

Optimizing Conditions for Ultrasound-Assisted Extraction of the Betulinic Acid, Oleanolic Acid, and Ursolic Acid from the Jujube using Response Surface Methodology (RSM)

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Abstract. Pentacyclic triterpenic acids have potential effects in treating human diseases. Thus, it seems necessary to have an effective method to extract and separate triterpenic acids from plants and fruits such as jujube. To this end, this study optimized ultrasound-assisted extraction of Betulinic acid (BA), Oleanolic acid (OA), and Ursolic acid (UA) from Iranian jujube using response surface methodology (RSM) and quantified by high-performance liquid chromatography (HPLC). Box-Behnken Design (BBD) was used to model the response surface. The selected independent variables were ultrasonic bath temperature (T), sonication time (θ), and liquid to solid ratio (α). The P-value and R-squared (R^2) for all extraction efficiencies indicated a good correlation between the experimental results and those predicted by the quadratic model. The analysis of variance (ANOVA) results showed the significant impact of linear coefficients (T, θ , α), quadratic coefficients (T^2 , θ^2 , α^2), and interaction coefficients (T θ , T α , $\theta\alpha$) of the model on the extraction of three triterpenic acids. The predicted optimal temperature, sonication time, and liquid to solid ratio were 40.34 °C, 34.63 min, and 14.85 mL/g. The maximum yields for BA, OA, and UA were 304.14, 170.61, and 195.23 $\mu\text{g/g}$, respectively. Extraction was carried out by the calculated rounded up optimal values of T=40 °C, θ =35 min, and α =15 mL/g. The extraction efficiencies for BA, OA, and UA were 303.83 \pm 0.85, 169.52 \pm 0.86, and 195.84 \pm 0.75 $\mu\text{g/g}$, respectively. These results were comparable to those calculated under model-optimized conditions, indicating the accuracy of our model.

Keywords: Betulinic acid; oleanolic acid; ursolic acid; ultrasound-assisted extraction; Box-Behnken design.

Resumen. Los ácidos triterpénicos pentacíclicos tienen potencialmente efectos en el tratamiento de enfermedades humanas. Por ello es necesario disponer de un método eficaz para extraer y separar los ácidos triterpénicos de plantas y frutos como el jujube (*Ziziphus jujuba*). Con este fin, en este estudio se optimizó la extracción asistida por ultrasonido de los ácidos betulínico (BA), oleanólico (OA) y ursólico (UA) del jujube iraní utilizando la metodología de superficie de respuesta (RSM) y cuantificada por cromatografía líquida de alta resolución (HPLC). Se utilizó el diseño Box-Behnken Design (BBD) para modelar la superficie de respuesta. Las variables independientes seleccionadas fueron la temperatura del baño ultrasónico (T), el tiempo de sonicación (θ) y la proporción de líquido a sólido (α). El valor P y R-cuadrática (R^2) para todas las eficiencias de extracción indicaron una buena correlación entre los resultados experimentales y los predichos por el modelo cuadrático. Los resultados del análisis de varianza (ANOVA) mostraron el impacto significativo de los coeficientes lineales (T, θ , α), los coeficientes cuadráticos (T^2 , θ^2 , α^2) y los coeficientes de interacción (T θ , T α , $\theta\alpha$) del modelo en la extracción de los tres ácidos triterpénicos. La temperatura óptima predicha, el tiempo de sonicación y la proporción de líquido a sólido fueron 40.34 °C, 34.63 min y 14.85 ml/g. Los rendimientos máximos para BA, OA y UA fueron 304.14, 170.61 y 195.23 $\mu\text{g/g}$, respectivamente. La extracción se llevó a cabo mediante los valores óptimos redondeados,

T = 40 °C, θ = 35 min y α = 15 ml/g. Las eficiencias de extracción para BA, OA y UA fueron $303.83 \pm 0,85$, $169.52 \pm 0,86$ y $195.84 \pm 0,75$ $\mu\text{g/g}$, respectivamente. Estos resultados fueron comparables a los calculados en condiciones del modelo optimizado, lo que indica la precisión del modelo propuesto.

Palabras clave: Ácido betulínico; ácido oleanólico; ácido ursólico; extracción asistida por ultrasonido; diseño Box-Behnken.

Introduction

Jujube is known as a natural medicine because of its bioactive components such as triterpenic acids, flavonoids, polysaccharides, and alkaloids. Pharmacologically, jujube has a unique effect on inflammation, cancer insomnia, and neuroprotection [1-3]. Recent studies have shown some biological activity of pentacyclic triterpenic acids. Betulinic acid (BA) has anti-HIV-1 (human immunodeficiency virus) activity and is also a potent inhibitor of tumor proliferation and angiogenesis [4,5]. Oleanolic acid (OA) and ursolic acid (UA) are anti-inflammatory, diuretic, anti-tumor, and hepatoprotective drugs [6-8].

There were recently some challenges in studying the methods for improved extractions of triterpenic acids from jujube [9,10]. Ultrasonic-assisted extraction is known as a green, efficient, and productive method for successful extraction of triterpenic acids from plants and Chinese jujube [10-17]. Several parameters such as temperature, sonication time, liquid to solid ratio, and solvent must be also investigated to achieve an optimal result and study the simultaneous effects of these parameters. Statistical methods such as response surface methodology (RSM) can help optimize such a multi-factor process [9-10,18].

On the other hand, there are reports on various chromatographic methods used to determine triterpenes in plants [20]. High-performance thin-layer chromatography [21], and high-performance liquid chromatography (HPLC), because of the fast and accurate separating and evaluating use, are the most powerful and suitable methods [22-25]. The literature shows optimized extraction of total triterpenoids from Chinese jujube through UV spectrophotometry determination [10]. Another study simultaneously determined BA, OA, and UA from Chinese jujube by HPLC [24]. However, there is no report on precise optimal extraction conditions of these three triterpenic acids individually from Iranian jujube with accurate determination.

This study optimizes the ultra-assisted extraction conditions of BA, OA, and UA from Iranian jujube using RSM and simultaneously determines these acids by the HPLC-UV method. The effective parameters include temperature, sonication time, and the liquid to solid ratio.

Experimental

Material and methods

Samples

Dried jujube was purchased from northeastern Iran. The samples were milled and passed through a 35-mesh sieve and stored at 4 °C.

Reagents and standards

BA, OA, and UA were purchased from Sigma Aldrich. Methanol used for liquid chromatography, sodium perchlorate, and anthracene were obtained from Merck Millipore. Ethanol (purity: 96 %) was purchased from Kimia Alcohol Zanjan Company. HPLC grade water was prepared in our laboratory using Aquaplust Ultra Water Purification System. Nylon filters (0.45 μm) were purchased from Merck Millipore.

Standard solutions preparation

Standard stock solutions of BA, OA, UA, and anthracene, as internal standard, were prepared by dissolving an appropriate amount of these compounds in ethanol 96 % to reach a final concentration of 0.45, 0.2, and 0.15

respectively for anthracene, BA, and OA and UA. Each stock solution was serially diluted from a concentration of 15.79 to 24.82, 9.56 to 15.03, and 9.22 to 16.92 $\mu\text{g/mL}$ respectively for BA, OA, and UA. All the solution were prepared with the 1.8 $\mu\text{g/mL}$ anthracene as an internal standard.

Sample extraction and pretreatment

For sample extraction, 666, 800, and 1000 mg of the sample were precisely weighed and transferred to a 50 mL centrifuge tube. Ten milliliters of the extraction solvent (ethanol 96 % v/v) were added to obtain different liquid to solid ratios. Moreover, the samples were extracted in 3 replicates at different times and temperatures by the ultrasonic cleaner (D.S.A. China, frequency: 40 kHz.) The extraction solutions were then passed through 0.45 μm nylon filters and transferred to a 10.0 mL measuring flask. Finally, the volume was calibrated using ethanol 96 % v/v in the presence of 1.8 $\mu\text{g/mL}$ anthracene as an internal standard.

High-performance liquid chromatography systems

An HPLC (Kenuar Azura) equipped with a quaternary pump, degasser, an automated sample injector, a column compartment, and a single wavelength UV detector was used to analyze the samples and standards. The analytical column was MN Nucleodur C18 gravity (250 mm \times 4.6 mm, 5 μm) coupled to an MN C18 guard column (10 mm \times 4.6 mm, 5 μm). Before use, the mobile phase contained methanol and sodium perchlorate buffer 0.2 % w/v (88:12, v/v), which was passed through a 0.45 μm nylon filter. The flow rate was 0.5 mL/min, and the detection wavelength was set at 210 nm. The injection volume of 50 μL at a column temperature of 22 $^{\circ}\text{C}$.

Precision and recovery

The intra-day and inter-day variations to determine the precision of the developed method were investigated by determining sample extraction in 5 replicates during a single day and three consecutive days under the optimal extraction conditions. BA, OA, and UA variations (in $\mu\text{g/g}$) were considered precision measures, expressed as relative standard deviations.

A recovery test was used to evaluate the accuracy of this method. Three aliquots of the standards were added to the treated sample solution under the optimal extraction conditions. The final concentrations of the standards were 1.0, 2.0, and 3.0 $\mu\text{g/mL}$ of BA, OA, and UA in the presence of 1.8 $\mu\text{g/mL}$ anthracene as an internal standard. Then, all the solutions were injected three times.

The recovery was calculated as follows:

$$\text{Recovery (\%)} = (A-B)/C \times 100\%$$

where A represents the detected concentration in the spiked sample, B the concentration of the sample without the added standards, and C is the added amount of the standards.

Experiment design

The extraction efficiency of the triterpenic acids from jujube based on RSM was conducted for experimental design. According to the ANOVA for the effective parameters, a three-level-three-factor Box-Behnken Design (BBD) (Design-Expert V11.1.1.0, Stat-Ease Inc.) was used for experimental design, model building, and data interpretation. The ultrasonic bath temperature (T), sonication time (θ), and liquid to solid ratio (α) were selected as independent variables (see Table 1). The amounts of BA, OA, and UA were chosen as dependent parameters. A total of 15 experimental runs were carried out with three replicates as the center points.

Table 1. Uncoded and coded levels of the independent variables of the extraction process.

Independent variables	symbol	Coded level		
		-1	0	1
Temperature ($^{\circ}\text{C}$)	T	40	50	60
sonication time(min)	θ	25	30	35
Solid to liquid ratio(ml/g)	α	10	12.5	15

Results and disussion

HPLC conditions and simultaneous quantification of BA, OA, and UA

A detection wavelength of 210 nm was chosen because of better absorption and sensitivity for BA, OA, UA. Several chromatographic parameters were investigated to obtain a suitable separation between BA, especially OA and UA. Sodium perchlorate (NaClO₄) is a more effective anionic ion-pairing reagent than water or ammonium acetate in the acidic pH of the mobile phase.

In this work, optimal conditions for the mobile phase were sodium perchlorate buffer 0.2 % w/v, and methanol (12:88, v/v) in terms of good shape and separation. The flow rate and temperature of the column are crucial and set at 0.5 mL/min and 22 °C, respectively.

Anthracene was chosen as an internal standard because of no interference with other peaks in the standard and sample chromatograms.

The sequence of the three triterpenic acid peaks in the chromatogram obtained from HPLC was as follows: BA, OA, and UA. The average retention time in repeated injections of the standard solutions was 16.9, 32.2, 36.3, and 38.1 min for anthracene, BA, OA, and UA, respectively.

The areas under each acid peaks in the standard and sample solutions were normalized by the internal standard peak area. The amounts of the triterpenic acids were quantified by the respective linearity equations. Table 2 reports fifteen sets of extraction conditions and the quantity of each acid in µg/g.

Table 2. The experimental results.

Run	(T) Temperature (°C)	(θ) Time (min)	(α) Liquid to solid ratio (ml/g)	The actual content (µg/g)		
				BA	OA	UA
1	60	30	15	245.06	151.85	153.96
2	40	25	12.5	226.09	126.21	167.79
3	40	30	15	289.1	162.07	189.19
4	50	25	10	228.43	123.12	140.78
5	50	35	10	200.35	106.96	124.88
6	40	30	10	220.09	120.09	130.52
7	40	35	12.5	257.68	143.93	168.56
8	60	35	12.5	214.97	121.6	165.03
9	50	30	12.5	243.71	133.66	165.39
10	60	30	10	236.07	124.67	149.84
11	50	30	12.5	242.33	134.76	164.19
12	50	35	15	270.4	155.49	179.98
13	50	30	12.5	244.39	135.59	168.63
14	60	25	12.5	241.82	143.65	150.96
15	50	25	15	237.75	143.98	147.79

Linearity, repeatability, and recovery

The calibration graphs for BA, OA, and UA were plotted from the normalized peak areas of the standard solutions as the response (Y) versus concentration (X). The correlation coefficients of all calibration curves were larger than 0.99 (see Table 3). The ANOVA for linear regressions (BA, OA, and UA calibration curves) were performed to validate the calibration models, and the results are summarized in Table 4. The ANOVA results show the P-value for the significance of BA, OA, and UA regressions and the lack of fit less

than 0.05 at a confidence interval of 95 %. Therefore, the null hypothesis regarding the significance of the regression is rejected, confirming the significance of the model.

Table 3. Summarized data of validation method.

Triterpenic acids	Linearity equation	LOD $\mu\text{g/ml}$	LOQ $\mu\text{g/ml}$	Correlation coefficient	Repeatability (RSD%)		Accuracy	
					Intra-day	Inter-day	Average recovery%	RSD %
Betulinic acid	$y = 21.746x + 121.53$	3.28	9.94	0.9937	1.52	2.74	96.18	1.51
Oleanolic acid	$y = 54.807x - 44.152$	2.18	6.6	0.9925	1.81	2.2	104.36	2.67
Ursolic acid	$y = 47.004x - 37.31$	1.04	3.15	0.9986	1.8	2.4	97.77	2.12

Table 4. ANOVA for linear regression of BA, OA, and UA with the lack of fit test.

BA						
Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	1	48147.3	48147.3	48147.3	1244.03	0.000
Conc	1	48147.3	48147.3	48147.3	1244.03	0.000
Error	8	309.6	309.6	38.7		
Lack-of-Fit	3	303.6	303.6	101.2	84.09	0.000
Pure Error	5	6.0	6.0	1.2		
Total	9	48457.0				
OA						
Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	1	112133	112133	112133	1031.96	0.000
Conc	1	112133	112133	112133	1031.96	0.000
Error	8	869	869	109		
Lack-of-Fit	3	849	849	283	70.03	0.000
Pure Error	5	20	20	4		
Total	9	113002				
UA						
Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	1	157334	157334	157334	4905.96	0.000
Conc	1	157334	157334	157334	4905.96	0.000
Error	8	257	257	32		
Lack-of-Fit	3	214	214	71	8.41	0.021
Pure Error	5	42	42	8		
Total	9	157591				

The relative standard deviations of the intra-day, inter-day precision tests, and average recovery rates were less than 2.8 %. The limit of detection (LOD) and limit of quantification (LOQ) for these three triterpenic acids were estimated using the new IUPAC definition [26] in the range of 1.04–3.28 and 3.15–9.94 $\mu\text{g/mL}$, respectively. Table 3 summarizes the results of method validation.

Single factor tests

Many factors such as extraction solvent, extraction time, liquid to solid ratio, and ultrasonic bath temperature could affect the ultrasound-assisted extraction yield of BA, OA, and UA from jujube [9, 10]. In these experiments, the active factors influencing the yield of BA, OA, and UA and their effects were first screened. Temperature (40–60 °C), sonication time (25–35 min), and liquid to solid ratio (10–15 mL/g) were selected and studied using a single factor. The content of the triterpenic acids was investigated for each factor while other factors were kept constant. Ethanol (96 % v/v) was used as the extraction solvent in all experiments. The first factor was the appropriate temperature. The extraction yields for each triterpenic acid were compared at different temperatures (40, 50, and 60 °C) at a constant sonication time (30 min), and liquid to solid ratio (12.5 mL/g). The results showed a maximum extraction yield at the lowest temperature of 40 °C. The second factor was the time required for ultrasonic extraction. The extraction yields of BA, OA, and UA were compared after ultrasonication for 25, 30, and 35 min at a constant temperature of 40 °C. The results indicated a maximum extraction efficiency after 35 min. The last factor investigated was the liquid to solid ratio. The liquid to solid ratios of 10, 12.5, and 15 mL/g were utilized at a constant ultrasonic extraction time of 35 min and a bath temperature of 40 °C. The extraction efficiency increased by increasing the liquid/solid ratio from 10 to 15 mL/g. The single factor tests indicated the desirable selected ranges for these extraction parameters in RSM experiments.

Fitting the response surface models.

According to the experimental design, 15 experiments were performed with 3 replicates (see Table 2). The extraction efficiencies of BA, OA, and UA (E_{BA} , E_{OA} , E_{UA}) were related to the following polynomials equations through multiple regression analysis of experimental data

$$E_{BA} = 243.48 - 6.88T + 1.16\theta + 19.67\alpha - 14.61T\theta + 15.18\theta\alpha - 15.00T\alpha + 2.51T^2 - 10.84\theta^2 + 1.60\alpha^2$$

$$E_{OA} = 134.67 - 1.32T - 1.12\theta + 17.32\alpha - 9.94T\theta + 6.92\theta\alpha - 3.70T\alpha + 3.23T^2 - 4.05\theta^2 + 1.77\alpha^2$$

$$E_{UA} = 166.07 - 4.53T + 3.89\theta + 15.61\alpha + 3.32T\theta + 12.02\theta\alpha - 13.64T\alpha + 2.27T^2 - 5.25\theta^2 - 12.46\alpha^2$$

Statistics

Table 5 summarizes the ANOVA results for BA, OA, and UA. As shown, P-values larger than 0.05 for the lack of fit show their insignificance, so the models were adequate to be predictive.

The excellent values of R^2 ($R^2 > 0.99$) for all responses indicate satisfactory quadratic models. R^2 , R^2 -adj (adjusted R^2), and R^2 -pred (predicted R^2) for BA, OA, UA in Table 6 are closer to each other, indicating a better correlation between the observed and predictive values.

Moreover, for each term of the models, P-value < 0.05 would imply a significant effect on the respective extraction efficiencies. Therefore, all coefficients of the equations affect the extraction yields of the three triterpenic acids. A large F-value and a small P-value would also imply a more significant effect on the respective response variable. Thus, the linear coefficient of the liquid to solid ratio (α) and interaction coefficients ($T\alpha$, $\theta\alpha$) have the most significant effects on the extraction yields of the three triterpenic acids (p-value < 0.0001).

Finally, a larger Adeq Precision (adequate precision > 4) in all models indicates a statistically credible and reliable quadratic model for the extraction yields of BA, OA, and UA.

Table 5. Analysis of variance (ANOVA) for the response surface quadratic model.

Triterpenic acid	BA		OA		UA	
	F-value	p-value	F-value	p-value	F-value	p-value
Model	1429.1	< 0.0001	900.72	< 0.0001	178.65	< 0.0001
Temperature(T1)	731.54	< 0.0001	35.28	0.0019	61.62	0.0005
Sonication time (θ)	20.94	0.006	25.66	0.0039	45.41	0.0011
Liquid to solid ratio (α)	5983	< 0.0001	6107.73	< 0.0001	730.81	< 0.0001
T θ	1649.89	< 0.0001	1006.49	< 0.0001	16.57	0.0096
T α	1740.53	< 0.0001	139.39	< 0.0001	278.81	< 0.0001
$\theta\alpha$	1782.16	< 0.0001	487.21	< 0.0001	216.68	< 0.0001
T ²	44.8	0.0011	98.05	0.0002	7.11	0.0445
θ^2	839	< 0.0001	154.35	< 0.0001	38.18	0.0016
α^2	18.26	0.0079	29.44	0.0029	214.82	< 0.0001
Lack of Fit	0.1161	0.9429	0.0319	0.9902	0.1764	0.9043
Adeq Precision	150.84		107.42		48.77	

Table 6. The values of R², R²-adj, and R²-pred for BA, OA, UA

Triterpenic acids	R ²	R ² -adj	R ² -pred
Betulinic acid	0.9996	0.9989	0.9983
Oleanolic acid	0.9994	0.9983	0.9982
Ursolic acid	0.9969	0.9913	0.9841

Optimization

As a powerful tool, the desirability approach can be used in multi-response systems. The combined application of RSM and desirability function, called desirability optimization methodology (DOM), has been successfully used to determine the optimal conditions. Optimization can be used to maximize, minimize, or obtain the target response value. The desirability functions range from 0 to 1, so 1 corresponds to optimal performance for studied parameters [27]. All responses in this study were maximized as the optimal solutions by implementing DOM. The optimum values of the extraction parameters were ultrasonic bath temperature: 40.34°C, sonication time: 34.63 min, and liquid to solid ratio: 14.85 mL/g. The maximum yields for BA, OA, UA were 304.14, 170.61, and 195.23 $\mu\text{g/g}$, respectively. Extraction was performed to validate the predicted results by the rounded-up optimal values of T=40 °C, θ =35 min, and α =15 mL/g. The obtained extraction efficiencies for BA, OA, UA were 303.83 \pm 0.85, 169.52 \pm 0.86, and 195.84 \pm 0.75 $\mu\text{g/g}$, respectively. Comparing the experimental and model optimal conditions indicates that the model was satisfactory and accurate.

The counterplots in Fig. 1 show the optimization zone with the interaction effects between temperature (T) and sonication time (θ) on BA (a), OA(b), and UA(c) yields.

More deformation of iso-space lines indicates the more significant interaction between the parameters so that the extraction yields of BA, OA, and UA were higher at low extraction temperatures and longer extraction times.

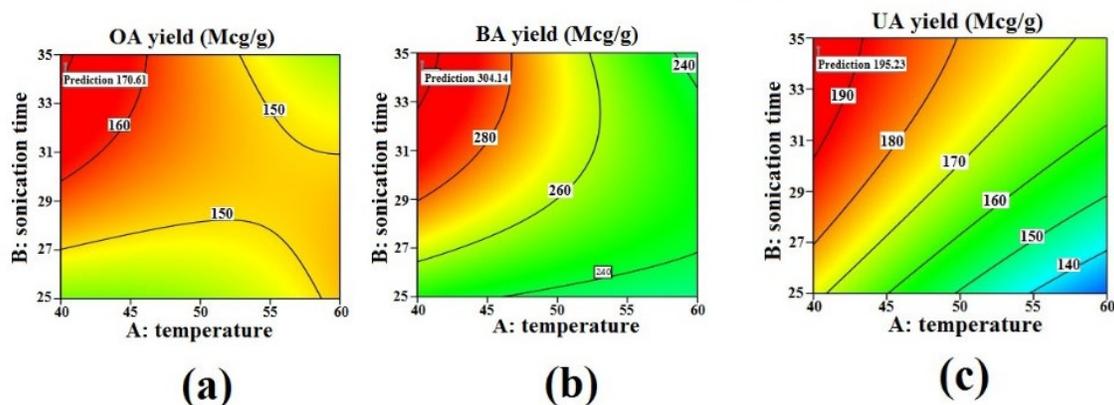


Fig. 1. Counterplots of the interaction effects between temperature (T) and sonication time (θ) on BA (a), OA (b), UA (c) yields at a constant liquid/solid ratio of 14.85 mL/g.

Conclusions

Response surface methodology was successfully employed to optimize ultrasound-assisted extraction of the three triterpenic acids individually from Iranian jujube. The ANOVA results indicated the significance of all three parameters including extraction temperature, sonication time, and liquid to solid ratio.

This is the first report on optimizing the conditions of ultra-assisted extraction of BA, OA, and UA from jujube.

The optimal conditions obtained from the model were validated by the rounded-up optimal conditions. The obtained extraction yields were comparatively accurate with the model-optimized values. The results showed that the model was well fitted with the experimental values.

The analytical HPLC-UV method was also accurately used for simultaneous determination of BA, OA, and UA in the ethanolic extract of jujube at a detection wavelength of 210 nm where the mobile phase was methanol: sodium perchlorate buffer 0.2 % w/v-methanol (88:12, v/v) in the MN Nucleodur gravity C18 (250 mm \times 4.6 mm, 5 μ m) column. The relative standard deviations for precise intra-day and inter-day determination of these three triterpenic acids were less than 2.8 %. The average recovery rates of BA, OA, and UA were 96.18 %, 104.36 %, and 97.77 %, respectively. These results provide validated data for modeling.

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