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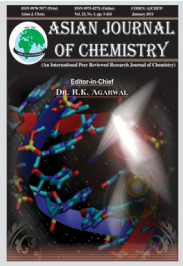

Optimization of Ultrasound-Assisted Aqueous Two-Phase Extraction of Flavonoids from *Erigeron breviscapus* by Response Surface Methodology

YUNTAO GAO*, YANLI FU, CHEN WANG, SHIYUE LI and YAOHONG YANG

School of Chemistry and Biotechnology, Yunnan University of Nationalities, Kunming, Yunnan 650031, P.R. China

*Corresponding author: Tel/Fax: +86 0871 5910071; E-mail: yuntaogao@sohu.com

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To evaluate the effectiveness of the combination of ultrasound-assisted extraction with aqueous two-phase separation and establish the optimal extraction conditions. Ultrasound- assisted propyl-alcohol ammonium sulfate aqueous two-phase extraction of flavonoids from Chinese herbal *Erigeron breviscapus* was investigated. Response surface methodology, based on a five levels, four variables central composite designs was employed to study the effect of extraction conditions on the extraction yield of total flavonoids. An experimental extraction yield of 6.12 % was achieved under the optimal extraction conditions of 59.3 % propyl-alcohol concentration, 25 of liquid/solid ratio, 25 min of extraction time and 29.5 % of ammonium sulfate concentration, which was agreement well with the optimal prediction value of extraction yield (6.15 %). Flavonoids content in extract of ultrasound-assisted aqueous two-phase extraction (36.4 %) was higher than that of conventional ultrasound -assisted extraction (28.3 %).

Key Words: Extraction, Flavonoids, *Erigeron breviscapus*, Ultrasound, Optimization.

INTRODUCTION

Erigeron breviscapus (Vant.) Hand-Mazz, belonging to the family of compositae, is one of the oldest heart herbal in Chinese traditional medicine[1-4]. Flavonoids is the main bioactive component of *Erigeron breviscapus*[5,6].

Various techniques have been developed for the extraction of flavonoids from plants, such as ultrasound-assisted extraction[7], microwave-assisted extraction[8] and pressurized solvent extraction[9]. Ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction methods. This technique has been proved to decrease extraction time and increase extraction yield significantly for many plant materials[10-13]. But ultrasound can also enhance the extraction of undesirable component due to its powerful extraction ability[14]. So, effective, economical and valuable methods need to be developed for the further separation of ultrasound-assisted extraction.

Aqueous two-phase extraction is an efficient method for the separation and purification of bioactive substances[15-17]. It has several advantages, such as bio-compatibility, environmentally friendly, poisonless and easy to scale-up. Conventional aqueous two-phase systems are prepared by mixture polymers or surfactant and salts, but, most of polymers and surfactants used are difficult to handle in the subsequent processes of aqueous two-phase extraction.

It has been recently introduced that a mixture of water-soluble alcohol, such as propyl-alcohol and water can also form aqueous two-phase system by adding inorganic salt[18]. The subsequent process becomes much easy for this new aqueous two-phase extraction technique because of no polymer or surfactant in the system,*e.g.*, the extracted target material and extraction solvent can be recovered by distillation. This new aqueous two-phase system could be an ideal technique to integrate with ultrasound-assisted extraction for further separation.

In the present work, the combination of ultrasound and propyl-alcohol ammonium sulfate aqueous two-phase system for extraction of flavonoids from *erigeron breviscapus* was investigated. The extraction conditions were optimized by response surface methodology (RSM)[19], with a five levels, four variables central composite design (CCD)[20]. The proposed method was also compared with conventional ultrasound-assisted extraction.

EXPERIMENTAL

Erigeron breviscapus was obtained from China Yunnan Yuxi Botanic Pharmaceutical Co., Ltd. and had been powdered to particle before the experiment. An ultrasound cleaner (AS10200AD, China Tianjin Autoscience Instrument Co., Ltd.) was used in this work, operating at a frequency of 60 kHz with output power of 320 W. The extraction vessel was put at

the center of the ultrasound cleaner in the experimental process. The temperature of extraction was controlled and maintained at 45 ± 1 ºC by circulating external water from a thermostated water bath. An UV-visible spectrophotometer (Unico (Shanghai) Instrument Co., Ltd.)was used for total flavonoids determination. Water was purified using a Milli-Q gradient system (Millipore Corporation, Genay Cedex, France).

Ultrasound-assisted aqueous two-phase extraction: Propyl-alcohol ammonium sulfate aqueous two-phase system[18] was adopted with some modifications. The aqueous two-phase system was prepared by weighing requisite ammonium sulfate into a mixture solution of propyl-alcohol and water. The desired system composition could be achieved by varying of the amount of ammonium sulfate and the volume of propyl-alcohol.

10.0 g dried *erigeron breviscapus* powder was soaked in the propyl-alcohol ammonium sulfate aqueous two-phase system (varying propyl-alcohol concentration from 40.0 to 80.0 %, v/v; varying ammonium sulfate concentration from 5.0 to 45.0 %, w/v; varying liquid/solid ratio from 10 to 30) for 2 h and then placed in the ultrasound cleaner and sonicated at 45 ºC for certain time (varying extraction time from 10 to 50 min). The extraction solution is filtered off through 0.45 μm microporous membrane, the filtrate was allowed to stand until the mixture was thoroughly separated into two phases. The samples of propyl-alcohol rich phase (top phase) and salt-water phase (bottom phase)were collected for total flavonoids content analysis, respectively.

Determination of total flavonoids content: The total flavonoids content was determined by a colorimetric method described by Kaijv *et al.*[21] and slightly modified in this research. 0.3 mL $NaNO_3$ solution (5 %, w/v), 0.6 mL $A1Cl_3$ solution (10 % w/v) and 2.0 mL NaOH solution (1.0 mol/L) were added to 1.0 mL sample. The final volume was adjusted to 10.0 mL with deionized water. The mixture was allowed to stand for 5 min and the absorbance was measured at 507 nm against the same mixture, without the example as a blank. The amount of total flavonoids was expressed as rutin equivalents (mg rutin/g sample) through the calibration curve of rutin. The calibration curve (Y = 9.27X - 0.0119, where Y is absorbance of sample, X is sample concentration) ranged 0.75-6.0 mg/mL (R^2 = 0.9993).

Experimental design: Response surface methodology was used to find out the optimal extraction conditions. The experiment was carried out according to a central composite design with four factors and five levels. The four independent variables selected for this work were propyl-alcohol concentration (X_1), liquid/solid ratio (X_2), extraction time (X_3) and ammonium sulfate concentration (X_4), the extraction yield of total flavonoids was the response variable (Y). The experimental range of each factor was based on the results of preliminary experiments.

Table-1 represents the coded and non-coded values of the experimental variables. A design of thirty experiments was formulated for four factorial designs with six replicates at the central points, eight axial points and fourteen points for second-order polynomial model. The chosen independent variables used in this work were coded according to eqn. 1:

TABLE-1
INDEPENDENT VARIABLES AND THEIR LEVELS USED FOR CENTRAL COMPOSITE DESIGN

Variables	Levels*				
	-α	-1	0	1	+α
Propyl-alcohol concentration (X_1, %)	40	50	60	70	80
Liquid/solid ratio (X_3)	10	15	25	30	35
Extraction time (X_2, min)	10	20	30	40	50
Ammonium sulfate concentration (X_4, %)	5	15	25	35	45

*α = 2.0

$$x_i = (X_i - X_0)/\Delta X \quad (1)$$

where x_i is the coded value of the variable X_i, X_0 is the value of X_i at the centre point and ΔX is the step change. The experimental design along with the extraction yield is given in Table-2. The behaviour of the system is explained by the following second degree polynomial equation:

$$Y = \sum A_0 + \sum_{i-1}^{4} A_i X_i + \sum_{i-1}^{4} A_{ii} X_i^2 + \sum_{i-1}^{3} \sum_{j-1+1}^{4} A_{ij} X_i X_j \quad (2)$$

where Y is the response variable, A_0 is constant, A_i, A_{ii} and A_{ij} are the coefficients estimated by the model. X_i and X_j are the levels of the independent variable. They represent the linear, quadratic and interaction effects of the X_1, X_2, X_3 and X_4 on the response variable, respectively.

TABLE-2
RESPONSE SURFACE ANALYSIS OF THE EXTRACTION YIELD OF TOTAL FLAVONOIDS FROM *Erigeron breviscapus* WITH FACTORS*

No.	Propyl-alcohol (%)	Liquid/ solid ratio	Extraction time (min)	Ammonium sulfate (%)	Yield (%)
1	70.0	15	40	15.0	5.29
2	50.0	25	40	15.0	4.27
3	50.0	15	20	15.0	4.99
4	70.0	25	20	35.0	5.24
5	80.0	20	30	25.0	4.78
6	70.0	25	40	35.0	5.47
7	60.0	30	30	25.0	6.12
8	60.0	20	30	5.0	4.32
9	40.0	20	30	25.0	4.08
10	60.0	20	10	25.0	5.22
11	60.0	20	30	25.0	6.17
12	70.0	25	20	15.0	4.66
13	60.0	20	50	25.0	5.83
14	60.0	20	30	25.0	6.17
15	60.0	20	30	25.0	6.12
16	50.0	15	20	35.0	5.15
17	70.0	25	40	15.0	5.29
18	60.0	20	30	25.0	6.14
19	60.0	10	30	25.0	5.47
20	50.0	25	20	15.0	4.64
21	70.0	15	40	35.0	5.47
22	60.0	20	30	25.0	6.16
23	70.0	15	20	15.0	5.33
24	60.0	20	30	45.0	5.13
25	50.0	15	40	15.0	4.39
26	50.0	15	40	35.0	5.52
27	60.0	20	30	25.0	6.17
28	50.0	25	20	35.0	5.83
29	70.0	15	20	35.0	5.22
30	50.0	25	40	35.0	5.83

*Experiments were conducted in a random order.

The analysis of the experimental design and the calculation of predicted value were performed by DESIGN EXPERT software (version 7.0, Stat-Ease Inc., Minneapolis, MN, USA). The analyses of variance were performed by ANOVA procedure. The mean values were considered significantly different when $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of extraction conditios of total flavonoids: The value of response variable Y at different experimental conditions is given in Table-2. The percentage extraction yield of total flavonoids ranges from 4.08 to 6.19 %. The maximum value is found at the propyl-alcohol concentration 60.0 %, liquid/solid ratio 20, extraction time 0.5 h and ammonium sulfate concentration 25.0 %.

The statistical analysis shown in Table-3 indicates that quadratic terms as propyl-alcohol concentration ($p < 0.0001$), extraction time ($p < 0.0055$) and ammonium sulfate concentration ($p < 0.0001$), liner term as ammonium sulfate concentration ($p = 0.0008$) and the interactive propyl-alcohol concentration with ammonium sulfate concentration ($p = 0.0073$) for the extraction yield are highly significant ($p < 0.01$). It may be found that the operational parameter turned out to be significant ($p < 0.05$) for the extraction yield is the linear term as propyl-alcohol concentration ($p = 0.0203$). While the linear terms of extraction time ($p = 0.2490$) and liquid/solid ratio ($p = 0.3660$), the quadratic terms of liquid/solid ratio ($p = 0.0791$) and the interactive terms of propyl-alcohol concentration with extraction time ($p = 0.2710$), propyl-alcohol concentration with liquid/solid ratio ($p = 0.0992$), liquid/solid ratio with extraction time ($p = 0.6180$), liquid/solid ratio with ammonium sulfate concentration ($p = 0.0753$) and extraction time with ammonium sulfate concentration ($p = 0.2490$) are not significant ($p > 0.05$).

TABLE-3
ANALYSIS OF VARIANCE (ANOVA) FOR RESPONSE SURFACE QUADRATIC MODEL

Source	Sum of squares	df	Mean square	F-value	Prob > F
Model	9.364	14	0.669	9.871	<0.0001
X_1-Propyl-alcohol	0.457	1	0.457	6.737	0.0203
X_2-Liquid/Solid ratio	0.059	1	0.059	0.871	0.3660
X_3-Extraction time	0.098	1	0.098	1.439	0.2490
X_4-Ammonium Sulfate	1.193	1	1.193	17.601	0.0008
$X_1 X_2$	0.089	1	0.089	1.306	0.2710
$X_1 X_3$	0.209	1	0.209	3.089	0.0992
$X_1 X_4$	0.652	1	0.652	9.623	0.0073
$X_2 X_3$	0.018	1	0.018	0.259	0.6180
$X_2 X_4$	0.248	1	0.248	3.653	0.0753
$X_3 X_4$	0.098	1	0.098	1.441	0.2490
X_1^2	4.608	1	4.608	68.011	<0.0001
X_2^2	0.241	1	0.241	3.550	0.0791
X_3^2	0.712	1	0.712	10.512	0.0055
X_4^2	2.285	1	2.285	33.726	<0.0001
Residual	1.016	15	0.068	–	–
Lack of Fit	1.016	10	0.102	1524.088	<0.0001
Pure Error	0.000	5	0.000	–	–
Cor Total	10.381	29	–	–	–

The response variable and the test variables are related by a second-order polynomial equation by multiple regression analysis on the experimental data. All non-significant terms ($p > 0.05$) are eliminated to simplify the model. The simplified model is represented by the following equation:

$$Y = 6.13 + 0.138X_1 + 0.223X_4 - 0.202X_1X_4 - 0.410X_1^2 - 0.00150X_3^2 - 0.289X_4^2 \quad (3)$$

The statistical testing of the quadratic model was evaluated by the analysis of variance (ANOVA) as presented in Table-3. The result shows that experimental data for the extraction yield have a correlation coefficient (R^2) of 0.969 with the calculated model, indicating a high degree of correlation between the observed value and predicted value. In addition, the statistical analysis gives high significant level ($p = 0.00017$) for the goodness of fit of the model. This result suggests that the model can work well for the prediction of total flavonoids extraction by the proposed extraction method.

Based on the sum of squares, the importance of the independent variables on the extraction yield of total flavonoids can be ranked in the following order: ammonium sulfate concentration (X_4) > propyl-alcohol concentration (X_1) > extraction time (X_3) > liquid/solid ratio (X_2). The result suggests that the changes of ammonium sulfate concentration and propyl-alcohol concentration have a significant effect on the total flavonoids extraction.

The three-dimensional response surface profiles of multiple non-linear regression model are depicted in Figs. la-f. Fig. 1a gives the extraction yield as a function of propyl-alcohol concentration and extraction time. It shows that the extraction yield increases with the increase of propyl-alcohol concentration from 40.0 to 60.0 % at extraction time below 0.5 h. However, more than 0.5 h extraction time in propyl-alcohol concentration above 60.0 % appears to be disadvantaged on the extraction. Fig. 1b shows the effect of extraction time and ammonium sulfate concentration on the extraction yield, it can be seen that the extraction yield increases with the increase of ammonium sulfate concentration from 15.0 to 25.0 % and extraction time from 10 to 30 min. But, the extraction yield decreases with increasing ammonium sulfate concentration above 35.0 % and extraction time beyond 0.5 h. The effect of liquid/solid ratio and extraction time on the extraction yield (presented in Fig. 1c) shows that the extraction yield increases with the increase of liquid/solid ratio from 10 to 20 and extraction time from 10 to 30 min. The initial increase of the extraction yield is followed by a decrease after liquid/solid ratio more than 20 at extraction time above 0.5 h.

It can be observed from Figs. 1a-c that increase of extraction time from 10 to 30 min enhances the extraction of total flavonoids, whilst a decreasing effect can be observed as extraction time more than 0.5 h. This can be explained that as extraction time prolongs, the chemical decomposition of bioactive compound in extract may occurs, resulting in a decrease in the extraction yield[22].

The simultaneous effect of liquid/solid ratio and propyl-alcohol concentration on the extraction yield is shown in Fig. 1d, the extraction yield changes slightly with the change in liquid/solid ratio. Similarly, the extraction yield increases

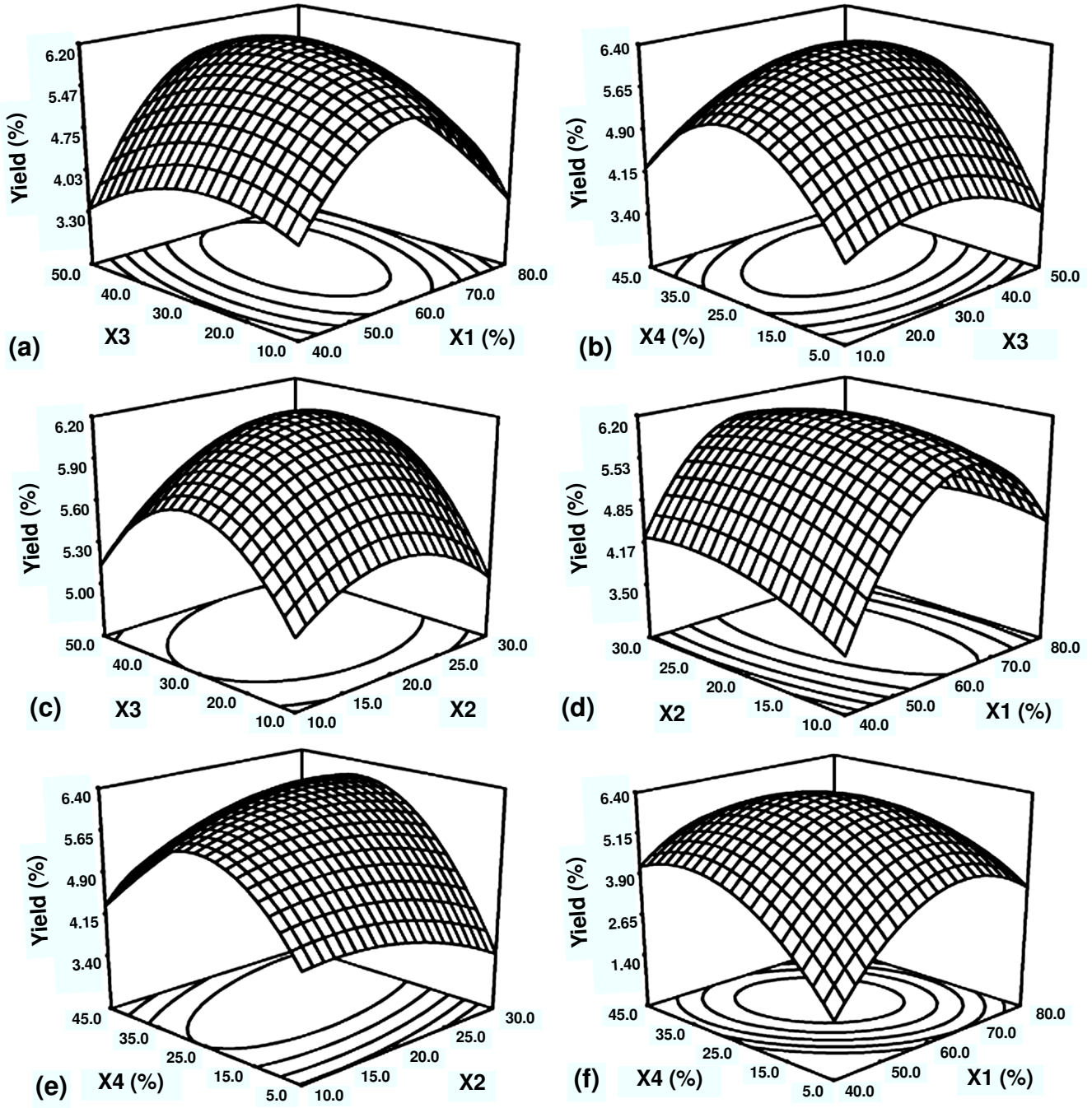

Fig. 1. Response surface (3D) showing the effect of propyl-alcohol concentration (X_1), liquid/solid ratio (X_2), extraction time (X_3) and ammonium sulfate concentration (X_4)on the response Y

slowly with the increase of liquid/solid ratio, as shown in Fig. 1e, which gives the extraction yield as a function of liquid/solid ratio and ammonium sulfate concentration. The result suggests that the extraction yield have no significant changes along with liquid/solid ratio.

The effect of propyl-alcohol concentration and ammonium sulfate concentration on the extraction yield shown in Fig. 1f demonstrates that the extraction yield increases rapidly with the increase of propyl-alcohol concentration, while the increase in ammonium sulfate concentration also leads to a marked increase of the extraction yield, the initial increase of the extraction yield is followed by a rapidly decrease after propyl-alcohol concentration more than 60.0 % at ammonium sulfate concentration above 25.0 %. This result implies that propyl-alcohol concentration and ammonium sulfate concentration are two significant factors to affect the extraction yield. Similar result has also been seen from Figs. 1a-d and 1d-e. This result can be explained by the facts that ammonium sulfate concentration and propyl-alcohol concentration have a significant effect on the formation of two phase system[18] and affect the composition of propyl-alcohol rich phase. Some important physical characteristics of propyl-alcohol rich phase, such as polarity, viscosity and surface tension would be changed[18,23] as the result. These changes have a significant effect on

TABLE 4
OPTIMUM CONDITIONS, PREDICTED AND EXPERIMENTAL VALUE OF RESPONSE AT THAT CONDITION

Optimum conditions				Extraction yield of flavonoids	
Propyl-alcohol concentration (%)	Extraction time (min)	Liquid/solid ratio	Ammonium sulfate (%)	Experimental* (%)	Predicted (%)
59.3	25	25	29.5	6.11 ± 0.062	6.15

*Means ± standard deviation (n = 3)

TABLE 5
COMPARISON WITH CONVENTIONAL ULTRASOUND-ASSISTED EXTRACTION

Method*	Extraction medium	Extraction yield of flavonoids** (%)	Flavonoids content in extract** (%)
Ultrasound-assisted extraction	60.0 % propyl-alcohol	5.94 ± 0.081	28.3 ± 0.27
Ultrasound-assisted aqueous two-phase extraction	59.3 % propyl-alcohol + 29.5 % ammonium sulfate	6.11 ± 0.062	36.4 ± 0.33

*Liquid/solid ratio 25 for two method; extraction time 25 min; extraction temperature 45 ºC; **Means ± standard deviation (n = 3)

sonication activity and the partition behaviour of flavonoids in aqueous two-phase system[24].

Ammonium sulfate concentration and propyl-alcohol concentration are two significant factors to affect the extraction yield according to the gradient of slope in the 3-D response surface plots (Fig. 1) and Prob > F in Table-3, followed by extraction time, liquid/solid ratio. Interaction between propyl-alcohol concentration and ammonium sulfate concentration can be observed in Fig. 1f, while the interactive effect of others on the extraction yield is insignificant.

Optimization by the desirability function: Central composite design (CCD) is able to function as optimal design for the desired response of the system with numerical optimization based on the model obtained and the input criteria. The desired goal was chosen for each factor and response from the menu. The possible goals are: maximize, minimize, target, within range, none (for responses only) and set to an exact value (factors only).

The optimization of the extraction yield was based on the maximum level, the level of propyl-alcohol concentration within range of 50.0-70.0 %, liquid/solid ratio within range of 15-25 and ammonium sulfate concentration within range of 15.0-35.0 % were set for maximum desirability, respectively. While extraction time was at the target goal of 25 min for the reason that the chemical decomposition of flavonoids may occurs as extraction time prolongs beyond its peak value (0.5 h) (Figs. 1a-c). Fig. 2 shows the ramp desirability that was

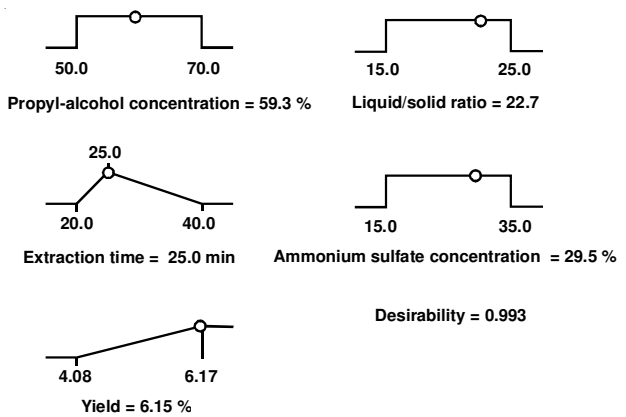

Fig. 2. Desirability ramp for numerical optimization

generated from eleven optimum points *via* numerical optimization. The best local maximum is found to be at propyl-alcohol concentration 59.3 %, extraction time of 25 min, liquid/solid ratio of 25 and ammonium sulfate concentration of 29.5 %. The prediction value of the extraction yield is 6.15 %. The obtained value of desirability (0.993) shows that the estimated function can represent the experimental model and the desired conditions.

Verification of predictive model and comparison with other extraction methods: The experimental rechecking was performed by the deduced optimal conditions to compare the predicted result with the practical value. A mean value of 6.11 ± 0.062 (n = 3), obtained from real experiments, is in agreement with the predictive value (6.15 %), which demonstrates the validation of the response surface model and confirms that the response surface model is adequate for reflecting the expected optimization (Table-4).

Erigeron breviscapus was also extracted by conventional ultrasound-assisted extraction to evaluate the efficiency of ultrasound-assisted aqueous two-phase extraction. The result is shown in Table-5.

Table-5 shows that ultrasound-assisted aqueous two-phase extraction can produce 6.11 % of the extraction yield, which is higher than that by conventional ultrasound-assisted extraction (5.94 %). Moreover, the flavonoids content in the extract by ultrasound-assisted aqueous two-phase extraction is 36.4 %, it is higher than that by ultrasound-assisted extraction (28.3 %).

The present work confirms that combination aqueous two-phase separation with ultrasound-assisted extraction to improve the extraction of total flavonoids is quite practicable, it is a simple, inexpensive and efficient alternatve to remove undesirable co-extraction component[19] from flavonoids extract. Flavonoids is alcohol-soluble and can be distributed mainly in propyl-alcohol rich phase, while water-soluble byproducts such as xylan, mannosans and glucosan, are main partition in salt-water phase, so the extraction and purification of flavonoids can be achieved in a single step by the proposed extraction method.

Conclusion

It was found that the extraction yield and the flavonoids content in the extract of ultrasound-assisted aqueous two phase extraction is higher than that of conventional ultrasound-

assisted extraction. The statistical testing and the 3-D response surface plots indicate that the changes of ammonium sulfate concentration and propyl-alcohol concentration have significant effect on the extraction yield of total flavonoids. An interaction between propyl-alcohol concentration and ammonium sulfate concentration can be observed. The prediction value optimized by desirability function is in agreement well with the experimental result.

This study confirms that the combination of ultrasound-assisted extraction with propyl-alcohol ammonium sulfate aqueous two-phase separation is an effective way to improve the quality of extract of flavonoids from *Erigeron breviscapus*. There is also a potential for the application of aqueous two-phase separation as a simple, inexpensive and efficient alternative to improve the quality of bioactive substances of ultrasound-assisted extraction.

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