Optimization of Ultrasound-Assisted Extraction of Arbutin from *Pyrus Communis L.* leaves by Response Surface Methodology

Armut Yapraklarından Arbutinin Ultrasonik Destekli Ekstraksiyonunun Yüzey Yanıt Metadolojisi ile Optimizasyonu

Research Article

İbrahim Bulduk*, Işıl Açıkgöz Sağlam
Uşak University, Department of Chemical Engineering, Uşak, Turkey.

ABSTRACT

Arbutin is a naturally occurring derivative of hydroquinone. It is found in various plant species belonging to diverse families, such as *Lamiaceae*, *Ericaceae*, *Saxifragaceae* and *Rosaceae*. It inhibits tyrosinase and has been employed as a cosmetic skin whitening agent. In this study, the ultrasound assisted extraction of arbutin from *Pyrus Communis L. leaves* was modeled using responce surface methodology. A three-level-three-factor Box–Behnken design was employed to optimize three extraction variables, including extraction temperature (X1), extraction time (X2), and methanol concentration (X3), for the achievement of high extraction yield of the arbutin. The optimized conditions are extraction temperature of 43.37°C, methanol concentration of 56.81%, extraction time of 29.66 min. Under this optimized conditions, the experimental yield of arbutin is 3.10%, which is well matched with the predicted yield of 3.12%.

Key Words
Pyrus Communis L.; Arbutin; Extraction; Optimization; RSM.

ÖZET

Arbutin doğal olarak oluşan bir hidrokinon türevidir. Ballıbabagiller, fundagiller, taşkırangiller, gülgiller gibi farklı familyalara ait farklı bitki türlerinde bulunur. Arbutin tirozinazı engeller ve cilt beyazlatma ajanı olarak kullanılır. Bu çalışmada Yüzey Yanıt Metodolojisi kullanılarak armut yapraklarından arbutinin ultrasonik destekli özütlemesi modellenmiştir. Arbutinin yüksek özütleme veriminin elde edilmesi için özütleme sıcaklığı (X1), özütleme zamanı (X2) ve metanol derişimi (X3) gibi üç özütleme değişkenini optimize etmek için üç-düzeyli üç-faktörlü Box-Behnken tasarımı kullanılmıştır. Optimize koşullar; 43.37°C özütleme sıcaklığı , %56.81 metanol derişimi ve 29.66 dakika özütleme zamanıdır. Bu optimize koşullar atında arbutinin deneysel verimi %3.10'dur. Bu değer tahmin edilen %3.12 değeri ile uyumludur.

Anahtar Kelimeler
Pyrus Communis L.; Armut, Arbutin; Özütleme; Optimizasyon; RSM.

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Correspondence to: İ. Bulduk, Uşak University, Department of Chemical Engineering, Uşak, Turkey.
Tel: +90 542 455 8916 Fax: +90 276 221 2145 E-Mail: ibrahim.bulduk@usak.edu.tr

INTRODUCTION

Pyrus Communis, known as the European pear or common pear, is a species of pear native to central and Eastern Europe and southwest Asia [1]. The plants are medium-sized trees that can reach 5 m in height. The leaves are glosssy green and oval. The pear leaves are useful for treatment of inflamation of the bladder, bacteriuria, high blood pressure and urinary stones. They also have diuretic properties [2].

The leaves of this tree contain a considerable amount of arbutin (hydroquinone-β-D-glucopyranoside), a naturally occurring derivative of hydroquinone [3]. Arbutin is found in various plant species belonging to diverse families, such as the *Ericaceae*, *Lamicaceae*, *Saxifragaceae* and Rosaceae [4]. Its tyrosinase-inhibiting qualities have made arbutin (4-hydroxyphenyl glucopyranoside) to be widely used as a whitening agent in many cosmetics [5–9]. Arbutin inhibits tyrosinase and has been employed as a cosmetic skin-whitening agent in humans [10]. It has been shown to have antioxidant and free radical scavenging properties [11], as well as bactericidal and antifungal effects [10]. Extracting arbutin from pear has recently attracked considerable interest. Species and parts of pear from which arbutin has been extracted are Pyrus pyrifolia Nakai (fruit peel) [12] P. pyrifolia Niitaka (fruit peel), [13] Pyrus biossieriana Buhse (leaves) [14,15] four species of oriental pear (*Pyrus bretschnrideri, P. pyrifolia, Pyrus ussuriensis,* and *Pyrus sinkiangensis*), and one species of occidental pear (the flowers, buds, and young fruits of *Pholiota communis* [16].

The content of arbutin was determined in plant extracts by many methods: spectrophotometric [7], capillary zone electrophoresis [18], densitometric [19], GC/MS [20]. Reversed-phase HPLC was found to be the more suitable chromatographic method for arbutin sepa→ration [17,21,22]. To our knowledge, there is no single validated HPLC method which was developed for the quantification of arbutin in many different plant extracts.

Many factors such as solvent composition, extraction time, extraction temperature [23], solvent to solid ratio [24] and extraction pressure [25], among others, may significantly influence the extraction efficacy. In general, optimization of a process could be achieved by either empirical or statistical methods; the former having limitations toward complete optimization. The traditional one-factor-at-a-time approach to process optimization is time consuming. Moreover, the interactions among various factors may be ignored hence the chance of approaching a true optimum is very unlikely. Thus, one-factor-at-a-time procedure assumes that various parameters do not interact, thus the process response is a direct function of the single varied parameter. However, the actual response of the process results from the interactive influence of various variables. Unlike conventional optimization, the statistical optimization procedure allows one to take interaction of variables into consideration [26].

Response surface methodology (RSM), originally described by Box and Wilson [27], enables evaluation of the effects of several process variables and their interactions on response variables. Thus, RSM is a collection of statistical and mathematical techniques that has been successfully used for developing, improving and optimizing processes [28]. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. Therefore, it is less laborious and time consuming than other approaches required to optimize a process. Response surface methodology has been successfully used to model and optimize biochemical and biotechnological processes related to food systems [29-34) including extraction of phenolic compounds from berries [24,29] and evening primrose meal [23], anthocyanins from black currants [24] and sunflower hull [35] and vitamin E from wheat germ [36], among others.

In present work, conditions of extraction and chromatographic parameters have been combined in order to establish a simpler, faster and cheaper method for the extraction and HPLC determination of arbutin in *Pyrus Communis* leaves. Optimization of experimental conditions that results in the highest arbutin content of *Pyrus Communis* leaves extracts was conducted (Figure 1).

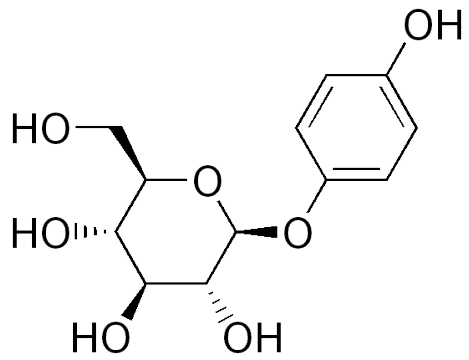

Figure 1. The molecular structure of arbutin.

MATERIALS AND METHODS

Reagents and materials:

Pyrus Communis leaves were collected in the city of Uşak in western Turkey in July 2014. The leaves were dried at room temperature in a dark room for fifteen days. Dried leaves were ground to the size of 80–100 mesh before extraction.

All chemicals used in experiments were analytical grade and all solvents used for chromatographic purposes were of HPLC grade. 0.45μm membranes (Millipore, Bedford, MA, USA) were used for filtering the all solutions. Arbutin Standard (of at least 98% purity) was purchased from Sigma Chemical Co.

Ultrasound Assisted Extraction

Ultrasound assistant extraction was carried out using Bandelin Sonorex brand ultrasonic bath with 50 kHz frequency. For the standard ultrasonic conditions, erlenmeyer flasks were placed inside the ultrasonic bath. Water that inside the ultrasonic bath was circulated in order to keep the temperature stable. Solvent level in the erlenmeyer flask and water level in the ultrasonic bath were kept the same. After the extraction process had been completed, mixture was filtered with Whatman filter paper in order to prevent capillary blockage first and then filtered with 0.45 micron membrane filter (Millipore, Bedford, MA, USA).

HPLC Analysis

Identification and quantitative determination of arbutin was established by Agilent 1260. Chromatographic system equipped with auto sampler, quaternary pump, column compartment and a UV-VIS detector. Final quantification was performed on a 250 mm×4.6 mm id, 5 μm particle size, ACE 5 C-18 column. The mobile phase was a solution of 7% methanol in water, The mobile phase filtered through 0.45 μm Millipore filters. The flow rate was 1.2 ml/min and the injection volume was 5 μL. The column temperature was maintained at 30 °C and detection was carried out at 280 nm. Chromatographic analysis was carried out using a single-column isocratic reverse phase method.

Analytical Method Validation

The method has been validated in terms of linearity, precision, accuracy and stability according to ICH guidelines, taking into account the recommendations of other appropriate guidelines. Results obtained from testing different parameters during validation of the analytical method were given in Table 1.

Standard Solution and Calibration Curves

Standard stock solution in water of arbutin was prepared at the final concentration of 1000 μg/ml for arbutin. Before calibration, the stock solution was diluted with water. The standard curve was prepared over a concentration range of 50-250 μg/ml for arbutin with five different concentration levels. Linearity for arbutin was plotted using linear regression of the peak area versus concentration. The coefficient of correlation (R^2) was used to judge the linearity. The dedection limits (LOD) and quantitation limits (LOQ) for tested compound were determined by the signal to noise (S/N) ratio (Table 1).

Response Surface Methodology (RSM)

The RSM with the Box-Behnken design was then employed to design the experiment to investigate the influence of three independent parameters, temperature, time and methanol concentration on the extraction of arbutin. Optimal ranges of temperature (30-60^{0}C), time (15-45 min) and methanol concentration (25-75%) were determined based on preliminary experiments. The independent variables and their code variable levels are shown in Table 2. To express the arbutin content as a function of the independent variables, a second order polynomial equation was used as follows and previously described.

Table 1. Results obtained from testing different parameters during validation of the analytical method.

Parameters		Arbutin
Specifity	Peak Purity Ratio	0.0010
Linearity	Concentration Range µg/mL	50-250
	Correlation Coefficient	0.9997
	Intercept	2.5220
	Slope	1.5926
LOD (ppm)		3.3731
LOQ (ppm)		7.7390
Retention Time min.		4.4500

Table 2. Treatment variables and their coded and actual values used for optimization of arbutin extraction from *Pyrus Communis* by using Box-Behnken design.

Independent Parameters	Units	Symbol of the parameters	Coded Levels		
Extraction Temp.	^{0}C	(X1)	30	45	60
Extraction Time	min	(X2)	15	30	45
Methanol Concentration	%	(X3)	25	50	75

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} {X_i}^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + e \quad (1)$$

Where various X_i values are independent variables affecting the response Y: β_0, β_i, β_{ii} and β_{ij} are the regression coefficient fort he intercept and the linear, quadratic and interaction terms, respectively and k is the number of variables.

Statistical analysis

Statistical analysis on the means of triplicate experiments was carried out using the analysis of variance (ANOVA) procedure of the Instat® software version 3.0 (GraphPad, San Diego, CA, USA). Anova test was applied to identify the interaction between the variables and the response using Design-Expert program. Three replication analyses were carried out for each sample. ANOVA test was applied for identifying the interaction between the variables and the response by using Design-Expert program. The results of HPLC analysis were expressed as means of extraction efficiency.

RESULTS AND DISCUSSION

Effect of process variables on the UAE performance

Experimental conditions of Box-Behnken design runs designed with Design Expert 8.0.7.1 are shown in Table 2. Table 3 also displays the effects of extraction temperature, extraction time and methanol concentration on the extraction efficiency obtained by UAE.

Effect of extraction time on the UAE performance

The influence of the extraction time on the extraction efficiency of arbutin was examined over a range of 15-45 min and the results are shown in Table 3. The experiment results showed that 30 min is the optimum extraction time of the arbutin, as shown in Figure 2. When extraction time increased, the cell walls of *Pyrus Communis* leaves got fully fall apart and arbutin got into

Table 3. Box-Behnken Design of the independent variables (X1, X2, X3) and experimental results for the EY.

Run	Ext. Temperature	Ext. Time	Methanol Concentration	Arbutin Yield
	^{0}C	min	%	%
1	45.00	45.00	50.00	3.03
2	30.00	30.00	50.00	2.31
3	45.00	45.00	75.00	2.7
4	45.00	45.00	25.00	2.54
5	30.00	30.00	50.00	2.34
6	60.00	60.00	50.00	2.24
7	60.00	60.00	25.00	2.26
8	45.00	45.00	50.00	3.13
9	45.00	45.00	50.00	3.09
10	60.00	60.00	50.00	2.23
11	45.00	45.00	25.00	2.41
12	60.00	60.00	75.00	2.23
13	45.00	45.00	50.00	3.11
14	45.00	45.00	75.00	2.76
15	30.00	30.00	75.00	2.66
16	30.00	30.00	25.00	2.37
17	45.00	45.00	50.00	3.07

*Data are expressed as the mean (n=3) .

material liquid diffusion so that the extraction yield is relatively rapid. During long extraction time, *Pyrus Communis* leaves overheating was prone to cause thermal decomposition of arbutin, because of the unstable chemical bonds of arbutin molecular, such as unsaturated bonds. And then the arbutin content was decreased. Therefore, 30 min is favorable for extracting the arbutin.

Effect of extraction temperature on the UAE performance

Extraction process was carried out using extraction temperature from 30 to 60˚C. As shown in Figure 3, extraction temperature has obvious effects on yield of arbutin. When extraction temperature increased, the extraction yield increased rapidly and reached a maximum at 45˚C. In general, extractions at higher temperatures increase mass transfer and extraction performance because of enhanced solute desorption from the active sites of plant matrix. When extraction temperature went above 45˚C, the extraction yield started to decrease. At initially, extraction yield increasing with the rising of temperature may be that elevated temperature accelerated the arbutin chemical bond rupture and speeded molecular motion, so that a large number of arbutin in cell dissolution into the solution. when heating temperature greater than 45˚C, high temperature caused the destruction of arbutin structure, accelerated the degradation reaction, and lost arbutin activity, and then arbutin content is rapidly reduced. Therefore, 45˚C is favorable for extracting the arbutin.

Effect of methanol concentration on the UAE performance

Extraction process was carried out using methanol concentration from 25% to 75%. The effect of methanol concentration on extraction yield of

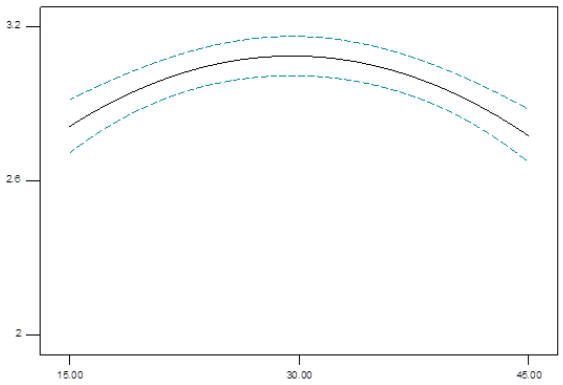

Figure 2. The influence of extraction time on extraction performance.

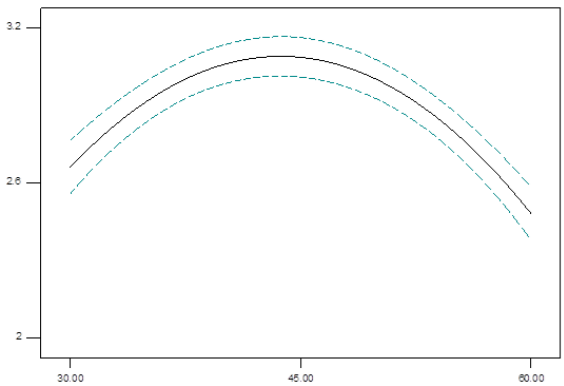

Figure 3. The influence of extraction temperature on extraction performance.

Table 4. The analysis of variance (ANOVA) for Response Surface Quadratic Model.

Source	Sum of Squares	df	Mean Square	f Value	p-Value Prob > F	
Model	1.95	9	0.220	41.46	< 0.0001	significant
X1-Ext. Temperature	0.065	1	0.065	12.41	0.010	significant
X2-Ext. Time	$2.812x10^{-3}$	1	$2.81x10^{-3}$	0.54	0.487	
X3-Methanol Concentration	0.074	1	0.074	14.20	0.007	significant
X1X2	$1.00\ 10^{-4}$	1	$1.00x10^{-4}$	0.019	0.894	
X1X3	0.026	1	0.026	4.90	0.062	
X2X3	$1.23x10^{-3}$	1	$1.23x10^{-3}$	0.230	0.643	
X12	1.11	1	1.11	213.28	< 0.0001	significant
X22	0.360	1	0.360	68.65	< 0.0001	significant
X32	0.150	1	0.150	29.65	0.001	significant
Residual	0.037	7	$5.22x10^{-3}$			
Lack of Fit	0.031	3	0.010	6.90	0.047	significant
Pure Error	$5.92x10^{-3}$	4	$1.48x10^{-3}$			

arbutin is shown in Figure 4. In the initial stage, along with the methanol concentration increased from 25% to 60%, the extraction yield of arbutin increased rapidly; while methanol concentration greater than 60% arbutin extraction yield was showing slow decreasing trend, and peak at 60% methanol concentration. This is because the increase of methanol concentration leads to enhanced mass transfer dynamics, solvents and *Pyrus communis* leaves getting full access, and then the contents of arbutin dissolved increased. When the methanol concentration reached a certain level, some of arbutin was difficult to be dissolved by high concentration of methanol, and also lead to the increase of the alcohol-soluble impurity content, resulting in a loss of arbutin content. Moreover, the greater of methanol concentration, the more difficult to refine arbutin and it will cause wasted and the cost of production increased. Therefore, the methanol concentration of 60% is good for the arbutin extraction. Figures 6,7 and 8 shows the interactive effect of different parameters for arbutin yield. The corresponding contour plots have also been depicted in Figures

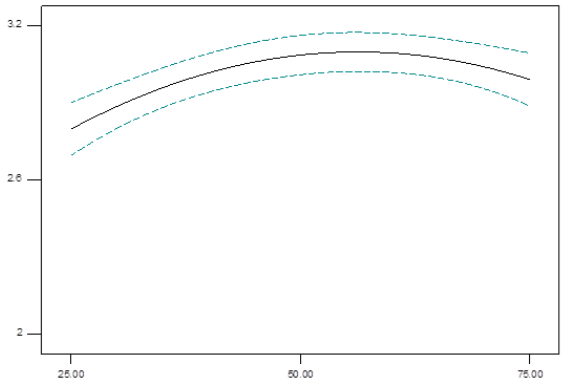

Figure 4. The influence of extraction temperature on extraction performance.

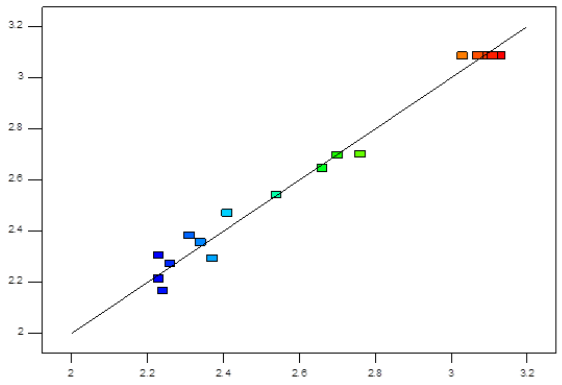

Figure 5. The correlation between the experimentally obtained values of the extraction yields versus the calculated values using the model equation.

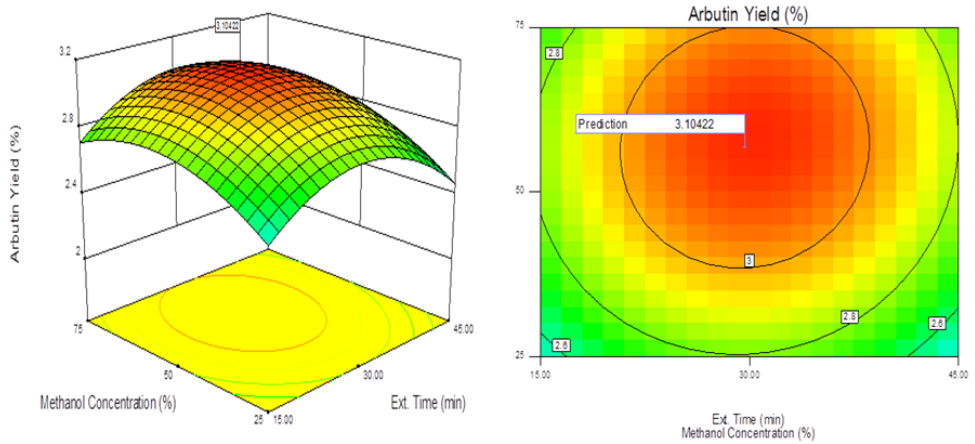

Figure 6. Three-dimensional response surface and contour plots for arbutin extraction showing the interactive effects of the methanol concentration and extraction time.

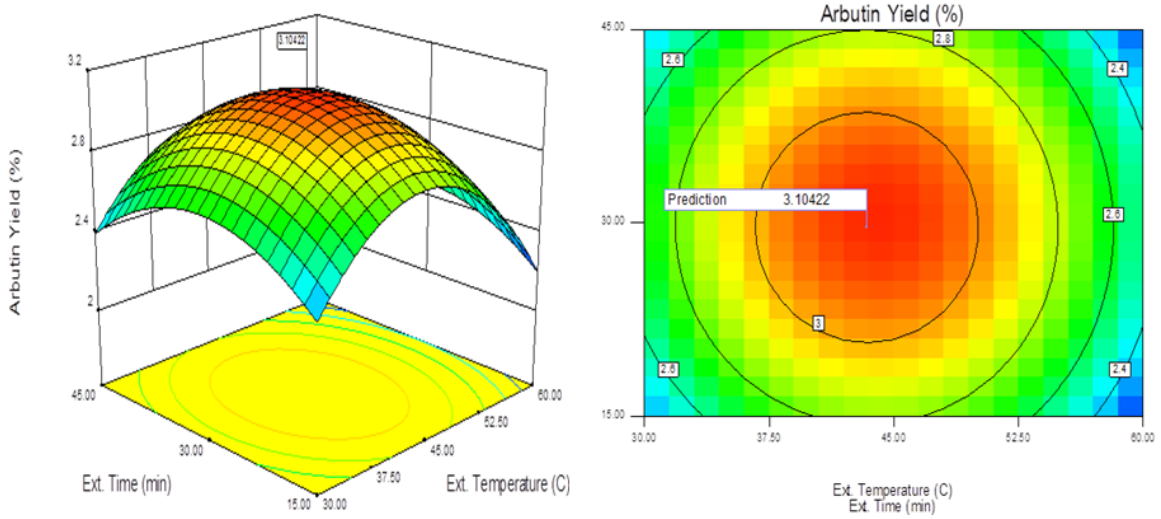

Figure 7. Three-dimensional response surface and contour plots for arbutin extraction showing the interactive effects of the extraction time and extraction temperature.

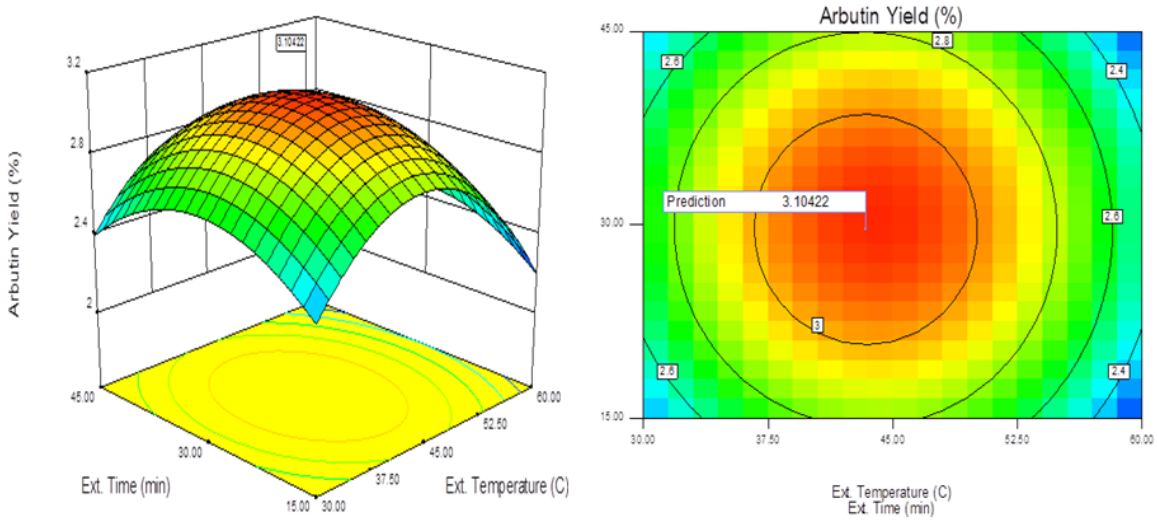

Figure 8. Three-dimensional response surface and contour plots for arbutin extraction showing the interactive effects of the methanol concentration and extraction temperature.

6,7 and 8.

Optimisation of UAE by RSM

Individual effects of process variables, which is also known as one-factor at-atime approach was applied in previous section. This classical approach ignores the possible interactions of process variables with each other, which may result in misleading conclusions. Response surface methodology (RSM) considers the probable interactions between operation parameters. Table 2 shows the three parameters (methanol concentration, time and temperature) including minimum, centre, maximum points. Seventeen experiment were run and chosen randomly by the design expert software, and the responses were recorded (Table 3). Using response surface methodology owing to the software, a quadratic model applying with not only forward stepwise but also backward elimination regressions for EY were obtained.

Using responce surface methodology from the software, a quadratic model given below was derived:

$$A = -3.80125 + 0.21103\,X1 + 0.21103\,X2 + 0.042730\,X3 - 2.22222\times10^{-5}\,X1X2 - 2.13333\times10^{-4}\,X1X3 + 4.66667\times10^{-5}\,X2X3 - 2.28556\times10^{-3}\,X1^2 - 1.29667\times10^{-3}\,X2^2 - 3.06800\times10^{-4}\,X3^2 \quad (2)$$

In Table 4, X2, X1X2, X1X3, X2X3, X3X4 are not significant effects for the model. After excluding their regression coefficients, new model may be given for better explanation of new condition.

$$A = -3.80125 + 0.21103\,X1 + 0.042730\,X3 - 2.28556\times10^{-3}\,X1^2 - 1.29667\times10^{-3}\,X2^2 - 3.06800\times10^{-4}\,X3^2 \quad (3)$$

Theoretical recovery values for arbutin calculated from this equation were plotted against practical ones. These relationships were shown in Figure 5.

The optimal extraction conditions were found by using optimization choice in design expert software to maximize the response. This value was measured at 56.81 of methanol concentration, 29.66 min of extraction time, 43.37°C of extraction temperature. The maximum response was found as (3.10%) under these operating conditions.

After finding optimal conditions, real sample extraction experiments were repeated 6 times and then, average with relative standard deviation was calculated.

Average: 3.11%
Standard Deviation: 0.02
Relative Standard Deviation: 0.45
Arbutin Yield (mg / 200 mg sample): 3.11 ± 0.02

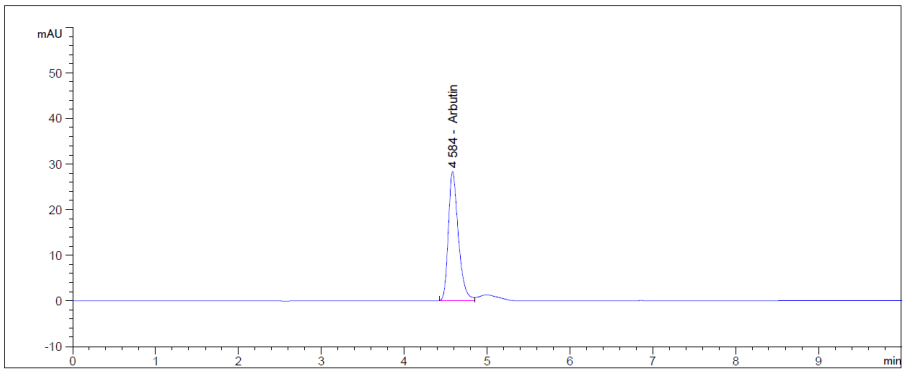

Figure 9. Chromatograme of arbutin standard solution.

Model fitting

The analysis of variance (ANOVA) for the quadratic equations of Design Expert 8.0.7.1 for the responses of EY are given in Table 4. In order to have the most suitable set of variables, stepwise regression was used. According to this process, given variables are tested and assessed within the given alpha levels (0.1) using both backward and forward techniques. Backward techniques include all the variables to estimate parameters, and then any variables with a non significant parameter at alpha levels are removed from the equation. This process continues until there are no significant variables left. Similar to backward technique, forward technique also assess the given variables within the given alpha levels. Unlike backward technique, forward technique starts with no variables included in the equation. The significant variable with the highest value of standardized beta ($p<0.05$) will be added to the equation. Then the next variable with the highest standardized beta value is assessed. If the variable is significant, it is added to the equation. This process continues until no significant variables left. Two of these regressions gave the same results [16].

The ANOVA for the quadratic equations of Design Expert 8.0.7.1 for the response is given in Table 4. Regression analysis was done at 95 % of confidence interval. F-value of the obtained model is 41.46 and $p < 0,0001$ indicate that derived model is significant. (X1), (X3), ($X1^2$), ($X2^2$), ($X3^2$) are significant model terms in the confidence interval (Table 4). The closer and higher multiple coefficients (R-Squared, Adj R-Squared and Pred R-Squared) points out the higher accuracy of the model. Adj R-Squared also shows that a high degree of correlation between actual and predicted data. As seen in Table 4 methanol concentration (X3) is the most significant variable on the response. The 'F-value' of 'Lack of fit' (6.90) shows that the lack of fit is significant.

In our study, R-Squared (0.9816); Adj R-Squared (0.9579) and Pred R-Squared (0.7485) values for EY display good accuracy of the derived model. Thus, the response surface modeling can be achieved sufficiently to predict EY from *Pyrus Communis* L. Leaves with UAE. Also, the coefficient value of variation (C.V. %) is found as 2.76 respectively. The lower coefficient of variation value indicates a higher precision and reliability of the experimental results [17].

The regression equation coefficients were calculated and the data was fitted to a second-order polynomial equation. The response, arbutin extraction from *Pyrus Communis* dried leaves, can be expressed in terms of the following regression equation:

$$A = - 3.80125+0.21103\ X1+0.042730\ X3-2.28556\ 10\text{-}3\ X1^2 - 1.29667\ 10^{-3}\ X22\ -3.06800\ 10^{-4}\ X3^2 \quad (3)$$

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.9816 (a value > 0.75 indicates fitness of the model). This was an estimate of the

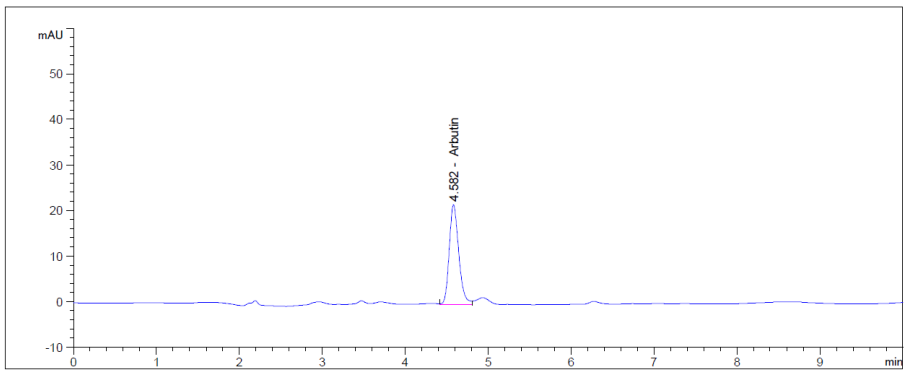

Figure 10. Chromatogram of arbutin standard solution (Concentration: 150 ppm).

fraction of overall variation in the data accounted by the model, and thus the model was capable of explaining 98.16% of the variation in response. The 'adjusted R^2' is 0.9579 and the 'predicted R^2' was 0.7485, which indicates that the model was good (for a good statistical model, the R^2 value should be in the range of 0–1.0, and the nearer to 1.0 the value was, the more fit the model was deemed to be). The 'adequate precision value' of the present model was 16.597, and this also suggests that the model can be used to navigate the design space. The 'adequate precision value' was an index of the signal-to-noise ratio, and values of higher than 4 are essential prerequisites for a model to be a good fit. At the same time, a relatively lower value of the coefficient of variation (CV = 2.76 %) indicated a better precision and reliability of the experiments carried out.

Thus, the responce surface modelling can be achieved sufficiently to predict EY from *Pyrus Communis L. Leaves* with UAE. The lower value of coefficient of variation indicates a higher precision and reliability of the experimental results [18,19]. The coefficient value is found 2.76 in our study. Figure 9 exhibits the corelation between the experimental and predicted data calculated from Equation 2 concerning the EY of *Pyrus comminus* leaves extracts obtained by UAE. It can be seen that the predicted date calculated from the model is in good agreement with the experimental data in the range of operating conditions. Figure 9 exhibits chromatogrames of arbutin standard solution. Figure 10 exhibit chromatogrames of *Pyrus comminus* leaves extract.

CONCLUSION

Response surface methodology was successfully used to investigate the optimum extraction parameters for extraction of arbutin from *Pyrus Communis* leaves. To optimize various parameters for extraction of arbutin from *Pyrus Communis* leaves three parameters viz temperature, time, temperature, solvent composition were tested by using Box-Behnken design criteria and three parameters time, temperature solvent composition showed significant effect on extraction of arbutin. The extraction parameters were optimized by applying Box-Behnken design and the parameters for best extraction of arbutin from *Pyrus Communis* leaves was found to be extraction time (29.66 minutes), temperature (43.37°C) and solvent composition (56.81% methanol in methanol-water mixture). The second order polynomial model was found to be satisfactory for describing the experimental data. The maximum arbutin from *Pyrus Communis* leaves was 3.10% dry weight. Linear coefficient of extraction temperature and methanol concentration and square coefficient of extraction temperature, extraction time and methanol concentration have the most significant effect on the EY obtained by UAE. After finding optimal conditions, real sample extraction experiments were repeated 6 times and then, average with relative standard deviation was calculated. Arbutin (%): 3.11 ± 0.02. Results is appropriate for the statistical evaluation.

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