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Research Article

Optimized Ultrasonic-assisted Extraction of Flavonoids from *Osmanthus fragrans* Lour. Residues

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Abstract: The extraction of flavonoids from *Osmanthus fragrans* residues was optimized using ultrasonic device. The reaction parameters studied were the ethanol concentration (60-100%), the extraction time (1.5-2.5 h) and temperature (70-90°C) using response surface methodology based on Box-Behnken design. A mathematical model was developed to show the effects of each variable and their combinatorial interactions on extraction yield of flavonoids. A high coefficient of determination (R^2 = 99.57%) indicated good agreement between the experimental and predicted values of extraction yield. The optimal values of the variables were as the followings: ethanol concentration, 82%; extraction time, 2.1 h; and temperature 82°C. The ultrasound-assisted extractions supported higher extraction yields as compared to traditional extraction and could be recommended as an alternative method for extraction of flavonoids compounds from *O. fragrans* residue.

Keywords: Box-Behnken design, flavonoids, *Osmanthus fragrans*, response surface methodology

INTRODUCTION

Osmanthus fragrans Lour. is an evergreen tree, which is widely distributed in China, Japan and the Himalayas. *O. fragrans* is regard as one of the most famous traditional flowers of China and is widely cultivated as an ornamental plant today (Wang *et al*., 2006; Tsai *et al*., 2007). Its flower is especially valuable as an additive for food, tea and other beverages in the Far East. Moreover, the essential oil of the flower is considered as excellent natural essences, which is only used in the most expensive perfumes and cosmetics (Wang *et al*., 2006; Leffingwell, 2002). Besides, *O. fragrans* has a number of pharmacological effects such as anti-inflammation, anti-oxidation, anti-tussive, nitric oxide scavenging, nitric oxide-suppressing and neuroprotection (Deng *et al*., 2004; Lee *et al*., 2007; Wu *et al*., 2009).

There is a growing demand in the development of essential oil for use in the medicine and cosmetics industry. Therefore, the plants which produce essential oil of the *O. fragrans* in China have increased year by year. The extraction process of essential oil is known to generate large quantities of *O. fragrans* residue, which retains high levels of flavonoids. The efficient utilization of these flavonoids compounds is of great importance not only for minimizing environment impact but also for higher profitability.

Recently, ultrasound-assisted extraction (UAE) has drawn much wider attention, for it can more easily be scaled up for commercial production (Ma *et al*., 2012). And the UAE has been described to be an alternative to conventional extraction methods due to its several advantages such as higher reproducibility, shorter extraction time and less solvent consumption (Jiao and Zuo, 2009; Zuo *et al*., 2004). Response surface methodology (RSM) is a very useful statistical tool, which was first introduced by Box and Wilson (1951). It has been reported that RSM can be used to optimize the total flavonoid compound from many medicinal plants (Liu *et al*., 2010). In this study, ultrasonic device was applied to help extraction of flavonoids from *O. fragrans*. The effects of several experimental parameters, such as ethanol concentration, extraction time and temperature, on the extraction efficiency of flavonoids from *O. fragrans* were optimized by RSM.

MATERIALS AND METHODS

Materials: The samples were by-products of the essential oil extraction process of *O. Fragrans* and provided by TianYuan Medicinal Oil Refinery (JiAn, China). The dried *O. fragrans* residues were milled to about 250 μm and stored at a desiccator at room temperature. Rutin was purchased from Sigma Chemicals Co. All other chemical reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. The water used in the experiments was double distilled.

Ultrasound-assisted extraction of flavonoids: For the routine extraction of flavonoids, 5.0 g *O. fragrans*

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Table 1: Factors and levels tested for the designed experiment

	Coded levels		
Independent variables	-1	0	1
Ethanol concentration (X_1, %)	60	80	100
Extraction time (X_2, h)	1.5	2.0	2.5
Temperature (X_3, °C)	70	80	90

residues powder was placed into a 250 mL conical flask, soaked with a series of aqueous EtOH solution (0-100%) with different ratios of solid-liquid (g/mL) (1:10 to 1:50) for 30 min and then placed in the temperature-controlled ultrasonic device (SY360, Ninson Ultrasonic Instrument Company, Shanghai, China) at 40 kHz for a given time (0.5 to 3.0 h), while the temperature was controlled from 40 to 90°C. The extract was filtered and the filtrate was collected for the determination of the flavonoids.

Traditional extraction methods of flavonoids: Enzymolysis extraction (EE) was conducted in a water bath at 45°C. 5.0 g drug powder were placed into a 250 mL glass flask with 95 mL 50% (v/v) aqueous ethanol and 5 mL 0.1% cellulase and extracted for 4 h.

Mechanical shaking extraction (MSE) with 100 mL 50% (v/v) aqueous ethanol was performed on 5.0 g drug powder, placed in a shaking incubator for 8h.

Repeated Freezing and Thawing Extraction (RFTE) was conducted in an refrigerator. 5 g drug powder were place into a 250 glass flask with 100 mL 50% (v/v) aqueous ethanol, extracted utilizing freeze-thaw for 6 h.

Experimental design: In this study, response surface methodology (RSM) and Box-Behnken experimental design (BBD) with three factors and three levels was chosen to optimize and investigate the influence of process variables such as ethanol concentration (0-100 %), extraction time (0.5-3.0 h) and temperature (40-90°C) on the extraction yield of flavonoids from *O. fragrans* residues. All factors and levels tested were reported in Table 1. The complete design consisted of 17 experimental points including five replications of the centre points. All the experiments were done in triplicate and the average extraction yield values were taken as the responses. All data was analyzed using the Design Expert statistical software (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, USA).

Analytical procedures: Aluminum chloride assay method was used to determine the total flavonoid content (Li *et al*., 2011). Briefly, 1.0 mL diluted sample was transferred to 25 mL volumetric flask and then 3 mL 0.1 mol/L $AlCl_3$ was added. The final volume was adjusted to 25 mL with 95% (v/v) ethanol. The solution was mixed well and the absorbance was measured at 410 nm. The amount of flavonoids was expressed as rutin equivalents through the calibration curve of rutin. All samples were analyzed in triplicates and the average values were calculated.

RESULTS AND DISCUSSION

Single factor analysis method: To determine the single factor experiment condition, four main factors, including ethanol concentration, liquid-solid ratio, extraction time and temperature were studied in our test.

Extraction of flavonoids from *O. fragrans* residues was performed at different ethanol concentrations (0, 20, 40, 60, 80 and 100%, respectively), while other conditions were fixed: temperature, 60°C; extraction time, 1.0 h; and the solid-liquid ratio, 1:20. As the results (Fig. 1) shown, the extraction yields began to increase with increasing ethanol solutions concentrations and a ethanol concentration of 80% supported a highest extraction yield, which was followed by a decrease with a further increase of ethanol concentration. Facts showed that the optimal ethanol concentration of flavonoids from *O. fragrans* residues was 80%~100%.

The effects of the solid-liquid ratios (1:10, 1:20, 1:30, 1:40 and 1:50) on the extraction yield were investigated, while other conditions were fixed: temperature, 60°C; extraction time, 1.0 h; and the ethanol concentration, 60%. As depicted in Fig. 2, the effect of various liquid-solid ratios on the extraction yields of flavonoids were obvious. When the solid-liquid ratio rose to 1:40, the extraction yield of flavonoids was improved rapidly and then the increase in the extraction yield seemed to reach a plateau slowly after 1:40. Therefore, solid-liquid ratio of 1:40 was used in the succedent experiments.

Data on the amounts of flavonoids extracted by 60% ethanol for periods up to 3 h of incubation was depicted in Fig. 3, while other conditions were fixed: temperature, 60°C; and the solid-liquid ratio, 1:20. The extraction time of 2.0 h resulted in the maximal extraction yields and then rapidly decreased with prolonged incubation due to the loss of activities. The

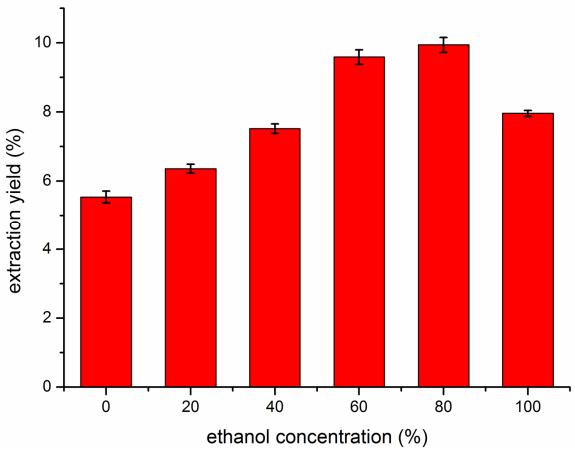


Fig. 1: Effect of ethanol concentration on the extraction yield of flavonoids from the *O. fragrans* residues

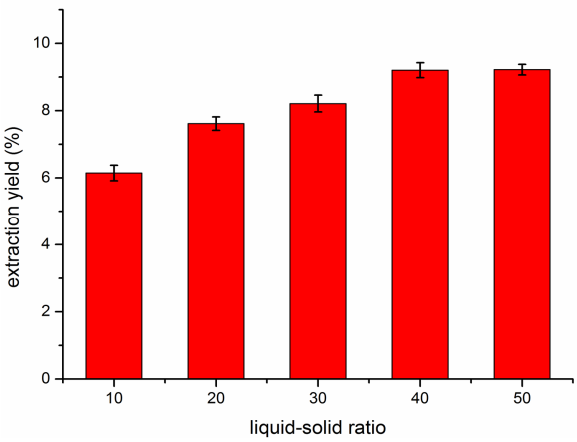


Fig. 2: Effect of liquid-solid ratio on the extraction yield of flavonoids from the *O. fragrans* residues

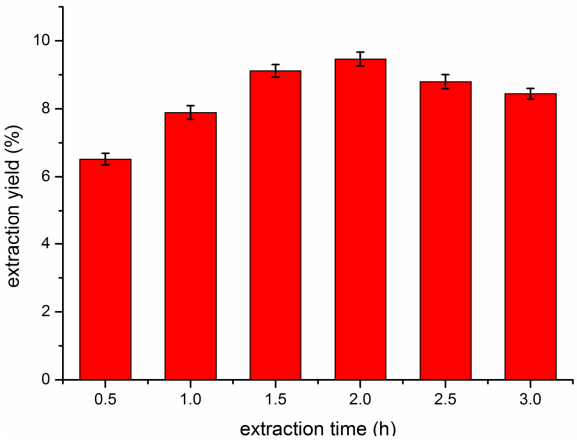


Fig. 3: Effect of extraction time on the extraction yield of flavonoids from the *O. fragrans* residues

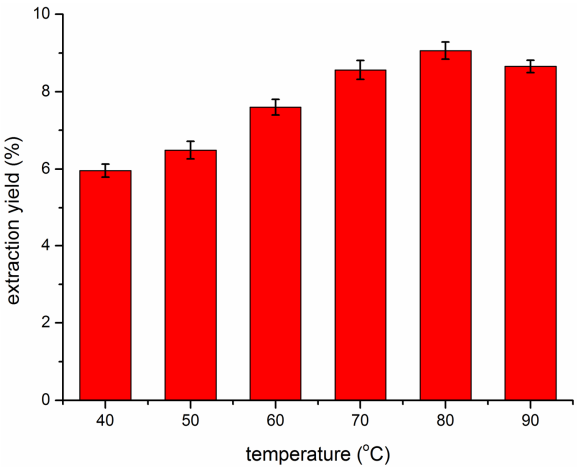


Fig. 4: Effect of temperature on the extraction yield of flavonoids from the *O. fragrans* residues

present experiment found that the optimal extraction time of flavonoids from *O. fragrans* residues was 1.5-2.5 h.

Table 2: Factors and levels in the response surface Box-Behnken design arrangement and experimental results and results

Run	X_1	X_2	X_3	Extraction yield (%)
1	0	1	1	12.07
2	1	1	0	9.91
3	0	0	0	12.68
4	0	0	0	12.52
5	1	-1	0	9.68
6	0	-1	-1	11.07
7	-1	-1	0	7.68
8	0	0	0	12.59
9	1	0	-1	9.72
10	0	0	0	12.62
11	0	1	-1	11.32
12	-1	0	-1	8.38
13	-1	1	0	8.87
14	0	0	0	12.56
15	-1	0	1	9.21
16	0	-1	1	11.35
17	1	0	1	9.82

The effect of temperature on the extraction yield was investigated (Fig. 4), while the process was carried out at the solid-liquid ratio of 1:20 for 1.0 h at ethanol concentration of 60%. The amounts of astaxanthin were improved with rising temperature to the maximum at 80°C, after which the extraction yield decreased. It remained to be proved that 70-90°C was chosen for the further optimization of extraction conditions.

Optimization of extraction conditions by using Box-Behnken design: The combined effect of three independent variables, ethanol concentration (X_1, %); extraction time (X_2, h); and temperature (X_3, °C) for flavonoids from *O. fragrans* residues was examined using Box-Behnken design. The experimental results for the Box-Behnken design are shown in Table 2. The analysis of variance (ANOVA) for extraction yield is presented in Table 3.

A second-order polynomial quadratic model was generated by the multiple regression analysis using the Design Expert software. It was designed to correlate the independent variables and to explain the behavior of the system in the design space. Thus, the following regression equation presented below shows the relative extraction yield as a function of the test variables:

$$Y = 12.59+0.62X_1+0.30X_2+0.24X_3-0.24X_1X_2-0.18X_1X_3+0.12X_2X_3-2.86X_1^2-0.69X_2^2-0.45X_3^2$$

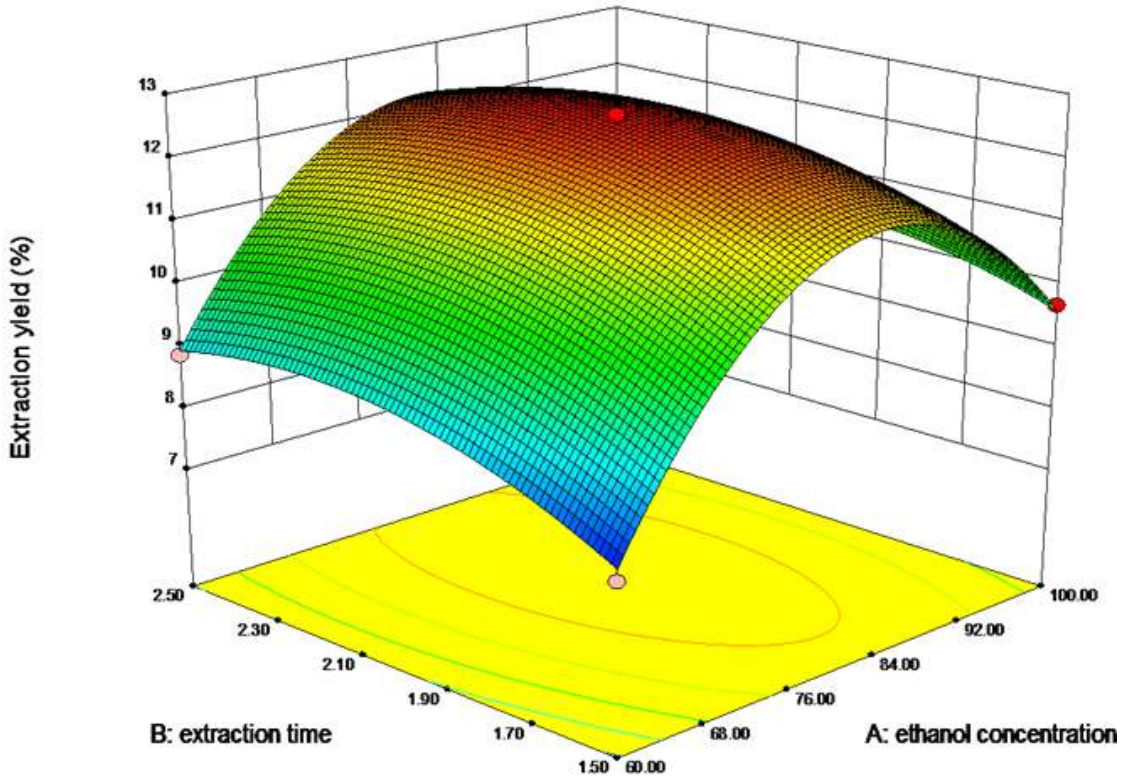
Table 3 showed the results of the response surface model fitting in the form of analysis of variance (ANOVA). The ANOVA of regression model demonstrated that the model was highly significant, as evident from the Fisher's F test ($F_{model} = 180.52$) with a very low probability value ($p_{model} < 0.0001$). The results showed that the critical F value was very less than the calculated F values of 180.52 and it suggested that the computed Fisher's variance ratio at this level was large enough to justify a very high degree of adequacy of the quadratic model and significance of the variables

combinations (Yetilmezsoy *et al*., 2009). The goodness of fit of the model can be checked by the determination coefficient (R^2). R^2 value for the model was 0.9957 which indicated that 99.57% of variation observed in the extraction yield of flavonoids could be attributed to ethanol concentration, extraction time, temperature and the interactions among these variables. A signal to noise ratio of 37.386 as estimated by adequate precision measure indicated an adequate signal for the model. The predicted R^2 value (0.9362) was in reasonable agreement with the adjusted R^2 value (0.9902), which indicated a good agreement between the experimental and predicted values of extraction yield. Values of 'probability > F' less than 0.05 indicated X_1, X_2, X_3, X_1^2, X_2^2, X_3^2 and X_1X_2 were the model terms which significantly influenced extraction yield of flavonoids. A low value of coefficient of the variance (C.V.%) (1.54) clearly indicated a high degree of precision and reliability of the experimental values.

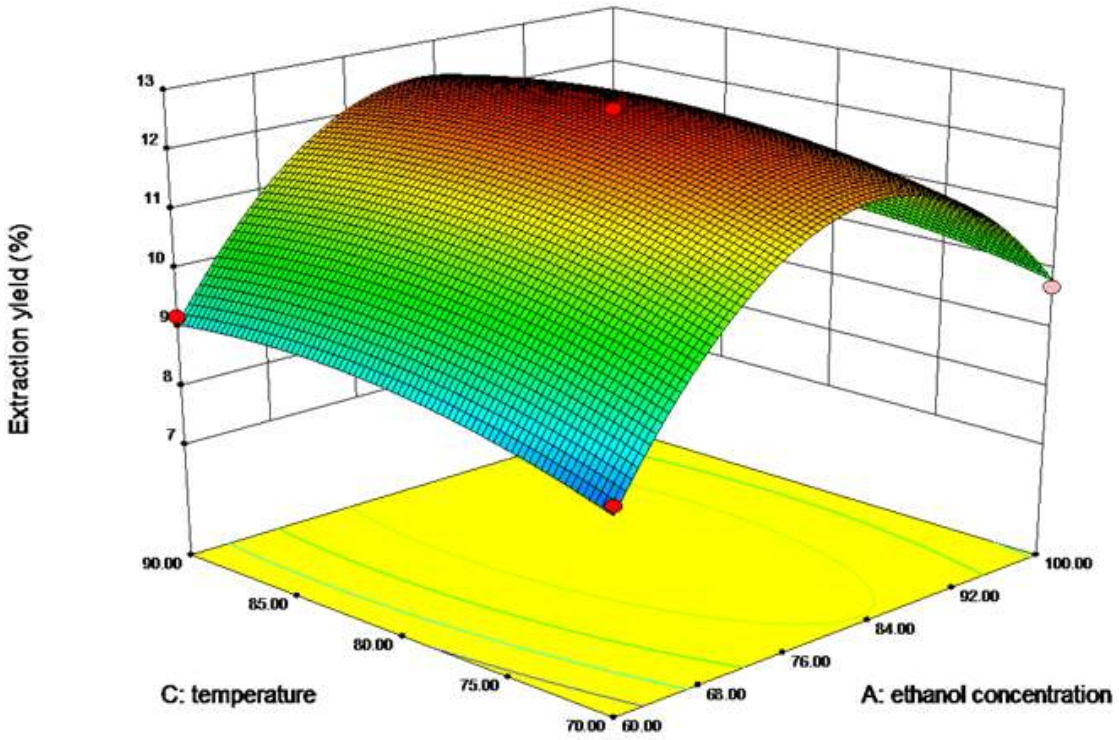
In order to determine the best extraction conditions supporting maximum extraction yield, response surface methodology was adopted. The three dimensional response surface curves (Fig. 5) showed the relative effect of any two independent variables, while keeping

Table 3: Analysis of variance (ANOVA) for the regression equation

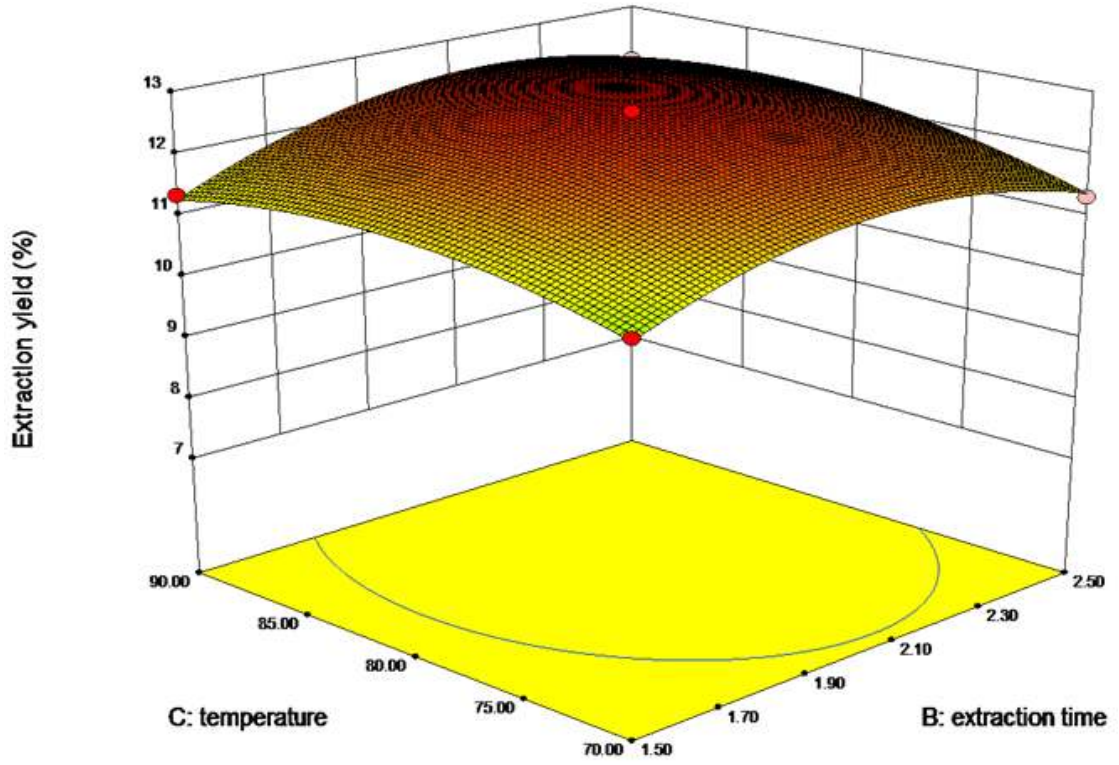
Source	SS	df	Mean square	*F* value	*p* value
Model	44.05	9	4.89	180.52	<0.0001
X_1	3.11	1	3.11	114.80	<0.0001
X_2	0.71	1	0.71	26.33	0.0014
X_3	0.48	1	0.48	17.71	0.0040
X_1X_2	0.23	1	0.23	8.50	0.0225
X_1X_3	0.13	1	0.13	4.91	0.0622
X_2X_3	0.055	1	0.055	2.04	0.1966
X_1^2	34.55	1	34.55	1274.23	<0.0001
X_2^2	2.03	1	2.03	74.90	<0.0001
X_3^2	0.84	1	0.84	31.03	0.0008
Residual	0.19	7	0.027		
Lack of fit	0.18	3	0.058	15.86	0.0110
SD	0.16	R^2	0.9957		
Mean	10.71	Adjusted R^2	0.9902		
C. V. %	1.54	Predicted R^2	0.9362		
PRESS	2.82	Adequate precision	37.386		



(a)



(b)



(c)

Fig. 5: Response surface graphs for the effects of ethanol concentration, extraction time and temperature on the extraction yield, (a) ethanol concentration (X_1) and extraction time (X_2), (b) ethanol concentration (X_1) and temperature (X_3) and (c) extraction time (X_2) and temperature (X_3)

Table 4: Flavonoids extraction: Comparison UAE/conventional extractions

Method	UAE	EE[a]	MSE[b]	RFTE[c]
Time (h)	2	4	8	6
Extraction yield (%)	12.70	11.86	11.64	8.94

[a]: Enzymolysis extraction; [b]: Mechanical shaking extraction; [c]: Repeated freezing and thawing extraction

the third independent variable at constant level. Figure 5a showed the interaction effect of X_1 and X_2 on extraction yield. An increase of X_1 and X_2 result in an initial increase of astaxanthin yield, which then decrease with the rising of X_1 and X_2. A higher ethanol concentration may lead to lower solubility of flavonoids and prolonged incubation time may result in degradation of extracts. Figure 5b and c showed the similar change rule to Fig. 5.

The model predicted a maximal extraction yield of 12.68%, while the optimal extraction conditions were as follows: ethanol concentration, 82%; extraction time, 2.1 h; and temperature 82°C. To verify the model prediction, tests were carried out in five replicates under the above optimized conditions and an average yield value of 12.70% extremely near to the predicted value was obtained. This confirms the closeness of the model to the experimental data and the aptness of the model.

Compared with traditional extraction methods: Based on optimum experimental conditions defined previously for the *O. fragrans* residues, a comparison of the four extraction methods was performed in terms of flavonoids extraction. From Table 4, it appears that UAE supported higher extraction yields as compared to traditional extraction with a shorter incubation time. These results confirm that presence of ultrasonic can improve the rate of flavonoids compounds extraction with obvious reduction of the reaction time.

CONCLUSION

In this study, an attempt was made to optimize the ethanol concentration, extraction time and temperature in order to maximize the extraction yields of flavonoids from the *O. fragrans* residues. The findings of the experiments suggested that the optimum conditions for the extraction yields were 82% of ethanol concentration, 2.1 h of extraction time and temperature 82°C. Compared to the traditional methods, UAE process reduced extraction time and obtained high extraction yields of flavonoids. This showed great potential for industrial application in the near future.

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