3 centers points was used to conduct 27 sets of tests to determine ideal conditions for UA-ATPS extraction. The results are shown in table 1.

Table 1. Factors and level table of RSM.

Level	Factors			
	X1 The ratio of solid to liquid	X2 Ultrasonic time (min)	X3 Ultrasonic power (w)	X4 Ultrasonic temperature (°C)
-1	1: 30	30	300	50
0	1: 35	35	360	60
1	1: 40	40	420	70

2.5. Statistical Analysis
All results were averaged from three repeated experiments. SPSS 23.0, Microsoft Excel 2020 and Design Expert8.0.6 were used for data collation, analysis and mapping.

3. Results and Discussion

3.1. Effect of the Ratio of Material to Solvent
Figure 1 shows the effect of hawthorn leaf addition on flavonoid extraction rates. The results showed that with the increase of solid-liquid ratio, the flavonoid yield gradually increased from 1:15 to 1:35, and the flavonoid yield began to decrease after 1:35.

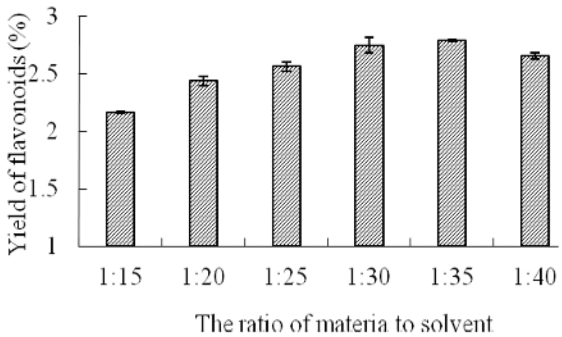

Figure 1. Effect of solid-liquid ratio on class yield.

3.2. Effect of Ultrasonic Time

As shown in figure 2, in the range of 20-35 min, the flavonoids yield increased with time, but after more than 35 min, the yield of flavonoids decreased gradually. This is because the release of flavonoids in hawthorn leaves into the solvent is promoted at the beginning of ultrasonication, and the flavonoids are lost for too long, thereby reducing the yield of extraction [11].

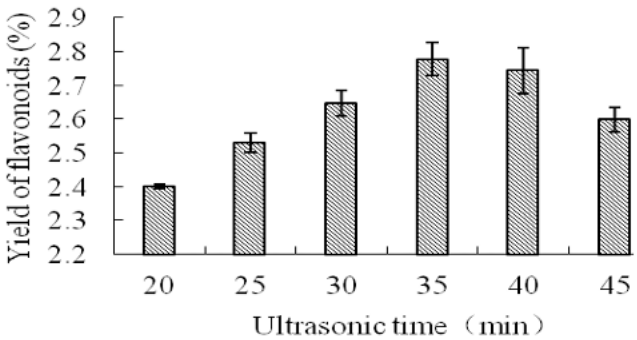

Figure 2. Effect of ultrasonic time on class yield.

3.3. Effect of Ultrasonic Power

It can be seen from figure 3 that the effect of ultrasonic power on the extraction yield of flavonoids. Between 300 and 360 W, the extraction rate of flavonoids increased with the increase of ultrasonic power, and between 360 and 540 W, the extraction rate of flavonoids tended to decrease. More flavonoids are released. When the ultrasonic output increases, the amplitude will increase, resulting in the degradation of flavonoids [12].

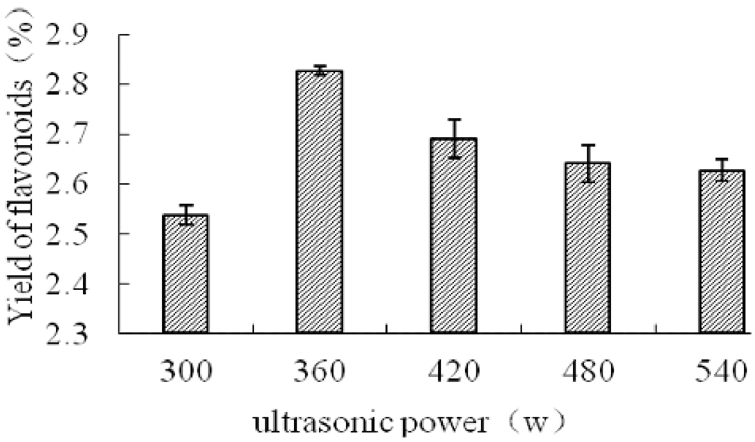

Figure 3. Effect of ultrasonic power on the class yield.

3.4. Effect of Ultrasonic Temperature

The effect of ultrasonic temperature on the yield of flavonoids is shown in figure 4. Between 30 and 60°C, the production of flavonoids increased with increasing temperature. The yield of flavonoids decreased above 60℃ ($P>0.05$). This phenomenon may be due to acoustic cavitation and diffusion through the cell wall. Both phenomena increase with increasing temperature. However, if the temperature is too high, the structure of flavonoids will be destroyed [13].

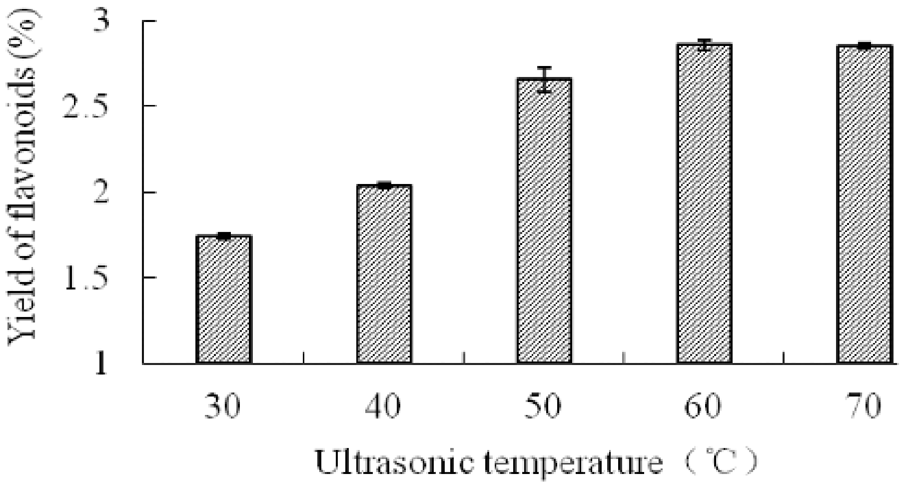

Figure 4. Effect of ultrasonic temperature on class yield.

3.5. Optimization of Extraction Conditions and Analyses of RSM

Based on the above experimental results, a BBD experiment was designed and represented by X1, X2, X3, and X4 and total flavonoid yield (%) for the response value (Y), using Design Expert8.0.6 software to analyze the test results, get regression model parameters and variance analysis, the results were shown in table 2 and table 3.

Table 2. Results of RSM experiment.

Run	Factor				Yield of flavonoids (%)
	X1	X2	X3	X4	
1	1:35	30	300	60	2.765
2	1:35	35	300	50	2.518
3	1:35	35	420	50	2.339
4	1:35	35	360	60	2.82
5	1:40	35	300	60	2.847
6	1:40	30	360	60	2.831
7	1:35	40	360	70	2.875
8	1:35	30	360	70	2.531
9	1:35	35	360	60	2.833
10	1:35	40	360	50	2.517
11	1:40	35	360	50	2.847
12	1:35	35	360	60	2.86
13	1:30	35	360	70	2.859
14	1:35	40	300	60	2.683
15	1:40	35	360	70	2.861
16	1:35	35	420	70	2.804
17	1:35	40	420	60	2.71
18	1:35	30	360	50	2.545
19	1:30	40	360	60	2.722
20	1:30	30	360	60	2.654
21	1:40	35	420	60	2.736
22	1:30	35	360	50	2.462
23	1:40	40	360	60	2.888
24	1:35	30	420	60	2.599
25	1:35	35	300	70	2.545
26	1:30	35	420	60	2.697
27	1:30	35	300	60	2.447

Table 3. ANOVA of RSM for the yield of flavonoids.

Source	Sum of squares	df	Mean square	F value	P-value (Prob>F)
Model	0.61	14	0.043	10.22	0.0001
X1	0.11	1	0.11	26.82	0.0002
X2	0.018	1	0.018	4.34	0.0594
X3	0.00001	1	0.00001	0.13	0.7292
X4	0.13	1	0.13	30.52	0.0001
X1X2	0.00001	1	0.00001	0.00001	0.9341
X1X3	0.033	1	0.033	7.67	0.017
X1X4	0.037	1	0.037	8.64	0.0124
X2X3	0.00001	1	0.00001	2.19	0.1644
X2X4	0.035	1	0.035	8.15	0.0145
X3X4	0.048	1	0.048	11.29	0.0057
$X1^2$	0.00001	1	0.00001	0.1	0.752
$X2^2$	0.017	1	0.017	4.12	0.0652
$X3^2$	0.099	1	0.099	23.23	0.0004
$X4^2$	0.096	1	0.096	22.68	0.0005
Residual	0.051	12	0.00001		
Lack of fit	0.05	10	0.00001	12.04	0.0791

In this case, X1, X4, X1X3, X1X4, X2X4, X3X4, $X3^2$ and $X4^2$ were all significant for the yield of flavonoids. The final predictive equation was obtained by the means of neglecting the insignificant factors, using Yield of flavonoids as the response value, establish a quadratic response surface model, as shown below:

Y1=-4.30612+0.172303*X1+0.10816*X4-0.000105*X1*X3-0.001915*X1*X4+0.000146*X2*X4-0.000017*X3*X3+0.000266*X3*X4-0.001098*X4*X4

The response surface diagram was shown in figure 5.

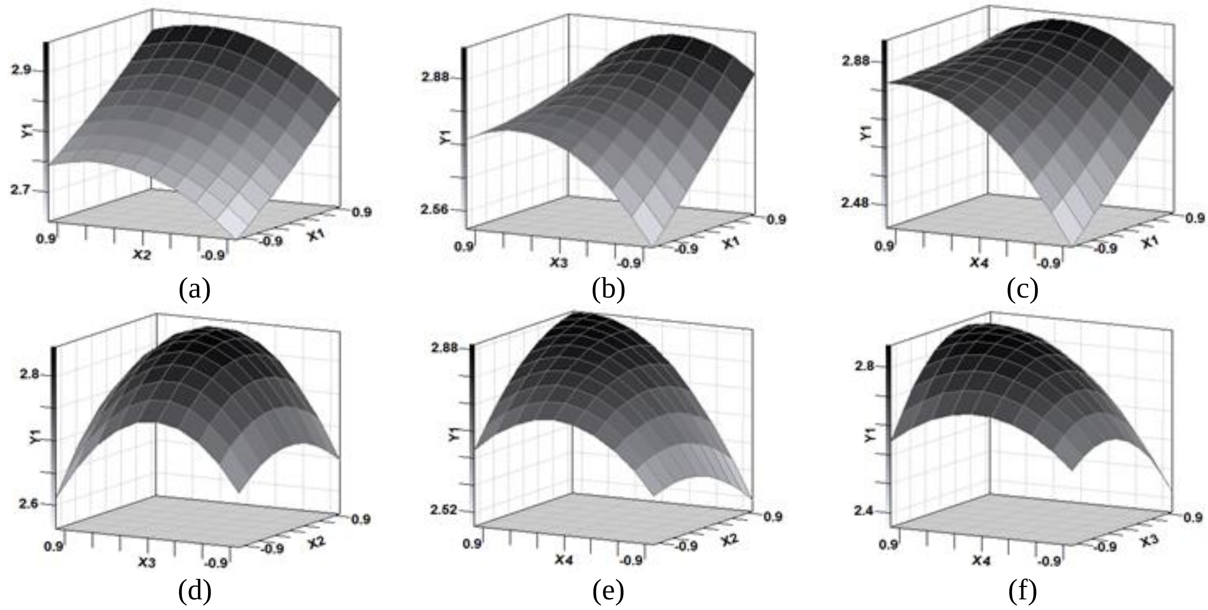

Figure 5. Response surface plots for the extract yield of flavonoids between different factors.

From figure 5(a), the yield of flavonoids increases slightly with the increase of solid-liquid ratio and sonication time. Figure 5(b) shows that the sonication time is fixed; the production of flavonoids increases with the solid-liquid ratio. Figure 5(c) shows the same trend as figure 5(b). Figure 5(d) shows that flavonoids first increased and then decreased with the increase of sonication time and sonication efficiency. As shown, figures 5(e) and 5(f) show that the production of flavonoids increased with increasing temperature between 50 ℃ and 60 ℃.

According to the surface reaction curve method, the ideal process conditions for the extraction of conventional flavonoids from leaves are the solid-liquid ratio of 1:37.35, the ultrasonic time of 40.40 minutes, the ultrasonic power of 381.5 W, and the ultrasonic temperature of 67 degrees. The expected result is 2.921%. To facilitate the experiment, the following conditions were selected to confirm the surface pattern of the reaction curve: the ratio of solid to liquid was 1:37, the ultrasonic time was 40 minutes, the ultrasonic power was 360 W, and the ultrasonic temperature was 65 ℃. The extraction efficiency of all flavonoids was 2869 ± 0.0004%, in line with.

4. Conclusions

This study shows that the use of ATP combined with ultrasound to extract total flavonoids from hawthorn leaves has superiority and defecation. The invention not only saves extraction time, but also improves extraction efficiency. The optimal extraction conditions were that the ratio of solid to liquid was 1:35, the ultrasonic time was 40 min, the ultrasonic power was 360 W, and the ultrasonic temperature was 65 ℃. Under the optimum conditions, the yield of flavonoids was 2.869±0.0004 %. The UA-ATPS method shows the advantages of extracting flavonoids. The method provides a theoretical basis for the further purification of whole herb flavonoids, and can replace the traditional whole herb flavonoid extraction solvent method.

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