Optimization of simultaneous ultrasonic-assisted extraction of water-soluble and fat-soluble characteristic constituents from Forsythiae Fructus Using response surface methodology and high-performance liquid chromatography

Yong-Gang Xia*, Bing-You Yang*, Jun Liang, Di Wang, Qi Yang, Hai-Xue Kuang

School of Pharmacy, Key Laboratory of Chinese Materia Medica (Heilongjiang University of Chinese Medicine), Ministry of Education, Harbin, 150040, P.R. China

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ABSTRACT

Background: The compounds (+)-pinoresinol-β-glucoside (1) forsythiaside, (2) phillyrin (3) and phillygenin (4) were elucidated to be the characteristic constituents for quality control of Forsythiae Fructus extract by chromatographic fingerprint in 2010 edition of Chinese Pharmacopoeia due to their numerous important pharmacological actions. It is of great interest to extract these medicinally active constituents from Forsythiae Fructus simultaneously. **Materials and Methods:** In this study, a new ultrasound-assisted extraction (UAE) method was developed for the simultaneous extraction of biological components 1-4 in Forsythiae Fructus. The quantitative effects of extraction time, ratio of liquid to solid, extraction temperature, and methanol concentration on yield of these four important biological constituents from Forsythiae Fructus were investigated using response surface methodology with Box-Behnken design. The compounds 1-4 extracted by UAE were quantitative analysis by high-performance liquid chromatography-photodiode array detect (HPLC-PAD), and overall desirability (OD), the geometric mean of the contents of four major biological components, was used as a marker to evaluate the extraction efficiency. **Results:** By solving the regression equation and analyzing 3-D plots, the optimum condition was at extraction temperature 70°C, time 60 min, ratio of liquid to solid 20, and methanol concentration 76.6%. Under these conditions, extraction yields of compounds 1-4 were 2.92 mg/g, 52.10 mg/g, 0.90 mg/g and 0.57 mg/g, respectively, which were in good agreement with the predicted OD values. In order to achieve a similar yield as UAE, soxhlet extraction required at least 6 h and maceration extraction required much longer time of 24 h. Established UAE method has been successfully applied to sample preparation for the quality control of Forsythiae Fructus. Additionally, a quadrupole time-of-flight mass spectrometry was applied to the structural confirmation of analytes from the complex matrices acquired by UAE. **Conclusion:** The results indicated that UAE is an effective alternative method for extracting bioactive constituents, which may facilitate a deeper understanding of the extract of active constituents in Forsythiae Fructus from the raw material to its extract for providing the theoretical references.

Key words: Box-Behnken design, Forsythiae Fructus, *Forsythia suspensa,* response surface methodology, ultrasound-assisted extraction

Address for correspondence:
Prof. Hai-Xue Kuang, School of Pharmacy, Key Laboratory of Chinese Materia Medica (Heilongjiang University of Chinese Medicine), Ministry of Education, Harbin, 150040, P.R. China.
E-mail: hxkuang@hotmail.com

*These authors contributed equally to this work

INTRODUCTION

Forsythiae Fructus, the fruit of *Forsythia suspensa* (Oleaceae),[1] is one of the most famous Chinese herbal medicines listed in the Pharmacopoeia of the People's Republic of China.[2] Forsythiae Fructus has been utilized as an antipyretic, detoxicant, antioxidant and anti-inflammatory

agent for the treatment of various infectious diseases.[3,4] Furthermore, Forsythiae Fructus extract have been used for a long time as traditional Asian medicines to treat gonorrhea, erysipedas, inflammation, and pharyngitis.[5,6] More than 40 Chinese medicinal preparations containing Forsythiae Fructus or its extract are listed in 2010 edition of Chinese pharmacopoeia, such as "Shuanghuanglian Injection," "Kangbingdu oral solution," "Yin Qiao tablet," etc.,[2] Many studies have elucidated that caffeoyl glycosides, and lignans are the main bioactive components responsible for the various biological activities of Forsythiae Fructus.[7,8] For example, forsythoside is responsible for the antibacterial and the antioxidant activities of the herb.[3,9,10] Phillyrin could enhance immunological function and alleviate delayed hypersensitivity in mice.[6,11] Phillygenin and (+)-pinoresinol-β-glucoside showed their protective effects against peroxynitrite-induced oxidative stress in LLC-PK1 cells.[12] Therefore, (+)-pinoresinol-β-glucoside, (1) forsythiaside, (2) phillyrin (3) and phillygenin (4) possess important pharmacological activities and may become promising phytopharmaceuticals.

Extraction and determination of the compounds 1-4 in Forsythiae Fructus are also essential for the quality evaluation of the traditional Chinese medicine.[8,13-15] By our previous studies, the compounds 1-4 were elucidated to be the characteristic constituents for quality control of Forsythiae Fructus extract by chromatographic fingerprint in 2010 edition of Chinese pharmacopoeia due to their numerous important pharmacological actions.[8] Besides, a number of studies found that the quality of Forsythiae Fructus often varied considerably according to it's the harvest season, storage, geographic origin or other growing conditions.[8,13-15] Though determining the contents of 1-4 have been widely described no study has been reported on the extraction of these four "characteristic constituents" simultaneously. Conventional soxhlet extraction (SE) and maceration extraction (ME) are very time-consuming and require relatively high extraction temperature for SE, which have been used for several decades.[16,17] Thus, there is an increasing demand for a novel extraction technique with the shortened extraction time, reduced extraction temperature, and increased extraction efficiency.

Ultrasonic technique is being used widely in analytical chemistry, facilitating different steps in the analytical process, particularly in sample preparation.[18-20] Ultrasonic-assisted extraction (UAE) is an expeditious, inexpensive, and efficient alternative to traditional extraction techniques.[21,22] UAE may enhance the extraction efficiency due to disruption of cell walls, particle-size reduction, and enhancing mass transfer of the cell contents as a result of cavitation bubble collapse.[20-22] UAE of phillyrin from the seeds of *F. suspensa* has been performed by classical univariate approach (one-variable-at-a-time),[23] but this method cannot determine the interactions between parameters and find the most suitable UAE condition, so some "experimental design" was adopted to detect the influencing factors while the number of trials can be kept to a minimum. So far, there is no report for the simultaneous extraction of compounds 1-4 using ultrasonic technique coupled with Box-Behnken statistical design (BBD). BBD provides efficient solutions compared with a three-level full-factorial design, reducing the number of required experiments by confounding higher-order interactions, which becomes more significant as the number of factors increases.[24-26] It provides information about the relative significance of main effects, as well as information about interaction effects that cannot be predicted by univariate techniques. The model results are easily interpreted and visualized in response surface plots.[27,28]

The goals of this study are to optimize the UAE condition for extracting these four characteristic compounds 1-4 from Forsythiae Fructus coupled with BBD. The overall desirability (OD), the geometric mean of the contents of four major biological components was used as a marker to evaluate the extraction efficiency.[29,30] BBD was applied to fit and to exploit a mathematical model representing the relationship between the responses and the variables (i.e, extraction time, ratio of liquid to solid, temperature, and methanol concentration). This study may facilitate a deeper understanding of the extract of these active constituents from the raw material to its extract and provide theoretical references for industrial production of Forsythiae Fructus extract.

MATERIALS AND METHODS

Materials and reagents

The unripe fruits of *F. suspensa* were collected in March 2010 from Henan Province, China and identified by Prof. Wang Zhen-Yue of Heilongjiang University of Chinese Medicine, The voucher specimen (2010013) was deposited at Herbarium of Heilongjiang University of Chinese Medicine, Harbin, China. The crude drug was pulverized into powder form by a disintegrator (Weinengda Instrument Company, Lanxi, China), and then sieved with stainless steel sieves to classify the particle size. The powdered sample was kept in a dry and dark place until use.

All organic solvents used for extraction were of analytical grade and purchased from Tianjin Chemical Factory, Tianjin, China. The high-performance liquid chromatography (HPLC) grade methanol was purchased from Dikama Technology Corporation (Richmond Hill, USA). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

Standards, namely (+)-pinoresinol-β-glucoside, forsythiaside, phillyrin, and phillygenin were isolated by the author from the fruits of *F. suspensa*. Structures of the standards are shown in Figure 1.

UAE

For the UAE experiments, an ultrasonic bath was used as an ultrasound source. The bath (KQ-500DB, Kunshan Ultrasound Co. Ltd., China) was a rectangular container (540 mm × 320 mm × 350 mm). The bath power rating was 500 W on the scale of 4-10. The extraction temperature was controlled and maintained at the desired value by circulating external water from a thermostatic water bath into the cleaning bath. The sample beakers were immersed into the ultrasonic cleaning bath for irradiation under different extraction conditions including solvents: Ethanol, methanol, acetonitrile, and water; percentage of methanol in water of 20-100%; solvent to solid ratio of 20-80 mL/g; temperature of 30-70°C; extraction time of 15-60 min; particle size of 40-60 mesh. Finally, extracts were filtered off through 0.22 μm membrane filter and the filtrate was collected for HPLC analyses. All samples were prepared and analyzed in triplicate.

ME

ME method was performed with 1.0 g (50 mesh) of dried samples and 20 mL of 76.6% methanol extracted 3 times, each for 24 h, and then mixed them at room temperature. The extracts were combined and concentrated by a rotary vacuum evaporator. All solutions were filtered through 0.22 μm membrane filter before direct injection into the HPLC system. All samples were prepared and analyzed in triplicate.

SE

SE was performed in a soxhlet apparatus. The powders of 1.0 g (50 mesh) and 20 mL of 76.6% methanol were placed into the soxhlet apparatus. The exhaustive extraction was performed for 6 h at 70°C. All solutions were filtered with 0.22 μm membrane filter before the HPLC analysis. All samples were prepared and analyzed in triplicate.

HPLC analysis

The analyses were performed using waters e2695 liquid chromatography system, equipped with a quaternary solvent delivery system, a waters 600 controller, two waters 600 pumps, a 2695 auto sampler, a waters 2998

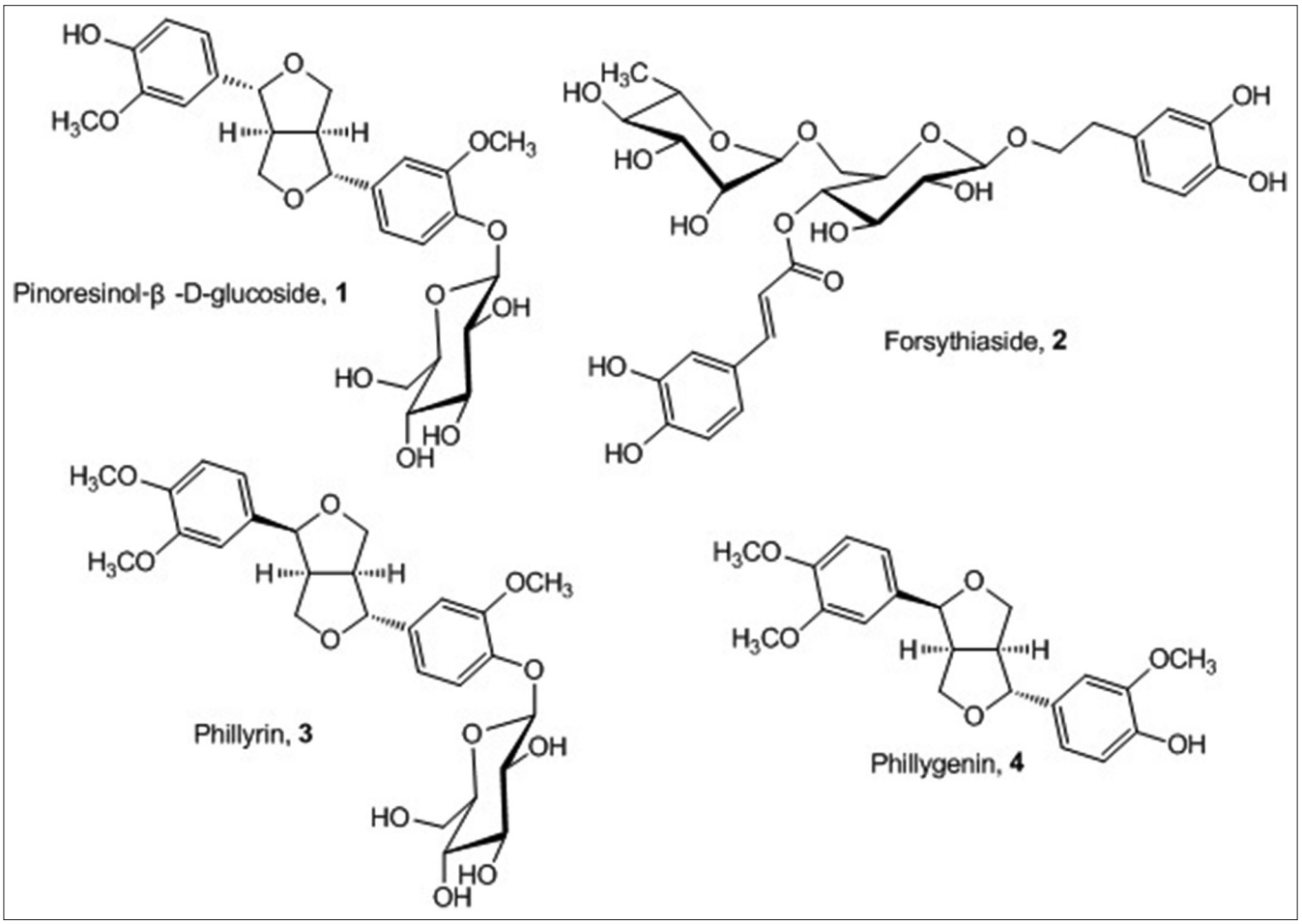

Figure 1: Chemical structures of pinoresinol-β-D-glucoside, (1) forsythiaside (2), phillyrin (3) and phillygenin (4)

photodiode array detector, and a waters 2695 column oven. The separation was carried out on waters symmetry C_{18} column (4.6 mm × 150 mm, 5 μm). The gradient elution was employed using solvent A (MeOH) and solvent B (water) at 30°C; the gradient program was used as follows: initial 0-10 min, linear change from A to B (10:90, v/v) to A-B (25:75, v/v); 10-40 min, linear change to A-B (35:65, v/v); 40-60 min, linear change to A-B (60:40, v/v). The flow rate was set at 1.0 mL/min, and the injection volume was 10 μL. Due to the different UV characteristic of these components, the detection wavelength was set at 332 and 270 nm for quantitative analysis of the caffeoyl glycoside and lignans, respectively. Quantification was carried out by the integration of the peak using external standard method by means of a six point calibration curve. The regression equations, correlation coefficient and linear range are listed in our previous published papers.[8] The extraction yields of (+)-pinoresinol-β-glucoside, forsythiaside, phillyrin, and phillygenin by UAE, ME and SE methods were calculated using the following equation: extraction yields (%) = weight of compounds extracted (g)/weight of dried sample (g) ×100%.

Qualitative analysis based on ultra performance liquid chromatography-quadrupole time-of-flight

The UPLC-MS analysis was performed on a Waters ACQUITY UPLC system (Waters Corporation, Milford, USA) coupled with a Waters Xevo QTOF equipped with electrospray ionization. For the reversed-phase UPLC analysis, the ACQUITY UPLC™ HSS C18 column (100 mm × 2.1 mm i.d., 1.7 μm, Waters Corp, Milford, USA) was used. The column temperature was maintained at 40°C; the flow rate of the mobile phase was 0.40 mL/min; the injection volume was fixed at 2.0 μL. Mobile phase A consisted of 0.1% formic acid in methanol while mobile phase B consisted of 0.1% formic acid in water. The column was eluted with a linear gradient of 2-18% B over initial to 6.0 min, 18-24% B over 6.0-13.0 min, 24-29% B over 13.0-18.0 min, 29-42% B over 18.0-23.0 min, 42-50% B over 23-24.5 min, 50-98% B over 24.5-26 min, and returned to 2% B for 1.0 min and then held for 1.0 min at an eluent flow rate of 0.40 mL/min.

For the UPLC-Xevo QTOF analysis, the mass spectrometric full-scan data were acquired in the negative ion mode from 100 to 1000 Da with a 0.1 s scan time. Other conditions were as follows: capillary voltage of 2.4 kV, desolvation temperature of 400°C, sample cone voltage of 25 V, extraction cone voltage of 4.0 V, collision energy of 30 eV, source temperature of 120°C, cone gas flow of 50 L/h and desolvation gas flow of 400 L/h for negative ion mode. Data were centroided, and mass was corrected during acquisition using an external reference (Lock-Spray™) consisting of a 0.2 ng/ml solution of leucine-enkephalin infused at a flow rate of 20 μL/min via a lockspray interface, generating a reference ion for negative ion mode ($[M-H]^-$ =554.2615) to ensure the accuracy during the MS analysis.

Experimental design

After determining the preliminary range of the extraction variables through single-factor test, a 29-run BBD consisting of four variables at three levels was established to optimize the four marker constituents extraction conditions from *F. suspensa*, including extraction temperature, extraction time, solvent concentration, and ratio of liquid to solid, which significantly influenced the extraction yield. As shown in Table 1, the four factors chosen for this study were designated as X_1, X_2, X_3, and X_4 and prescribed into three levels, coded + 1, 0, −1 for high, intermediate and low value, respectively. The four variables were coded according to the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x}, i = 1,2,3,4 \quad (1)$$

Where X_i is a coded value of the variable; x_i is the actual value of the variable; x_0 is the actual value of the independent variable at the center point, and is the step change of the variable.

A 2nd-order polynomial model corresponding to the BBD was fitted to correlate the relationship between the independent variables and the response (extraction yield, polysaccharide yield and uronic acid yield) to predict the optimized conditions. The computer-generated quadratic model is given as:

$$Y = \beta_0 + \sum_{i=0}^{4} \beta_i X_i + \sum_{j=0}^{4} \beta_{ii} X_i^2 + \sum_{i=0}^{4} \sum_{j=0}^{4} \beta_{ij} X_i X_j \quad (2)$$

Where Y is the predicted response; X_i and X_j are the coded independent variables; β_0 is the intercept coefficient; β_i is the linear coefficient; β_{ii} is the squared coefficient, and β_{ij} is the interaction coefficient. Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (version 8.0, Stat-Ease, Inc., Minneapolis, USA).

Statistical analyses

Design-Expert 8.0, trial version was used for the analysis

Table 1: Factors and levels for BBD

Factors	Symbol	Coded levels		
		−1	0	1
Extraction time (min)	X_1	15	37.5	60
Ratio of liquid to solid (mL/g)	X_2	20	50	80
Extraction temperature (°C)	X_3	30	50	70
Methanol concentration	X_4	20	60	100

BBD: Box-Behnken statistical design; OD: Overall desirability

of variance (ANOVA) analysis of the experimental data obtained. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2, and the values of adjusted-R^2 of models were evaluated to check the model adequacies. The significance of each term in the equation is to estimate the goodness of fit in each case. The ANOVA tables were generated, and effect and the regression coefficients of individual linear, quadratic and interaction terms were determined. The *P* values of less than 0.05 were considered to be statistically significant. The regression coefficients were then used to make the statistical calculation to generate contour and dimensional maps from the regression models.

RESULTS AND DISCUSSION

Single-factor experiments

Optimizing UAE conditions should consider the interaction of different extraction factors and the linear relationship between response and variables. In order to reveal the complicated interaction and relationship, a statistical analysis method, BBD was selected to optimize UAE parameters. The OD, the geometric mean of the contents of 4 target compounds was used as a marker to evaluate the extraction efficiency.[29,30] Before BBD optimizing UAE parameters, a preliminary experiment has been performed. In the preliminary experiment, extraction factors including solvent type, particle size, ultrasonic power, temperature, time, methanol concentration and ratio of liquid to solid were studied.

The four different solvents (ethanol, methanol, acetonitrile and water) were tested under the same conditions: sample of 1.0 g, temperature of 70°C, solvent to solid ratio of 20 mL/g, ultrasound frequency 60 kHz, extraction time of 30 min and particle size of 50 meshes. The results showed that methanol gave the highest extraction yields, followed by ethanol, acetonitrile and water. The different extraction efficiencies of these solvents could be attributed to their different polarities and viscosities.

The effect of ultrasonic frequency on UAE was explored with solvent to solid ratio at 20 ml/g and other conditions fixed as mentioned previously (sample: 1.0 g, solvent: methanol, temperature: 70°C, extraction time: 30 min and particle size: 50 mesh). Results indicated an obvious

Table 2: BBD with the experimental values for extraction yield Y_1

Run	X_1	X_2	X_3	X_4	1[a]	2[a]	3[a]	4[a]	OD[b]
1	37.5 (0)	80 (1)	50 (0)	100 (1)	0.2781	4.72	0.0882	0.0602	0.63
2	37.5 (0)	80 (1)	50 (0)	20 (−1)	0.1986	4.19	0.0598	0.0530	0.37
3	60 (1)	20 (−1)	50 (0)	60 (0)	0.2671	4.55	0.0881	0.0538	0.58
4	37.5 (0)	80 (1)	70 (1)	60 (0)	0.2850	4.72	0.0896	0.0570	0.63
5	15 (-1)	20 (−1)	50 (0)	60 (0)	0.2594	4.25	0.0813	0.0528	0.51
6	37.5 (0)	20 (−1)	30 (−1)	60 (0)	0.2356	3.97	0.0809	0.0532	0.46
7	60 (1)	50 (0)	70 (1)	60 (0)	0.2675	4.48	0.0858	0.0539	0.56
8	37.5 (0)	50 (0)	70 (1)	20 (−1)	0.2204	3.88	0.0701	0.0424	0.35
9	37.5 (0)	20 (−1)	30 (−1)	60 (0)	0.2860	4.49	0.0867	0.0565	0.60
10	15 (-1)	80 (1)	50 (0)	60 (0)	0.2853	4.59	0.0855	0.0560	0.60
11	60 (-1)	50 (0)	50 (0)	100 (1)	0.2672	4.38	0.0779	0.0560	0.54
12	60 (1)	50 (0)	50 (0)	20 (−1)	0.1785	3.66	0.0591	0.0456	0.27
13	15(-1)	50 (0)	70 (1)	60 (0)	0.2704	4.40	0.0864	0.0549	0.56
14	37.5 (0)	50 (0)	50 (0)	60 (0)	0.2664	4.23	0.0793	0.0539	0.52
15	15 (-1)	50 (0)	30 (−1)	60 (0)	0.2656	4.28	0.0893	0.0538	0.55
16	37.5 (0)	50 (0)	50 (0)	60 (0)	0.2708	4.33	0.0812	0.0546	0.54
17	37.5 (0)	50 (0)	50 (0)	60 (0)	0.2688	4.30	0.0776	0.0536	0.52
18	37.5 (0)	20 (−1)	70 (1)	60 (0)	0.2768	4.58	0.0931	0.0584	0.63
19	60 (1)	80 (1)	50 (0)	60 (0)	0.2860	4.45	0.0805	0.0566	0.57
20	37.5 (0)	20 (−1)	50 (0)	100 (1)	0.2385	4.17	0.0726	0.0519	0.45
21	15 (−1)	50 (0)	50 (0)	20 (−1)	0.1659	3.59	0.0557	0.0457	0.24
22	15 (−1)	50 (0)	50 (0)	100 (1)	0.2578	4.28	0.0779	0.0549	0.52
23	37.5 (0)	50 (0)	30 (−1)	100 (1)	0.2341	3.89	0.0667	0.0511	0.40
24	37.5 (0)	20 (−1)	50 (0)	20 (−1)	0.1492	3.40	0.0512	0.0341	0.14
25	37.5 (0)	50 (0)	50 (0)	60 (0)	0.2898	4.35	0.0744	0.0563	0.54
26	37.5 (0)	50 (0)	50 (0)	60 (0)	0.3091	4.99	0.0725	0.0635	0.64
27	37.5 (0)	50 (0)	70 (1)	100 (1)	0.2591	4.35	0.0843	0.0568	0.55
28	37.5 (0)	50 (0)	30 (−1)	20 (−1)	0.1533	3.66	0.0517	0.0436	0.21
29	60 (1)	50 (0)	30 (−1)	60 (0)	0.2519	4.16	0.0801	0.0504	0.48

[a]Extraction yields (%)=weight of compounds extracted (g)/weight of dried sample (g)×100%; [b]Overall desirability; BBD: Box-Behnken statistical design

increase of extraction yields when the ultrasound frequency was increased from 40 kHz to 60 kHz. When the ultrasound frequency was increased from 60 kHz to 100 kHz, no significant differences between the extraction yields were detected ($P > 0.05$). Therefore, an ultrasound frequency of 60 kHz should be optimum to extract these four characteristic constituents.

Particles with the size 40, 50, 60 meshes were collected for the study. The result showed that there were no significant differences from 40 to 60 meshes for the extraction yields. Therefore, 50 meshes were selected for following experiments. Thus, the other four factors, extraction time, ratio of liquid to solid, extraction temperature, and methanol concentration were selected as BBD factors and the ultrasound frequency and particle size were set at 60 kHz and 50 mesh, respectively.

Statistical analysis and the model fitting

The effects of four process variables (i.e. extraction time [X_1], ratio of liquid to solid [X_2], extraction temperature [X_3] and methanol concentration [X_4]) were studied during the experimentation. An optimum process should be investigated in order to obtain high extraction yield. The results of 29 runs using BBD design are presented in Table 2 that includes the design, experimental values and the OD values. BBD with four factors and three levels, including five replicates at the center point, was used to fit a 2nd-order response surface in order to optimize the extraction conditions. The five center point runs were carried out to measure the process stability and inherent variability.

By applying the multiple regression analysis on the experimental data, the results of the BBD were fitted with a 2nd-order polynomial equation. Thus, a mathematical regression model for a total content fitted in the coded factors was given as following:

$$Y = 0.55 + (1.667 \times 10^{-3}) \times X_1 + 0.052 \times X_2 + 0.048 \times X_3 + 0.13 \times X_4 - 0.025 \times X_1 \times X_2 + 0.018 \times X_1 \times X_3 - (2.5 \times 10^{-3}) \times X_1 \times X_4 - 0.035 \times X_2 \times X_3 - 0.012 \times X_2 \times X_4 + (2.5 \times 10^{-3}) \times X_3 \times X_4 + 0.021 \times X_2^2 - (2.838 \times 10^{-3}) \times X_3^2 - 0.17 \times X_4^2 \quad (3)$$

where Y was the response, that was the OD of four phenolic compounds contents and X_1, X_2, X_3 and X_4 were the coded values of the test variables extraction time, ratio of liquid to solid, extraction temperature and methanol concentration, respectively. The significance of each coefficient was determined using P value, which is used as a tool to check the significance of each coefficient and the interaction strength between each independent variable.[31] If P value is the smaller it is the bigger the significance of the corresponding coefficient. The significance of the F value depends on the number of degrees of freedom in the model and is shown in the P value column (95% confidence level). In general, the effects lower than 0.05 are significant.

The ANOVA [Table 3] showed that this regression model was highly significant ($P < 0.0001$) with F value of 13.65. The F value of 1.05 for lack of fit implies that it is not significant comparing to the pure error. The fitness of the model was further confirmed by a satisfactory value of determination coefficient, which was calculated to be 0.9221,

Table 3: ANOVA for response surface quadratic model of extraction yield determined from BBD

Extraction yield (mg/g)	Sum of squares	DF	The mean square	*F* value	*P* value	Significant
Model	0.460	13.00	0.036	13.65	<0.0001	***
X_1	3.333×10^{-5}	1	3.333×10^{-5}	0.013	0.9117	
X_2	0.033	1	0.033	12.63	0.0029	**
X_3	0.028	1	0.028	10.70	0.0051	**
X_4	0.190	1	0.190	72.55	<0.0001	***
$X_1\times X_2$	2.500×10^{-3}	1	2.500×10^{-3}	0.95	0.3441	
$X_1\times X_3$	1.225×10^{-3}	1	1.225×10^{-3}	0.47	0.5045	
$X_1\times X_4$	2.500×10^{-5}	1	2.500×10^{-5}	9.546×10^{3}	0.9235	
$X_2\times X_3$	4.900×10^{-3}	1	4.900×10^{-3}	1.87	0.1915	
$X_2\times X_4$	6.250×10^{-4}	1	6.250×10^{-4}	0.24	0.6323	
$X_3\times X_4$	2.500×10^{-5}	1	2.500×10^{-5}	9.546×10^{3}	0.9235	
X_2^2	2.942×10^{-3}	1	2.942×10^{-3}	1.12	0.3060	
X_3^2	5.418×10^{-5}	1	5.418×10^{-5}	0.021	0.8876	
X_4^2	0.190	1	0.190	71.28	<0.0001	***
Residual	0.039	15	2.619×10^{-3}			
Lack of fit	0.029	11	2.655×10^{-3}	1.05	0.529	Not significant
Pure error	0.010	4	2.52×10^{-3}			
Cor total	0.050	28				

ANOVA: Analysis of variance; BBD: Box-Behnken statistical design; ** Significant at P<0.01,***Significant at P<0.001

Table 4: Fit statistics for the response values

Model parameter	Values
Standard deviation	0.0510
Mean	0.4900
Press	0.1600
R^2	0.9221
Adj R^2	0.8545
Pred R^2	0.6731
Adeq precision	12.314

indicating that 92.21% of the variability in the response could be predicted by the model [Table 3]. The value of the adjusted determination coefficient (adjusted $R^2 = 0.8545$) also confirmed that the model was highly significant. The "Pred R-Squared" of 0.6731 is in reasonable agreement with the "Adj R-Squared" of 0.8545 [Table 4]. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 12.314 indicates an adequate signal. This model can be used to navigate the design space. As shown in Table 3, the variable with the largest effect was the X_4 ($P < 0.0001$), followed by the other linear terms of X_2 and X_3 ($P < 0.01$). The other term coefficients were not significant ($P > 0.05$).

Analysis of response surfaces

The 3-D plot in Figure 2a showed the effects of extraction time (X_1) and ratio of liquid to solid (X_2) on extraction yield (Y) at fixed extraction temperature (50°C) and methanol concentration (60%). There was a rapid rise in extraction yield with an increase in extraction time (X_1) and ratio of liquid to solid (X_2). Figure 2b show the 3-D response surface plot at varying extraction time (X_1) and extraction temperature (X_3) at fixed the ratio of liquid to solid (50:1) and methanol concentration (60%). There was a slight upsurge in extraction yield (Y_1) with an increase in extraction time (X_1) and extraction temperature (X_3). Like Figure 2a-c showed the effects of extraction time (X_1) and methanol concentration (X_4) on extraction yield (Y) at fixed extraction temperature (50°C) and the ratio of liquid to solid (50:1). The extraction yield increased rapidly within the methanol concentration from 20% to 76.6%, but when beyond 76.6%, the extraction yield reached the plateau region where the yield was maximized and did not increase any more, and the yield increased slightly with the increase of the extraction temperature. In Figure 2d, when the 3-D response surface plot were developed for the extraction yield with varying ratio of liquid to solid (X_2) and extraction temperature (X_3) at fixed the extraction time (37.5 min) and methanol concentration (60%). It indicated that a rapid rise in extraction yield with an increase in the ratio of liquid to solid (X_2) and extraction temperature (X_3). In Figure 2e, when the 3-D response surface plot was developed for the extraction yield with varying ratio of liquid to solid (X_2) and methanol concentration (X_4) at fixed extraction temperature (50°C) and extraction time (37.5 min). It indicated methanol concentration (X_4) exhibited an important effect on the extraction yield, which did not continue to increase significantly until the methanol concentration was over 76.6%. And further increase in methanol concentration, results in slow decrease in the extraction yield. With respect to the ratio of liquid to solid (X_2), the influence of this parameter was not as significant as that of methanol concentration. As shown in this figure, extraction yield showed a slight rise with an increase in the ratio of liquid to solid (X_2). The 3-D response surface plot based on independent variables extraction temperature (X_3) and methanol concentration (X_4) are shown in Figure 2f while the other two independent variables, extraction time and ratio of liquid to solid were kept at 37.5 min and 50, respectively. It can be seen that the extraction yield increased with the increase of methanol concentration (X_4) from 20% to 76.6%, then dropped slightly from 76.6% to 100% and the yield increased very gently with the increase of the ratio of liquid to solid from 20 to 80.

Optimization of extracting parameters and validation of the model

By solving the regression equation (3) and analyzing 3-D plots, it can be concluded that the optimal extraction conditions from Forsythiae Fructus are extraction temperature 70°C, time 60 min, ratio of liquid to solid 20, and methanol concentration 76.6%. Among the four extraction parameters that have been studied, methanol concentration (X_4) was the most significant factor that affects the yield followed by the ratio of liquid to solid (X_2), extraction temperature (X_3) and extraction time (X_1) according to the regression coefficients significance of the quadratic polynomial model and gradient of slope in the 3-D response surface plot. A possible explanation is that the four characteristic compounds 1-4 possess different polarity and solubility; hence control of methanol concentration is very important to extract them simultaneously.

However, with the increase in the ratio of liquid to solid, the extraction yield of compounds 1-4 is down gradually, which is in agreement with previous results.[23] This phenomenon could be explained that some substances, which have dissolved in the extracting solvent, could increase the solubility of compounds 1-4. If the volume of extracting solvent was increased markedly, the concentrations of those substances dissolved in the extracting solvent would also decrease obviously, and the ability to increase the solubility of compounds 1-4 would also decrease distinctly, which results in a decreased extracting yield of compounds 1-4.

The predicted extraction yield (OD) that was given by the Design Expert software under the above conditions was

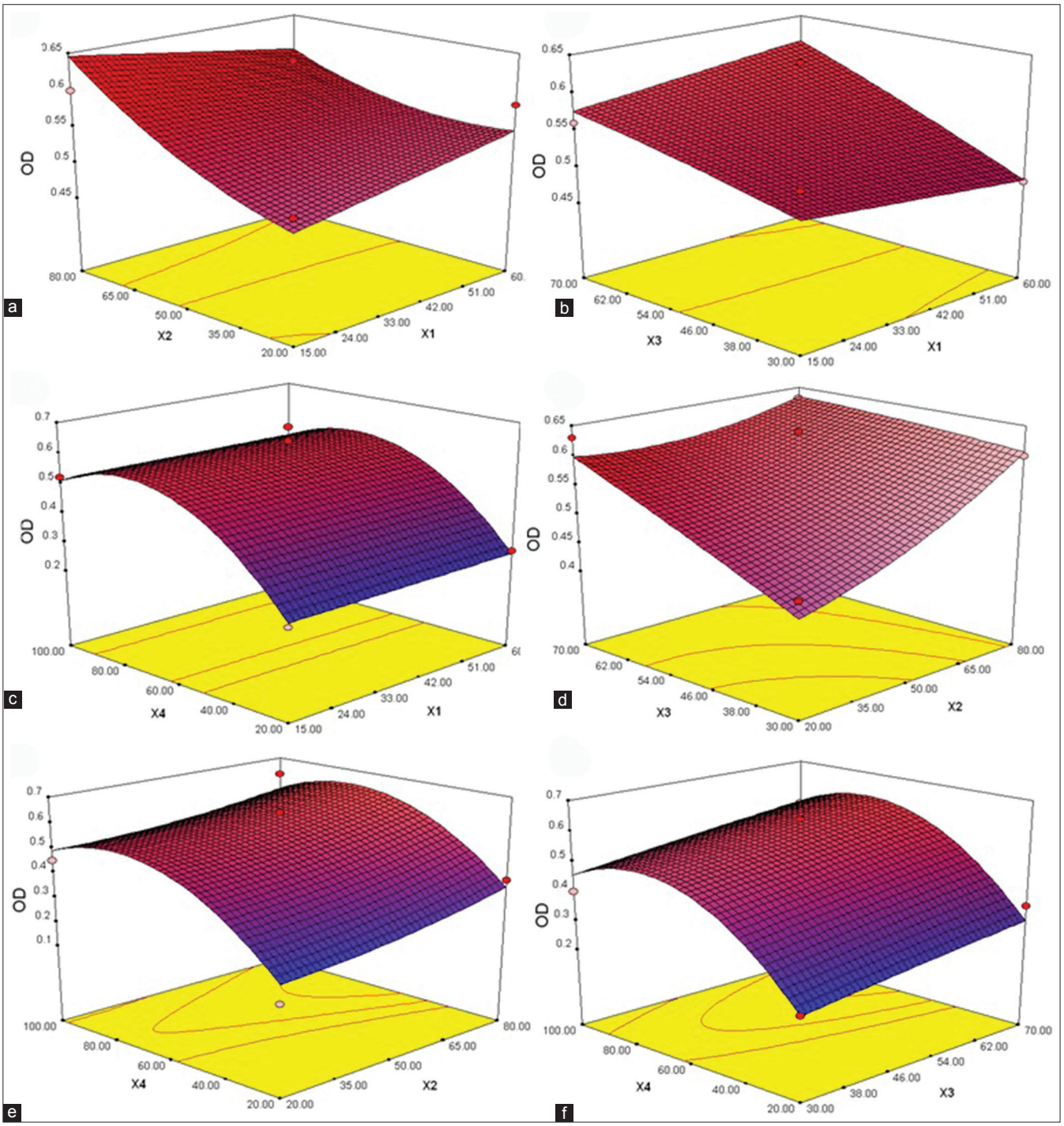

Figure 2: Response surface plots showing the predicted values of extraction yield: The effects of two variables on the response extraction yield (Y_1, mg/g), with the other two fixed at 0 level. (X_1: Extraction time, min; X_2: Ratio of liquid to solid, mL/g; X_3: Extraction temperature, °C; X_4: Methanol concentration)

0.672. The optimum extraction conditions were applied to 3 independent replicates to verify the prediction from the model. The mean experimental extraction yield of (+)-pinoresinol-β-glucoside, (1) forsythiaside, (2) phillyrin, (3) and phillygenin, (4) were 2.92 mg/g, 52.10 mg/g, 0.90 mg/g and 0.57 mg/g, respectively, namely OD of 0.670, which were in good agreement with the predicted value of the model equation, confirming that the response model was adequate for the optimization.

Besides, extraction temperature (30, 50, 70, and 90°C), extraction time (20, 40, 60, and 80 min), ratio of liquid to solid (10, 20, 30 and 40), and methanol concentration

(25%, 50%, 75% and 100%) were validated using univariate method. When one of the parameters, including temperature, time, ratio of liquid to solid and methanol concentration was optimized, the others were set at the predication optimization value (temperature 70°C, time 60 min, ratio of liquid to solid 20, and methanol concentration 76.6%). The results showed the optimization values of temperature, time and the ratio of liquid to solid were the same as the results of BBD [Figure 3a-c], but methanol concentration of univariate method was 75% [Figure 3d] because 75% ethanol concentration was a real experiment result rather than predicted value.

Comparison of UAE with ME and SE

The selection of an extraction method mainly depends on the advantages and disadvantages of the processes, such as extraction yield, production cost complexity, environmental friendliness, and safety. In general, ME and SE are the most frequently used traditional extraction methods. The results of extraction yields of UAE, ME and SE listed in Table 5 showed that ME obtained the highest yields of forsythiaside and phillygenin, and UAE got the highest yield of (+)-pinoresinol-β-glucoside and phillyrin. However, for total yield of these four characteristic constituents, ME and UAE had no significant differences ($P > 0.05$). Therefore, the UAE was more suitable for simultaneous extraction of (+)-pinoresinol-β-glucoside, forsythiaside, phillyrin, and phillygenin than ME and SE method, due to the fact that UAE can save a lot of time, reduce solvent consumption and bring the higher yield of total contents.

Application of UAE method to Forsythia samples coupled with HPLC for quality control

The developed UAE sample preparation method was then successfully applied to simultaneously determine the four characteristic components in 12 batches of samples obtained from different sources under the optimal UAE condition and quantified by HPLC, which was a validated and feasible method for evaluating and controlling quality of Forsythiae Fructus. The sample HPLC chromatography can be seen in Figure 4. A large variation of the contents of the four characteristic compounds was found among these samples from different origins and the harvest time. The percentage of forsythiaside ranging from 1.09% to 17.82% in Forsythiae Fructus was the highest, followed by (+)-pinoresinol-β-glucoside from 0.08% to 1.09%, phillyrin from 0.02% to 0.17% and phillygenin from 0.013% to 0.06%. The variation is possibly attributed to several factors, such as plant origins, harvesting time, storage conditions, etc.,[8,15] The variation in contents of these characteristic biological components may cause changes in clinical efficacy. To ensure the quality of Forsythiae Fructus extract, this suggests that each procedure involved should be standardized.

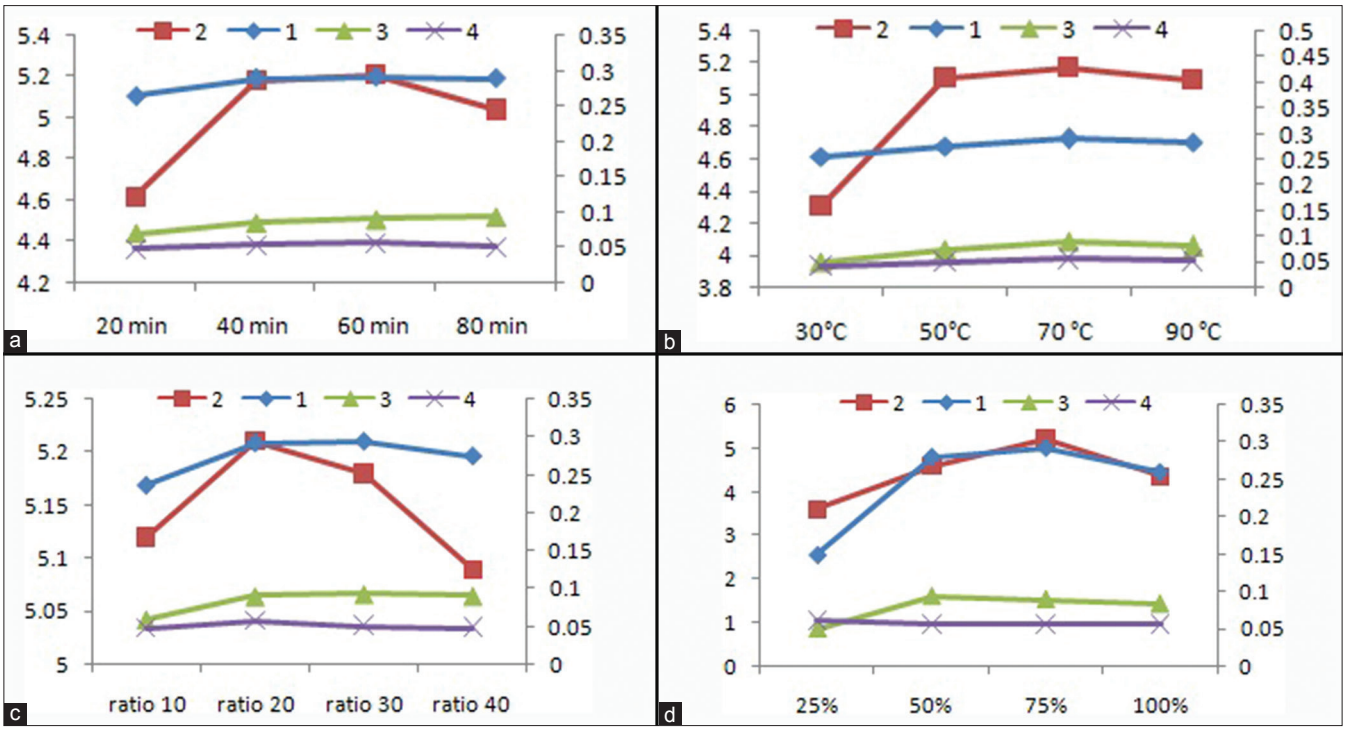

Figure 3: Validation of predication optimization values including extraction temperature, time, ratio of liquid to solid and methanol concentration. Condition: To determine one of the parameters, the others were set at the predication optimization value (extraction temperature 70°C, time 60 min, ratio of liquid to solid 20 and methanol concentration 76.6%)

Table 5: Comparison of UAE with other extraction methods

	Extraction yield (g/g×100%)				
	(+)-pinoresinol-β-glucoside	Forsythiaside	Phillyrin	Phillygenin	Total content
ME[a]	0.215	5.27	0.062	0.068	5.615
SE[b]	0.312	4.86	0.094	0.045	5.311
UAE[c]	0.292	5.21	0.090	0.057	5.649

[a]Conditions: 1.0 g sample 50 mesh, 20 ml of 76.6% methanol extracted three times, each for 24 h; [b]Conditions: 1.0 g sample 50 mesh, 20 ml of 76.6% methanol, 70°C, 6 h; [c]Conditions: 1.0 g sample 50 mesh, 20 ml of 76.6% methanol, 70°C, 1 h; ME: Maceration extraction; SE: Soxhlet extraction; UAE: Ultrasound-assisted extraction

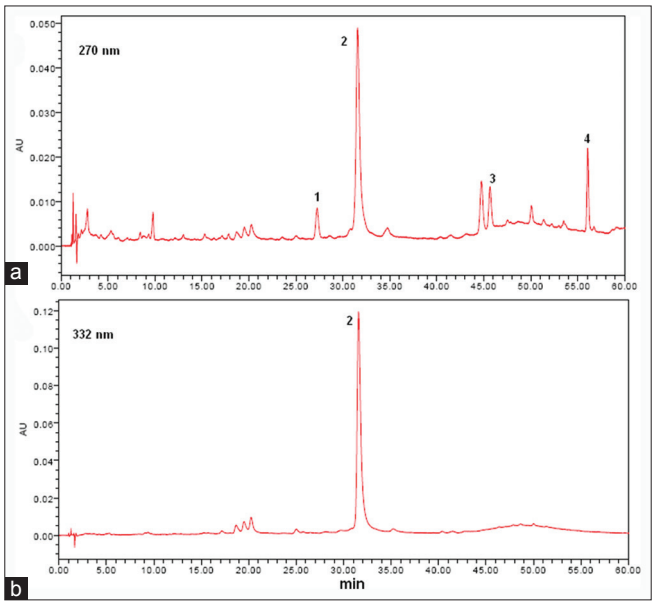

Figure 4: The high-performance liquid chromatography chromatograms of the samples. (a) crude extract by UAE from Forsythiae Fructus at 270 nm for (+)-pinoresinol-β-glucoside (1), phillyrin (3) and phillygenin (4), (b) crude extract by UAE from Forsythiae Fructus at 332 nm for forsythiaside (2)

Qualitative analysis of four characteristic constituents extracted by UAE based on quadrupole time-of-flight mass spectrometry

UAE method has been successfully applied to sample preparation for the quality control of Forsythiae Fructus. Being a high resolution mass spectrum, QTOF-MS could perform accurate mass measurement, which gives elemental composition of parent and fragment ions. Furthermore, the in-source collision induced dissociation technique was applied in our experiment to acquire sufficient structure information from QTOF-MS. The four targeted chromatographic peaks (1-4) were unambiguously identified as (+)-pinoresinol-β-glucoside, forsythiaside, phillyrin and phillygenin by comparison of their retention time, UV spectra and fragmentation behaviors with those of the reference. Peak 1 generated $[M-H]^-$ ions at m/z 519.1878 (calc. for $C_{26}H_{31}O_{11}$ 519.1866) indicating a molecular formula of $C_{26}H_{32}O_{11}$. The fragment ion at m/z 357.1313 $[M\text{-Glc-H}]^-$ was attributed to its aglycone of (+)-pinoresinol [Figure 5a]. Peak 2, forsythiaside, generated $[M-H]^-$ ions at m/z 623.1974 (calc. for $C_{29}H_{35}O_{15}$ 623.1976) indicating a molecular formula of $C_{29}H_{36}O_{15}$. A series of the diagnostic ions from the precursor ion at m/z 461.1633 (calc. for $C_{20}H_{29}O_{12}$ 461.4659), 477.1396 (calc. for $C_{23}H_{25}O_{11}$ 477.1397), 487.1459 (calc. for $C_{21}H_{27}O_{13}$ 487.1452), 443.1531 (calc. for $C_{20}H_{27}O_{11}$ 443.1553) and 315.1061 (calc. for $C_{14}H_{19}O_8$ 315.1080) were attributed to $[M\text{-Caffeoyl-H}]^-$, $[M\text{-Rha-H}]^-$, $[M\text{-phenylethanol-H}]^-$, $[M\text{-Caffeoyl-H}_2O\text{-H}]^-$ and $[M\text{-Caffeoyl-Rha-H}]^-$, respectively [Figure 5b]. Peak 3, phillyrin, gave $[M-H]^-$ ions at m/z 533.2042 (calc. for $C_{27}H_{33}O_{11}$ 533.2023) indicating a molecular formula of $C_{27}H_{33}O_{11}$. Two diagnostic ions from the pre-cursor ion at m/z 371.1461 (calc. for $C_{21}H_{23}O_6$ 371.1495) and 356.1200 (calc. for $C_{20}H_{20}O_6$ 356.1260) were observed, which can be identified as $[M\text{-Glc-H}]^-$ and $[M\text{-Glc-CH}_3\text{-H}]^-$, respectively [Figure 5c]. Peak 4 showed $[M-H]^-$ and $(2[M-H]-H)^-$ ions at 371.1461 (calc. for $C_{21}H_{23}O_6$ 371.1495) and 741.2953 (calc. for $C_{42}H_{45}O_{12}$ 741.2911) indicating a molecular formula of $C_{21}H_{24}O_6$. diagnostic ions $[M\text{-CH}_3\text{-H}]^-$ from the precursor ion at m/z 356.1218 (calc. for $C_{20}H_{20}O_6$ 356.1260) was observed, which can be identified as phillygenin [Figure 5d].

CONCLUSIONS

The UAE method reported here can offer an effective alternative for simultaneous extraction of (+)-pinoresinol-β-glucoside, forsythiaside, phillyrin, and phillygenin. It can be applied to sample preparation for the quality control of the Forsythiae Fructus. Additionally, it might serve as a promising industrial extraction protocol of the four biological characteristic compounds. Compared with ME and SE, UAE has been proved to be easy, efficient, and inexpensive method with low toxicity and high reproducibility. It is important to note that the methanol concentration was the most significant factor that affects the yield according to the regression coefficients significance of the quadratic polynomial model and gradient of slope in the 3-D response surface plot. The optimal UAE conditions found were: extraction temperature 70°C, time 60 min, ratio of liquid to solid 20, and methanol concentration 76.6%. The extraction yields of (+)-pinoresinol-β-glucoside, forsythiaside, phillyrin, and

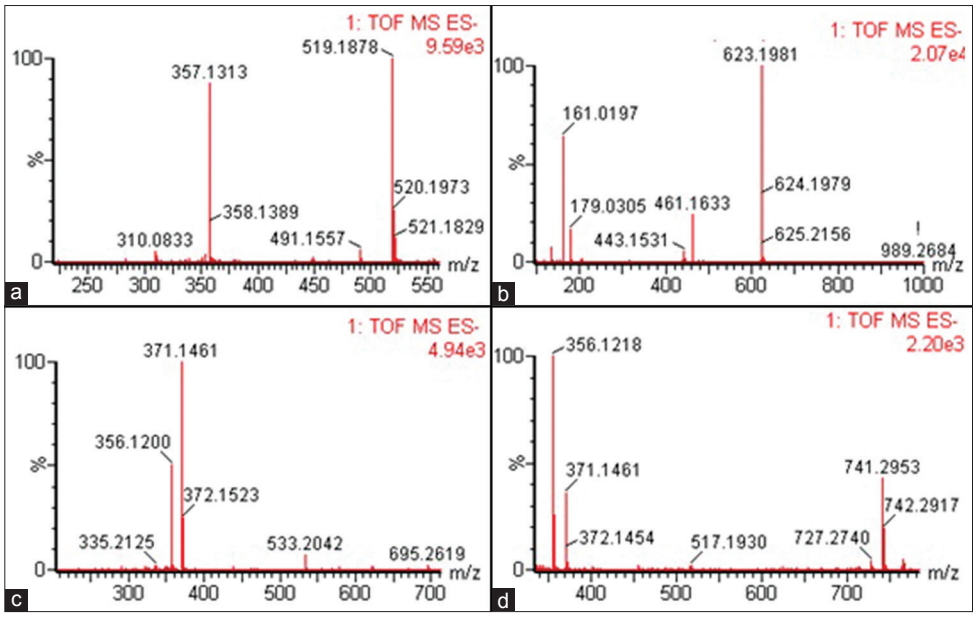

Figure 5: Mass spectra of 4 characteristic compounds obtained by UAE method (a), (+)-pinoresinol-*β*-glucoside, (b) forsythiaside, (c) phillyrin, (d) phillygenin

phillygenin were 2.92 mg/g, 52.10 mg/g, 0.90 mg/g and 0.57 mg/g, respectively. The results indicated that UAE may facilitate a deeper understanding of the extract of active constituents in Forsythiae Fructus from the raw material to its extract for providing theoretical references.

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