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Arbutin Analysis in Leaves, Fruit and Branches of Pyrus Anatolica, Method Optimization

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Arbutin 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. Pyrus anatolica, which is endemic to Turkey, is a species of plant in the Rosaceae family. It also contains arbutin like other members of the family. In this study, Analytical method was developed and optimized in order to analyze arbutin in leaves, fruits and branches of Pyrus anatolica. The response surface methodology was used for the extraction of arbutin from this endemic plant. Experimental design was performed using the Box–Behnken design, and the evaluated parameters were extraction temperature (X1), extraction time (X2) and methanol concentration (X3) for the achievement of high extraction yield of the arbutin. The optimized experimental conditions for extraction were extraction temperature of 43.72 °C, methanol concentration of 48.57% and extraction time of 39.33 min. By using this optimized conditions, the experimental yield of arbutin is 4.74%, which is well matched with the predicted yield of 4.69%. After finding optimal conditions, real sample extraction experiments were repeated 6 times and then, average with relative standard deviation was calculated. Results were appropriate for the statistical evaluation.

Keywords: optimization, extraction, Pyrus anatolica Browicz, arbutin

INTRODUCTION

Arbutin is a glycosylated hydroquinone. It inhibits tyrosinase and thus prevents the formation of melanin. It is used in the function of skin lightening and depigmentation in Japon and other Asian Countries [1]. There are two anomeric forms of arbutin; α- and β-arbutins [2]. Of these two arbutins, α-arbutin showed a stronger inhibitory activity than β-arbutin [3, 4]. Three kinds of preparative methods of arbutin have been reported; these are extraction from plants, plant cell culture and organic synthesis. Arbutin was synthesized from hydroquinone and sugars [2-5]. However, it is very difficult to manufacture the arbutin by synthesis and people in general have issues with synthetic compounds. Arbutin is naturally found in various

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plants such as *Ericaceae, Rosaceae,* and *Saxifragaceae* [6-9]. These plants have been commonly used for the treatment of urinary problems [10].

The pear is any of several tree and shrub species of genus *Pyrus*, in the family Rosaceae. The pear is native to coastal and mildly temperate regions of the Old World, from Western Europe and North Africa east right across Asia [11]. *Pyrus anatolica* Browicz, which is endemic to Uşak province in Turkey, is a species of plant in the Rosaceae family. Extracting arbutin from pear has recently attracked considerable interest. Species of pear from which arbutin has been extracted are *Pyrus pyrifolia* Nakai [12] *P. pyrifolia* Niitaka [13] *Pyrus biossieriana* Buhse [14,15] four species of oriental pear (*Pyrus bretschnrideri*, *Pyrus pyrifolia*, *Pyrus ussuriensis*, and *Pyrus sinkiangensis*), and one species of occidental [16].

There have been several reports on the determination of Arbutin from the plant extract, including the use of spectrophotometric [17], capillary zone electrophoresis [18], densitometric [19], GC/MS [20] and HPLC [21, 22, 17]. To our knowledge, there is no method for the determination of Arbutin from *Pyrus anatolica* Browicz.

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 is a direct function of the single varied parameter. However, the actual 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 was actually defined by Box and Wilson [27]. It makes possible evaluation of the effects of several process variables and their interactions on variables. Therefore, this methodology is a collection of statistical and mathematical techniques that has been successfully used for developing, improving and optimizing processes [28]. The main advantage of this methodology is the decreased number of experimental trials needed to evaluate multiple parameters and their interactions. Thus, it is less laborious and time consuming than other approaches required optimizing a process. surface methodology has been successfully used to model and optimize biochemical and biotechnological processes related to food systems [29, 30, 31, 32, 33, 34, 35 and 36].

In this study, a simple, fast and cheap HPLC method combined extraction procedure was developed for determination of arbutin in *Pyrus anatolica* Browicz. Optimization of experimental conditions that results in the highest arbutin content of *Pyrus anatolica* Browicz extracts was conducted.

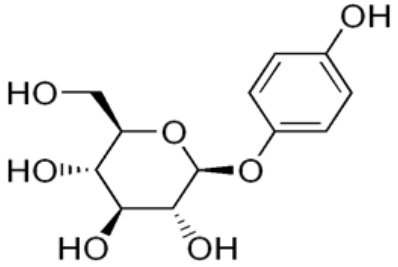

Figure 1: The molecular structure of arbutin.

EXPERIMENTAL

Reagents and materials:

Pyrus anatolica Browicz used in this study has collected from 5 km north of New Erice Village, Sivaslı Town, in Uşak Province in October 2015. The collection and identification of the plant was performed by Mehtap Dönmez Şahin. The plant sample was stored in Herbarium Material Warehouse of Uşak University. Its leaves and branches were dried at room temperature in a dark room for fifteen days. Dried leaves and branches were ground to the size of 80–100 mesh before extraction. Its fruit was grated before extraction.

All chemicals used in all experiments were analytical grade and all solvents used for chromatographic purposes were of HPLC grade. 0.45 μm membranes (Millipore, Bedford, MA, USA) were used for filtering the all solutions. Arbutin standard 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. Solvent level in the Erlenmeyer flask and water level in the ultrasonic bath were kept the same. The temperature and time value of the ultrasonic bath was set and extraction was carried out. 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

HPLC analysis of arbutin was established by Agilent 1260 chromatographic system equipped with auto sampler, quaternary pump, column compartment and a UV-VIS detector system. An ACE 5 C-18 column (250 mm × 4.6 mm Id, 5 μm) column was used as stationary phase at 30 ^{0}C. The system operates at 280 nm. The mobile phase assayed were methanol-water mixture 7% (v/v), The mobile phase filtered through 0.45 μm Millipore filters. The flow rate was 1.2 mL.min^{-1} and the injection volume was 10 μL.

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 solutions and calibration curves

Standard stock solution in water of arbutin was prepared at the final concentration of 1000 μg.ml^{-1} for arbutin. Before calibration, the stock solution was diluted with water. The standart curve was prepaerd over a concentration range of 40-200 μg.ml^{-1} for arbutin with five 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. Results obtained from testing different parameters during validation of the analytical method has been shown in Table 1.

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

Parameters		Arbutin
Specifity	Peak Purity Ratio	0.0010
Linearity	Range (ppm)	40-200
	Correlation Coefficient	0.99987
	Intercept	1.81524
	Slope	1.60321
LOD (μg.ml^{-1})		0.891
LOQ (μg.ml^{-1})		2.972
Retention Time (min.)		4.580

Response surface methodology

Box-Behnken design was then employed to design the experiment to investigate the influence of three independent parameters, temperature, time and methanol on the extraction of arbutin. Optimal ranges of temperature (30-60 ^{0}C), time (20-60 min) and methanol (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 by Vuong et al.

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

Where various X_i values are independent variables affecting the Y: β_0, β_i, β_{ii} and β_{ij} are the regression coefficient for the 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 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 by using Design-Expert program. The results of HPLC analysis were expressed as means of extraction efficiency.

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

Independent Parameters	Units	Symbols of the parameters	Coded Levels		
			-1	0	1
Extraction Temp.	^{0}C	(X1)	30	45	60
Extraction Time	min	(X2)	20	40	60
Methanol Conc.	%	(X3)	25	50	75

RESULTS AND DISCUSSIONS

Effect of process variables on the UAE performance

Experimental conditions of Box-Behnken design runs designed with Design Expert 9 are shown in Table 2. Table 3 also displays the effects of extraction temperature, extraction time and methanol 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 20-60 min and the results are shown in Table 3. The experiment results showed that 40 min is the optimum extraction time of the arbutin. When extraction time increased, the cell walls of the leaves of Pyrus anatolica Browicz got fully fall apart and arbutin got into material liquid diffusion so that the extraction yield is relatively rapid. During long extraction time, Pyrus anatolica Browicz 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, 40 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. When extraction temperature increased, the extraction yield increased rapidly and reached a maximum at 44 ˚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

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

Run	Ext. Temperature	Ext. Time	Methanol	Arbutin Yield
	^{0}C	min	%	%
1	45	40	50	4.60
2	30	20	50	3.50
3	45	60	75	3.67
4	45	20	25	3.86
5	30	60	50	3.56
6	60	60	50	3.39
7	60	40	25	3.42
8	45	40	50	4.75
9	45	40	50	4.69
10	60	20	50	3.38
11	45	60	25	3.65
12	60	40	75	2.95
13	45	40	50	4.72
14	45	20	75	3.76
15	30	40	75	3.61
16	30	40	25	3.60
17	45	40	50	4.65

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

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, 44 ˚C is favorable for extracting the arbutin.

Effect of methanol concentration on the UAE performance

Extraction process was carried out using methanol from 25% to 75%. In the initial stage, along with the methanol increased from 25% to 50%, the extraction yield of arbutin increased rapidly; while methanol greater than 50% arbutin extraction yield was showing slow decreasing trend and peak at 50% methanol. This is because the increase of methanol leads to enhanced mass transfer dynamics, solvents and *Pyrus anatolica* Browicz getting full access, and then the contents of arbutin dissolved increased. When the methanol reached a certain level, some of arbutin was difficult to be dissolved by high 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, the more difficult to refine arbutin and it will cause wasted and the cost of production increased. Therefore, the methanol concentration of 49% is good for the arbutin extraction. Figures 3, 4 and 5 shows the interactive effect of different parameters for arbutin yield. The corresponding contour plots have also been depicted in Figures 3,4 and 5.

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

$$A = -6.20500 + 0.32115X1 + 0.085888X2 + 0.090060X3 - 4.16667\ 10^{-5}X1X2 - 3.20000\ 10^{-4}X1X3 + 6.00000\ 10^{-5}X2X3 - 3.47667\ 10^{-3}X1^2 - 1.10563\ 10^{-3}X2^2 - 8.07600\ 10^{-4}X3^2 \quad (2)$$

In Table 4, X2, X3, 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.

$$A = -6.20500 + 0.32115X1 - 3.47667\ 10^{-3}X1^2 - 1.10563\ 10^{-3}X2^2 - 8.07600\ 10^{-4}X3^2 \quad (3)$$

Theoretical recovery values for arbutin calculated from this equation were plotted against practical ones. These relationships were shown in Figure 2.

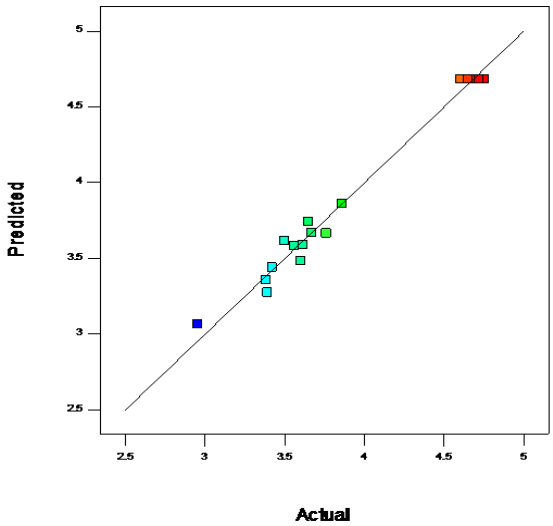

Figure 2: The correlation between the experimentally obtained values of the extraction yields versus the calculated values using the model equation.

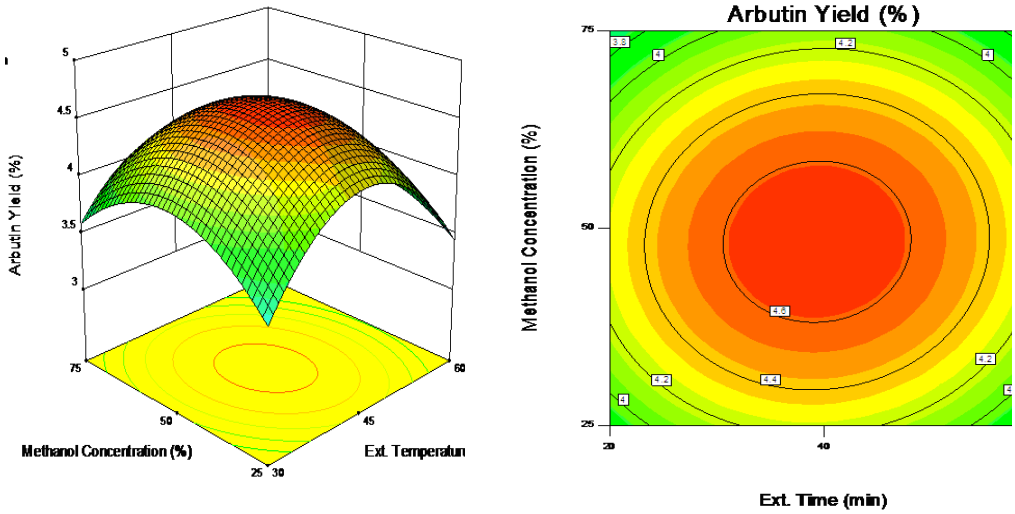

Figure 3: Three-dimensional surface and contour plots for arbutin extraction showing the interactive effects of the methanol concentration and extraction time.

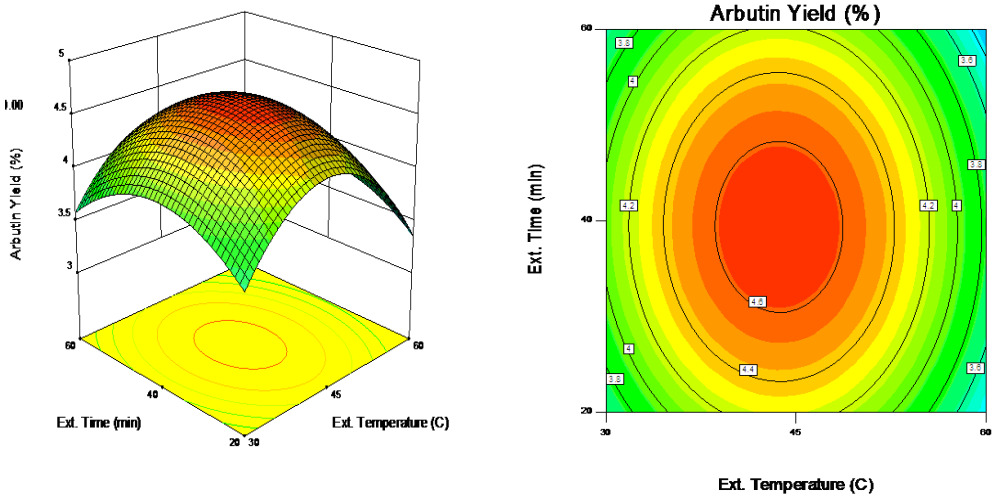

Figure 4: Three-dimensional surface and contour plots for arbutin extraction showing the interactive effects of the extraction time and extraction temperature.

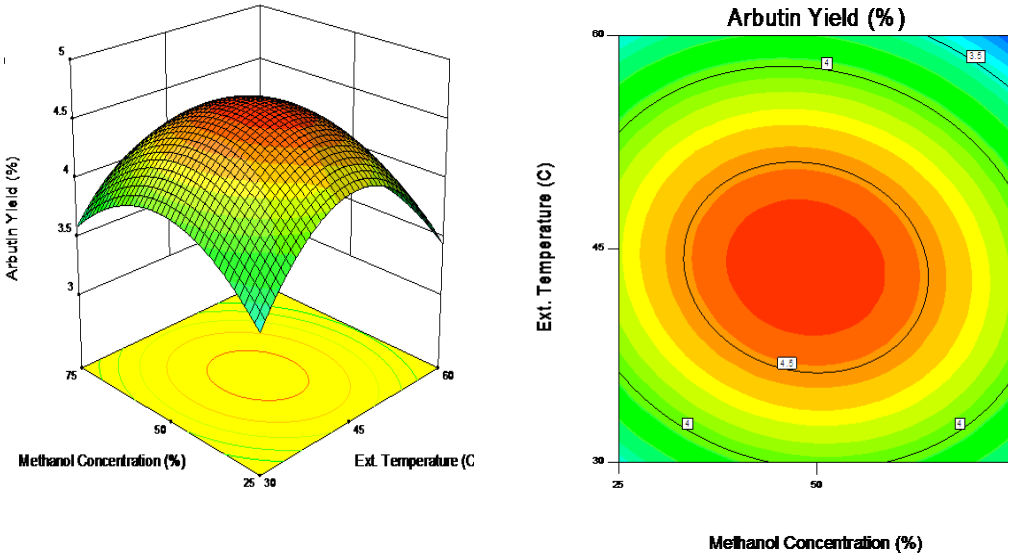

Figure 5: Three-dimensional surface and contour plots for arbutin extraction showing the interactive effects of the methanol concentration and extraction temperature.

The optimal extraction conditions were found by using optimization choice in design expert software to maximize the response. This value was measured at 48.57% of methanol concentration, 39.33 min of extraction time and 43.72 ^{0}C of extraction temperature. The maximum response was found as (4.69%) 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: 4.74%

Standard Deviation: 0.03

Relative Standard Deviation: 0.38

Arbutin Yield (mg / 200 mg sample): 4.74 ± 0.03

Model fitting

The analysis of variance (ANOVA) for the quadratic equations of Design Expert 9 for the s 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 9 for the is given in Table 4. Regression analysis was done at 95% of confidence interval. F-value of the obtained model is 46.72 and $p < 0.0001$ indicate that derived model is significant. (X1), ($X1^2$), ($X2^2$), ($X3^2$) 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 (X1) is the most significant variable on the . The 'F-value' of 'Lack of fit' (7.01) shows that the lack of fit is significant.

In our study, R-Squared (0.9836); Adj R-Squared (0.9626) and Pred R-Squared (0.7758) values for EY display good accuracy of the derived model. Thus, the response surface modeling can be achieved sufficiently to predict EY from *Pyrus anatolica* Browicz with UAE. Also, the coefficient value of variation (C.V.%) is found as 2.88 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. Arbutin extraction from *Pyrus anatolica* Browicz dried leaves can be expressed in terms of the following regression equation:

$$A = - 6.20500 + 0.32115X1 - 3.47667\ 10^{-3}X1^2 - 1.10563\ 10^{-3}X2^2 - 8.07600\ 10^{-4}X3^2 \quad (3)$$

The regression equation obtained from the ANOVA showed that the R2 (multiple correlation coefficient) was 0.9838 (a value > 0.75 indicates fitness of the model). This was an estimate of the 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 0.9630 and the 'predicted R^2' was 0.7784, 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 47.32, 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 = 2.86%) indicated a better precision and reliability of the experiments carried out.

Thus, the response surface modelling can be achieved sufficiently to predict EY from *Pyrus anatolica* Browicz 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 2 exhibits the corelation between the experimental and predicted data calculated from Equation 2 concerning the EY

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	5.22	9	0.580	46.72	< 0.0001	significant
X1-Ext. Temperature	0.160	1	0.160	12.86	0.0089	significant
X2-Ext. Time	$6.612\ 10^{-3}$	1	$6.612\ 10^{-3}$	0.530	0.4891	
X3-Methanol	0.036	1	0.036	2.940	0.1303	
X1X2	$6.25\ 10^{-4}$	1	$6.25\ 10^{-4}$	0.050	0.8288	
X1X3	0.058	1	0.058	4.640	0.0682	
X2X3	$3.600\ 10^{-3}$	1	$3.600\ 10^{-3}$	0.290	0.6068	
$X1^2$	2.580	1	2.580	207.65	< 0.0001	significant
$X2^2$	0.820	1	0.820	66.37	< 0.0001	significant
$X3^2$	1.070	1	1.070	86.46	< 0.0001	significant
Residual	0.087	7	0.012			
Lack of Fit	0.073	3	0.024	7.04	0.0453	significant
Pure Error	0.014	4	$3.47\ 10^{-3}$			

of *Pyrus anatolica* Browicz 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 6 exhibits chromatogram of arbutin standard solution. Figure 7 exhibit chromatogram of *Pyrus anatolica* Browicz leaves extract.

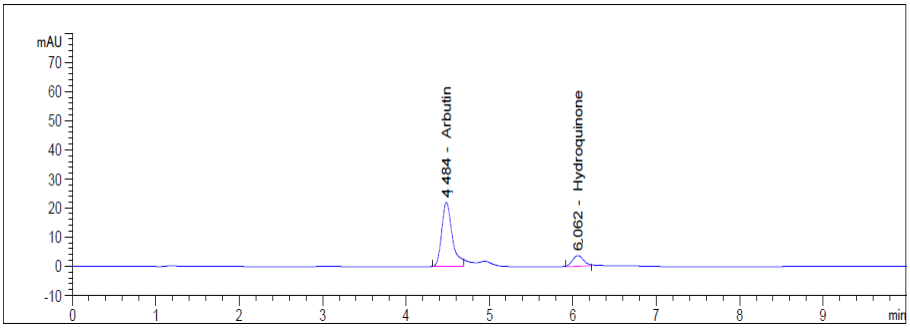

Figure 6: Chromatogram of arbutin standard solution (150 µg.ml^{-1})

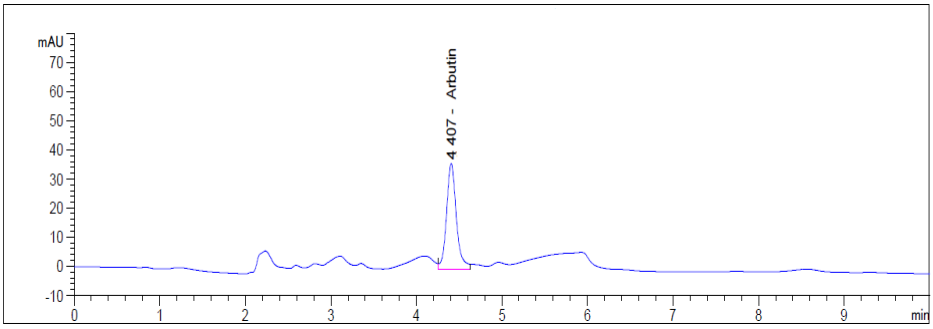

Figure 7: Chromatogram of *Pyrus anatolica* Browicz leaves extract.

After completion of the method optimization, arbutin analyses were made in leaves, fruit and branches of *Pyrus anatolica* Browicz. The results are given in the following table.

CONCLUSIONS

Response surface methodology was successfully used to investigate the optimum extraction parameters for extraction of arbutin from *Pyrus anatolica* Browicz leaves. To optimize various parameters for extraction of arbutin from *Pyrus anatolica* Browicz leaves three parameters via 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 anatolica* Browicz leaves was found to be extraction time (39.33 min), temperature (43.72 °C) and solvent composition (48.57% 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 anatolica* Browicz leaves was

Table 5. The results of arbutin analyses of leaves, fruit and branches of *Pyrus anatolica* Browicz.

Source	Arbutin%
Leaves	4.74
Branches	4.46
Fruits	0.109

4.69% dry weight. Linear coefficient of extraction temperature and methanol and square coefficient of extraction temperature, extraction time and methanol 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. Results is appropriate for the statistical evaluation.

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REFERENCES

1. Couteau C, Coiffard LJM. (2000). Photostability determination of arbutin, a vegetable whitening agent. *Farmaco 55*: 410-413
2. Funayama M, Arakawa H, Yamamoto R, Nishino T, Shin T, Murao S. (1995). Effect of α- and β-arbutin on activity of tyrosinases from mushroom and mouse melanoma. *Biosci. Biotech. Bioch. 59*: 143- 144
3. Seo DH, Jung JH, Ha SJ, Song MC, Cha JH, Yoo SH, Kim TJ, Baek NI, Park CS. (2009). Highly selective biotransformation of arbutin to arbutin-α-glycoside using amylosucrase from Deinococcus geothermalis DSM 11300. *J. Mol. Catal. B-Enzym. 60*: 113-118
4. Sugimoto K, Nomura K, Nishimura T, Kiso T, Sugimoto K, Kuriki T. (2005). Synthesis of α-arbutin-α-glycosides and a comparison of their inhibitory effects on human tyrosinase. *J. Biosci. Bioeng. 99*: 272- 276
5. Sugimoto K, Nishimura T, Nomura K, Sugimoto K, Kuriki T. (2003). Synthesis of arbutin-α-glycosides and a comparison of their inhibitory effects with those of α-arbutin and arbutin on human tyrosinase. *Chem. Pharm. Bull. 51*: 798-801
6. Robertson JA, Howard LA. (1987). Effect of carbohydrates on growth of Ureoplasma urealyticum and Mycoplasma hominis. *J. Clin. Microbiol. 25*: 160-161
7. Lamien-Meda A, Lukas B, Schmiderer C, Franz, Novak J. (2009). Validation of a quantitative assay of arbutin using gas chromatography in Origanum majorana and Arctostaphylos uvaursi extracts. *Phytochem. Anal. 20*: 416-420
8. Lubsandorzhieva PB, Zhigzhitov BS, Dargaeva TD, Bazarova ZG, Nagaslaeva LA. (2000). Chromatospectrophotometric determination of arbutin in the leaves of Bergenia crassifolia (L.) Fritsch. *Pharm. Chem. J. 34*: 261-264
9. Ferreira D, Guyot S, Marnet N, Delgadill I, Renard CM, Coimbra MA. (2002). Composition of phenolic compounds in a Portuguese pear (Pyrus communis L. var. S. Bartolomeu) and changes after sundrying. *J. Agr. Food Chem. 50*: 4537-4544
10. Pop C, Vlase L, Tamas M. (2009). Natural resources containing arbutin. Determination of arbutin in the leaves of Bergenia crassifolia (L.) Fritsch. Acclimated in Romania. *Not. Bot. Hort. Agrobot. Cluj 37*: 129-132
11. Cui T, Nakamura K, Ma L, Li JZ, Kayahara H. (2005). Analyses of arbutin and chlorogenic acid, the major phenolic constituents in Oriental pears. *J. Agr. Food Chem. 53*: 3882-3887.
12. Cho JY, Park KY, Lee KH, Lee HJ, Lee SH, Cho JA, Kim WS, Shin SC, Park KH, Moon JH. (2011). Recovery of arbutin in high purity from fuit peels of pear (Pyrus pyrifolia Nakai). *Food Sci. Biotechnol.20*: 801–807.
13. Lee BD, Eun JB. (2012). Optimum extraction conditions for arbutin from asian pear peel by supercritical fluid extraction (SFE) using Box-Behnken design. *J. Med. Plants Res.6*: 2348–2364.
14. Azadbakht M, Marstonm A, Hostettmann K, Ramezani M, Jahromi M. (2004). Biological activity of leaf extract and phenolglycoside arbutin of Pyrus boissieriana Buhse. *J. Med. Plants.3*: 9–14.
15. Shahaboddin ME, Pouramir M, Moghadamnia AA, Parsian H, Lakzaei M, Mir H. (2011). Pyrus biossieriana Buhse leaf extract: An antioxidant, antihyperglycaemic and antihyperlipidemic agent. *Food Chem. 126*: 1730–1733.
16. Cui T, Nakamura K, Ma L, Li JZ, Kayahara H. (2005). Analyses of arbutin and chlorogenic acid, the major phenolic constituents in oriental pear. *J. Agric. Food Chem. 53*: 3882–3887.

17. Pavlović R.D, Lakušić B, Došlov-Kokoruš Z, Kovačević N. (2009). Arbutin content and antioxidant activity of some *Ericaceae* species. *Pharmazie 64*: 656-659.
18. Glöckl I, Blaschke G, Veit M. (2001). Validated methods for direct determination of hydroquinone glucuronide and sulfate in human urine after oral intake of bearberry leaf extract by capillary zone electrophoresis. *J Chromatogr B: Biomed Sci Appl. 761*(2): 261-266.
19. Pyka A, Bober K, Stolarczyk A (2007). Densitometric determination of arbutinin cowberry leaves (*Vaccinium Vitis-idaeae*). *Acta Pol Pharm 63*(5): 395-400.
20. Lamien-Meda A, Lukas B, Schmiderer C, Franz Ch, Novak J (2009). Validation of a quantitative assay of arbutin using gas chromatography in *Origanum Majorana* and *Arctostaphylos uva-ursi* extracts. *Phytochem Anal 20*: 416-420.
21. Asaaf M, Ali A, Makboul M, Beck JP, Anton R (1986). Preliminary study of phenolic glycosides from *Origanum majorana*; quantitative estimation of arbutin; cytotoxic activity of hydroquinone. *Planta Med 53*: 343-345.
22. Parejo I, Viladomat F, Bastida J, Codina C (2001). A single extraction step in the quantitative analysis of arbutin in bearberry (*Arctostaphylos uva-ursi*) leaves by HPLC. *Phytochem Anal 12*(5): 336-339.
23. Wettasinghe, M., & Shahidi, F. (1999). Evening primrose meal: A source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals. *Journal of Agricultural and Food Chemistry 47*: 1801–1812.
24. Cacace, J. E., & Mazza, G. (2003). Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *Journal of Food Science 68*, 240–248.
25. Cacace, J. E., & Mazza, G. (2002). Extraction of anthocyanins and other phenolics from black currants with sulfured water. *Journal of Agricultural and Food Chemistry 50*, 5939–5946.
26. Haaland, P. O. (1989). Experimental design in biotechnology. New York: Marcel Dekker.
27. Box, G. E. P., & Wilson, K. B. (1951). On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society 13*,1–45.
28. Myers, R. H., & Montgomery, D. C. (2002). Response surface methodology: Process and product optimization using designed experiments (2nd ed.). New York: Wiley.
29. Cacace, J. E., & Mazza, G. (2003). Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering 59*, 379–389.
30. Parajo, J. C., Santos, V., Dominguez, H., & Vazquez, M. (1995). NH_4OH-based pretreatment for improving the nutritional quality of single-cell protein (SCP). *Applied Biochemistry and Biotechnology 55,* 133–150.
31. Senanayake, S. P. J. N., & Shahidi, F. (1999). Enzyme-assisted acidolysis of borage (Borage officinalis L) and evening primrose (Oenothera biennis L) oils: Incorporation of x-3 polyunsaturated fatty acids. *Journal of Agricultural and Food Chemistry 47*, 3105–3112.
32. Senanayake, S. P. J. N., & Shahidi, F. (2002). Lipase-catalyzed incorporation of docosahexaenoic acid (DMA) into borage oil: optimization using response surface methodology. *Food Chemistry 77*, 115–123.
33. Telez-Luis, S. J., Moldes, A. B., Alonso, J. L., & Vazquez, M. (2003). Optimization of lactic acid production by Lactobacillus delbrueckii through response surface methodology. *Journal of Food Science 68,* 1454–1458.
34. Vasquez, M., & Martin, A. (1998). Optimization of Phaffia rhodozyma continuous culture through response surface methodology. *Biotechnology and Bioengineering 57*, 314–320.
35. Gao, L., & Mazza, G. (1996). Extraction of anthocyanin pigments from purple sunflower hulls. *Journal of Food Science 61*, 600–603.
36. Ge, Y., Ni, Y., Yan, H., Chen, Y., & Cai, T. (2002). Optimization of the supercritical fluid extraction of natural vitamin E from wheat germ using response surface methodology. *Journal of Food Science 67*, 239–243.

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