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# Optimization of Ultrasound-Assisted Extraction of Arbutin from Leaves of *Pyrus elaeagnifolia* Pallas ssp. *elaeagnifolia (Rosaceae)* by Response Surface Methodology

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**ABSTRACT**
*Pyrus elaeagnifolia* Pallas. ssp. *elaeagnifolia* is a medicinal plant used in traditional medicine for the treatment of various diseases in Turkey. The leaves of *Pyrus elaeagnifolia* ssp. *elaeagnifolia* are a rich source of arbutin, which 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, Response Surface Methodology (RSM) using a Box Behnken Design (BBD) was employed to optimize the condition for extraction of arbutin from the leaves of *Pyrus elaeagnifolia* ssp. *elaeagnifolia*. Three influencing factors; methanol concentration, period of ultrasound-assisted extraction and extraction temperature were investigated in the ultrasonic aqueous extraction. The Response Surface Methodology was applied to optimize the extraction process focused on arbutin content with respect to the above influencing factors. The best combination of each significant factor was determined by RSM design and optimum pretreatment conditions for maximum arbutin content were established to be methanol concentration of 48.54 %, extraction time of 39.32 min. And extraction temperature of 43.71 $^0$C. Under these conditions 5.37 % of arbutin content was observed experimentally, similar to the theoretical prediction of 5.30 %.
***Keywords*** - Arbutin, Extraction, Optimization, *Pyrus elaeagnifolia* ssp. *elaeagnifolia*, RSM.

## I. INTRODUCTION

*Pyrus elaeagnifolia* ssp. *elaeagnifolia* is a species of pear that belongs to the plant family Rosacea It is native to Albania, Bulgaria, Greece, Romania, Turkey, and Ukraine's Crimea(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- ß-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 P. communis(16).

The content of arbutin was determined in plant extracts by many methods: spectrophotometric (17), capillary zone electrophoresis (18), densitometric (19), GC/MS (20). Reversed-phase HPLC was found to be the more suitable chromatographic method for arbutin separation (21, 22, 17). 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 procedures allow 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). Response surface methodology has been successfully used to model and optimize biochemical and biotechnological processes related to food systems (29, 30, 31, 32, 33 and 34) including extraction of phenolic compounds from berries (24 and 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 fort the extraction and HPLC determination of arbutin in a variety of raw material. Optimization of experimental conditions that results in the highest arbutin content of *Pyrus elaeagnifolia* ssp. *elaeagnifolia* leaves extracts was conducted. The molecular structure of arbutin has been shown in figure 1.

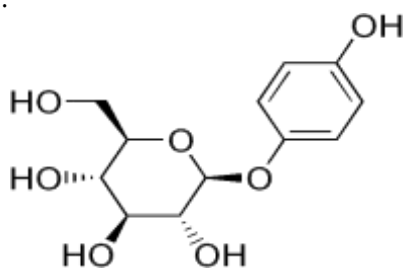


**Fig. 1** The molecular structure of arbutin.

## II. Material and Methods

### *2.1 Reagents and materials:*

The fresh fruits, branches and leaves of pear, *Pyrus elaeagnifolia* Pallas ssp. *elaeagnifolia* grown on Uşak City, Turkey, were harvested in October 2015 and identified by prof. Mehtap DONMEZ SAHIN, Health Care Education, Research and Application Center, Uşak University. A voucher sample was deposited in the herbarium of the laboratory. The leaves and branches of the tree 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. Its fruit was grated before extraction.

All chemicals used in 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.

### *2.2 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).

### *2.2 HPLC Analysis*

**A.** 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 ìm 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 ìm Millipore filters. The flow rate was 1.2 ml/min and the injection volume was 10 ìL. 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.

### *2.3 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.

**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 (ppm) | 40-200 |
| | Correlation Coefficient | 0.99987 |
| | Intercept | 1.81524 |
| | Slope | 1.60321 |
| | LOD ( ppm) | 0.891 |
| | LOQ ( ppm) | 2.972 |
| | Retention Time (min.) | 4.580 |

*2.3.1 Standard Solution and Calibration Curves*

Standard stock solution in water of arbutin was prepared at the final concentration of 1000 $\mu$g/ml for arbutin. Before calibration, the stock solution was diluted with water. The standard curve was prepared over a concentration range of 40-200 $\mu$g/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).

### *2.4 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 (20-60 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 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 response Y: $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are the regression coefficient fort he intercept and the linear, quadratic and interaction terms, respectively and k is the number of variables.

**Table 2.** Treatment variables and their coded and actual values used for optimization of arbutin extraction from *Pyrus elaeagnifolia* ssp. *elaeagnifolia* 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 Concentration | % | (X3) | 25 | 50 | 75 |

### *2.5 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.

## III. FIGURES AND TABLES

### RESULTS AND DISCUSSIONS

*3.1 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 concentration on the extraction efficiency obtained by UAE.

**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 | 40 | 50 | 5.20 |
| 2 | 30 | 20 | 50 | 3.96 |
| 3 | 45 | 60 | 75 | 4.15 |
| 4 | 45 | 20 | 25 | 4.36 |
| 5 | 30 | 60 | 50 | 4.02 |
| 6 | 60 | 60 | 50 | 3.83 |
| 7 | 60 | 40 | 25 | 3.87 |
| 8 | 45 | 40 | 50 | 5.37 |
| 9 | 45 | 40 | 50 | 5.30 |
| 10 | 60 | 20 | 50 | 3.82 |
| 11 | 45 | 60 | 25 | 4.13 |
| 12 | 60 | 40 | 75 | 3.33 |
| 13 | 45 | 40 | 50 | 5.34 |
| 14 | 45 | 20 | 75 | 4.25 |
| 15 | 30 | 40 | 75 | 4.08 |
| 16 | 30 | 40 | 25 | 4.07 |
| 17 | 45 | 40 | 50 | 5.26 |

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

*3.1.1 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, as shown in figure 2. When extraction time increased, the cell walls of the leaves of *Pyrus elaeagnifolia* ssp. *elaeagnifolia* got fully fall apart and arbutin got into material liquid diffusion so that the extraction yield is relatively rapid. During long extraction time, *Pyrus elaeagnifolia* ssp. *elaeagnifolia* 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.

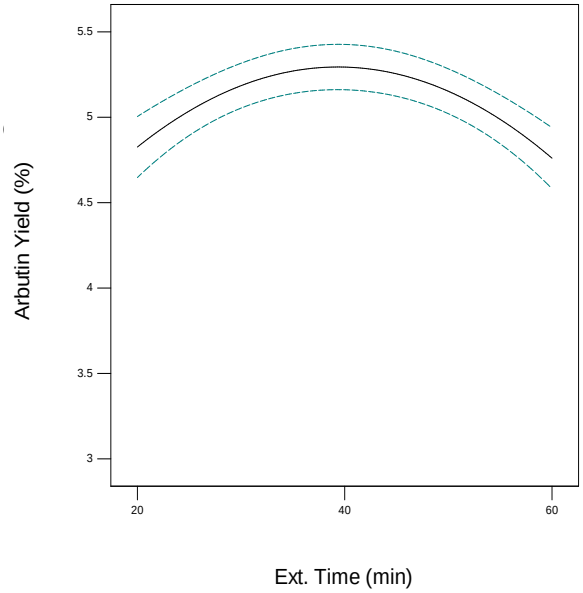


**Fig. 2** The influence of extraction time on extraction performance

*3.1.2 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 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 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.

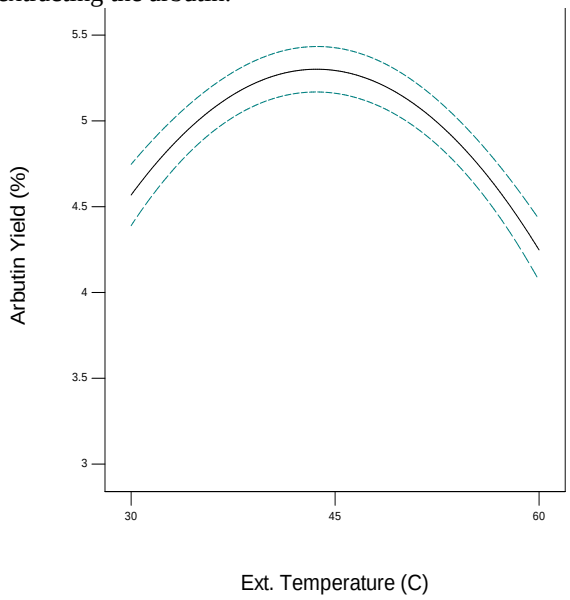


**Fig. 3** The influence of extraction temperature on extraction performance

*3.1.3 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 arbutin is shown in figure 4. In the initial stage, along with the methanol concentration increased from 25% to 50%, the extraction yield of arbutin increased rapidly; while methanol concentration greater than 50% arbutin extraction yield was showing slow decreasing trend, and peak at 50% methanol concentration. This is because the increase of methanol concentration leads to enhanced mass transfer dynamics, solvents and *Pyrus elaeagnifolia* ssp. *elaeagnifolia* 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 49 % 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 6,7 and 8.

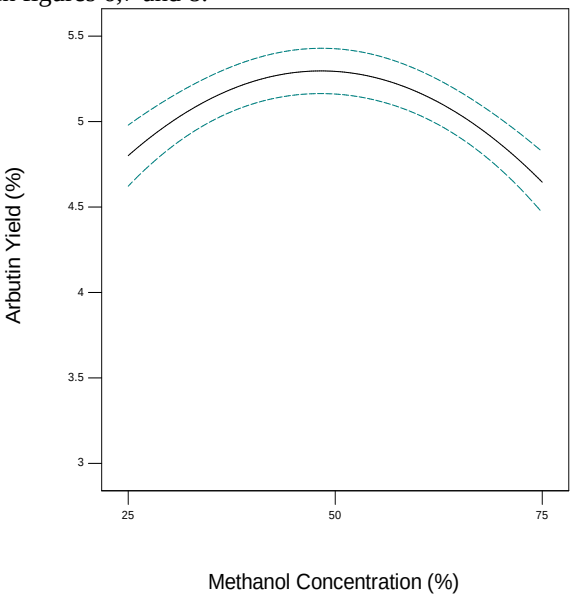


**Fig. 4** The influence of methanol concentration on extraction performance.

### *3.2 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:

$$A = -7.03375 + 0.36363\,X1 + 0.097150\,X2 + 0.10212\,X3 - 4.16667\,10^{-5}\,X1X2 - 3.66667\,10^{-4}\,X1X3 + 6.50000\,10^{-5}\,X2X3 - 3.93667\,10^{-3}\,X1^2 - 1.25188\,10^{-3}\,X2^2 - 9.13200\,10^{-4}\,X3^2 \quad (2)$$

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.

$A = -7.03375 + 0.36363\ X1 - 3.93667\ 10^{-3}\ X1^2 - 1.25188\ 10^{-3}\ X2^2 - 9.13200\ 10^{-4}\ X3^2$ (3)

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

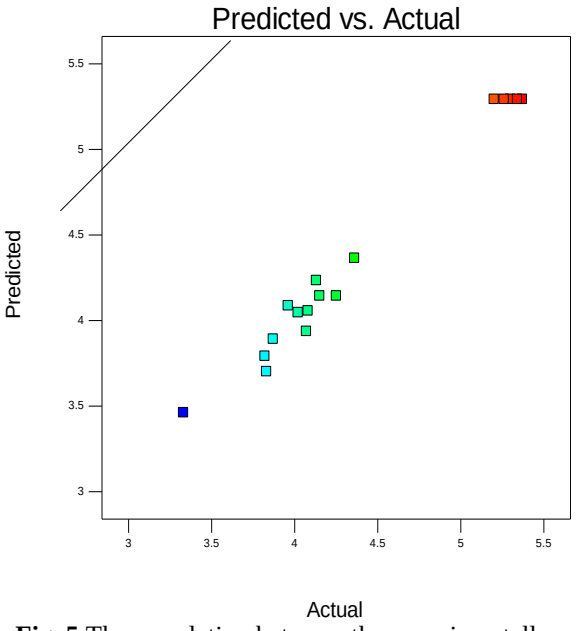


**Fig. 5** The correlation between the experimentally obtained values of the extraction yields versus the calculated values using the model equation.

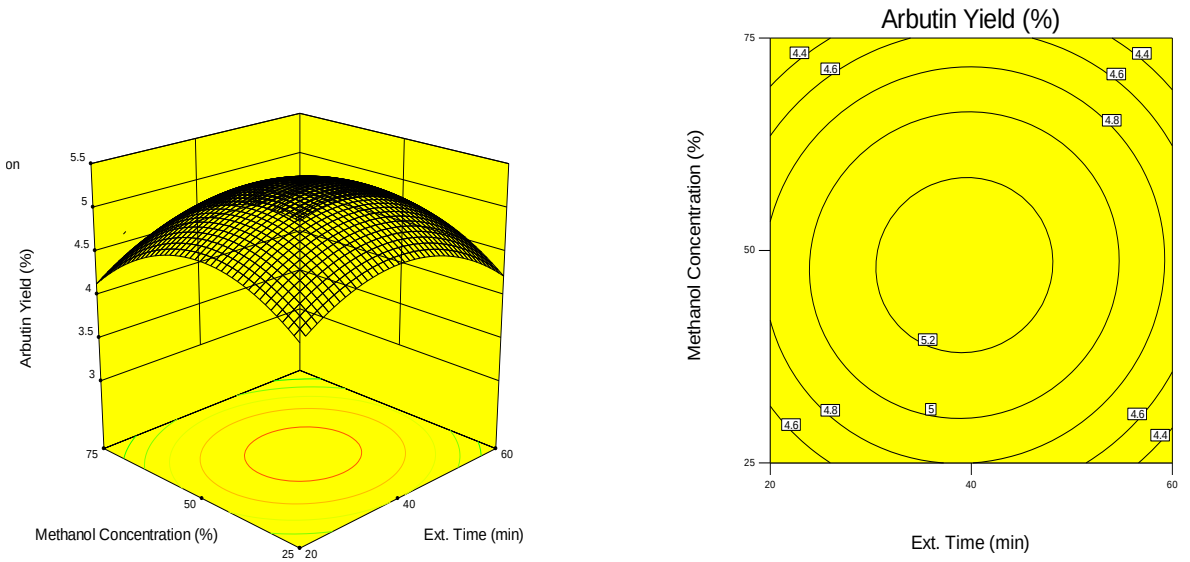


**Fig. 6** Three-dimensional **r**esponse surface and contour plots for arbutin extraction showing the interactive effects of the methanol concentration and extraction time.

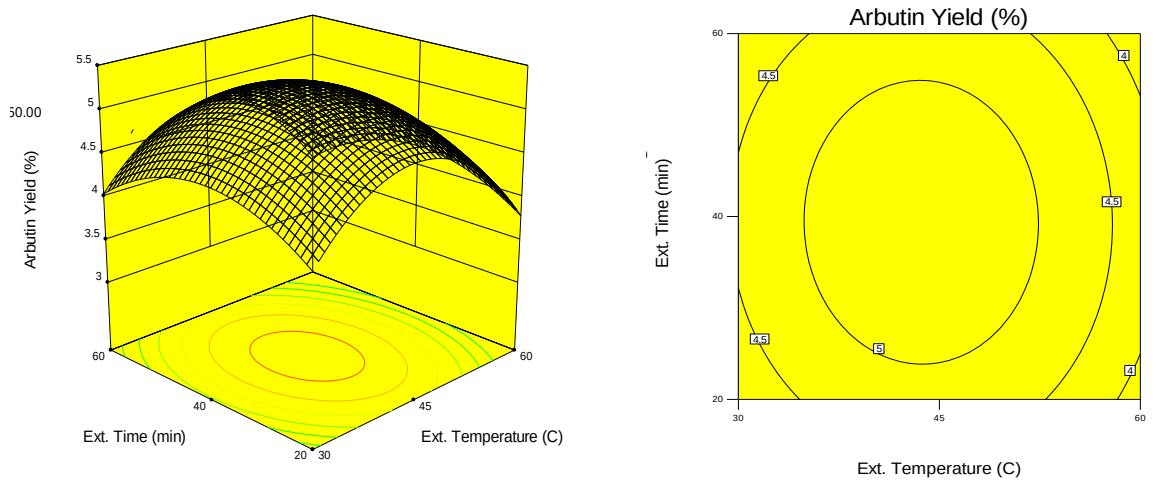


**Fig. 7** Three-dimensional **r**esponse surface and contour plots for arbutin extraction showing the interactive effects of the extraction time and extraction temperature.

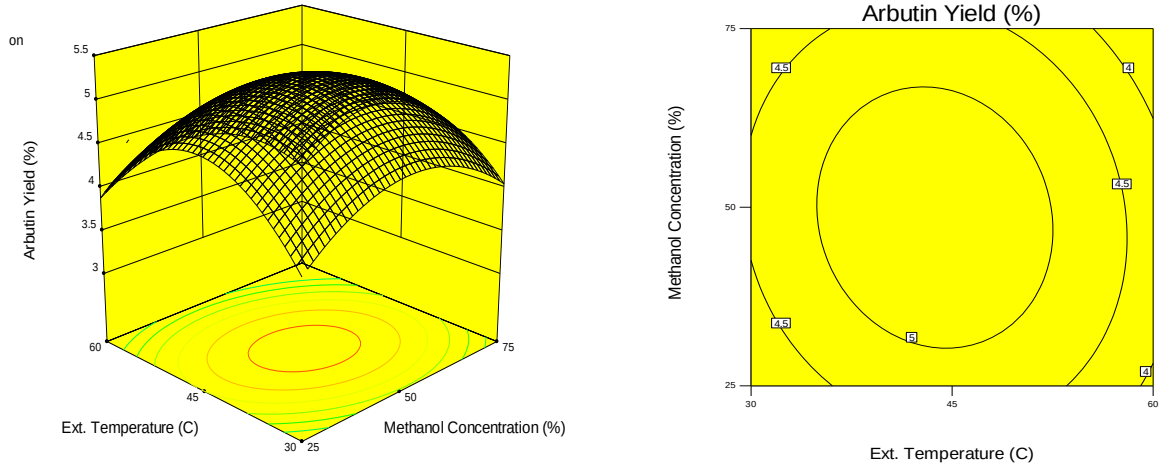


**Fig. 8** Three-dimensional **r**esponse surface and contour plots for arbutin extraction showing the interactive effects of the methanol concentration and extraction temperature.

The optimal extraction conditions were found by using optimization choice in design expert software to maximize the response. This value was measured at 48.54 of methanol concentration, 39.32 min of extraction time, 43.71 $^0$C of extraction temperature. The maximum response was found as (5.30 %) 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: 5.37 %
Standard Deviation: 0.04
Relative Standard Deviation: 0.35
Arbutin Yield (mg / 200 mg sample): 5.37 ± 0.04

### *3.3 Model fitting*

The analysis of variance (ANOVA) for the quadratic equations of Design Expert 9 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 9 for the response is given in Table 4. Regression analysis was done at 95 % of confidence interval. F-value of the obtained model is 47.21 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 extraction temperature (X1) is the most significant variable on the response. The 'F-value' of 'Lack of fit' (6.86) shows that the lack of fit is significant.

In our study, R-Squared (0.9838); Adj R-Squared (0.9630) and Pred R-Squared (0.7787) values for EY display good accuracy of the derived model. Thus, the response surface modeling can be achieved sufficiently to predict EY from *Pyrus elaeagnifolia ssp. elaeagnifolia* with UAE. Also, the coefficient value of variation (C.V. %) is found as 2.87 respectively. The lower coefficient of variation value indicates a higher precision and reliability of the experimental results [17].

**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 | 6.69 | 9 | 0.740 | 47.21 | < 0.0001 | significant |
| X1-Ext. Temperature | 0.200 | 1 | 0.200 | 13.01 | 0.0087 | significant |
| X2-Ext. Time | $8.450\ 10^{-3}$ | 1 | $8.450\ 10^{-3}$ | 0.540 | 0.4875 | |
| X3-Methanol Concentration | 0.048 | 1 | 0.048 | 3.050 | 0.1241 | |
| X1X2 | $6.25\ 10^{-4}$ | 1 | $6.25\ 10^{-4}$ | 0.040 | 0.8477 | |
| X1X3 | 0.076 | 1 | 0.076 | 4.810 | 0.0645 | |
| X2X3 | $4.225\ 10^{-3}$ | 1 | $4.225\ 10^{-3}$ | 0.270 | 0.6203 | |
| $X1^2$ | 3.300 | 1 | 3.300 | 206.89 | < 0.0001 | significant |
| $X2^2$ | 1.060 | 1 | 1.060 | 67.08 | < 0.0001 | significant |
| $X3^2$ | 1.370 | 1 | 1.370 | 87.15 | < 0.0001 | significant |
| Residual | 0.110 | 7 | 0.016 | | | |
| Lack of Fit | 0.062 | 3 | 0.031 | 6.86 | 0.0468 | significant |
| Pure Error | 0.018 | 4 | $4.48\ 10^{-3}$ | | | |

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

$$A = -7.03375 + 0.36363\ X1 - 3.93667\ 10^{-3}\ X1^2 - 1.25188\ 10^{-3}\ X2^2 - 9.13200\ 10^{-4}\ X3^2 \quad (3)$$

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 R2' is 0.9630 and the 'predicted R2' was 0.7787, which indicates that the model was good (for a good statistical model, the R2 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 19.04, 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 = 2.87 %) 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 elaeagnifolia* ssp. *elaeagnifolia* 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 5 exhibits the corelation between the experimental and predicted data calculated from Equation 2 concerning the EY of *Pyrus elaeagnifolia* ssp. *elaeagnifolia* 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 chromatogram of arbutin standard solution. Figure 10 exhibits chromatogram of Pyrus elaeagnifolia ssp. elaeagnifolia leaves extract.

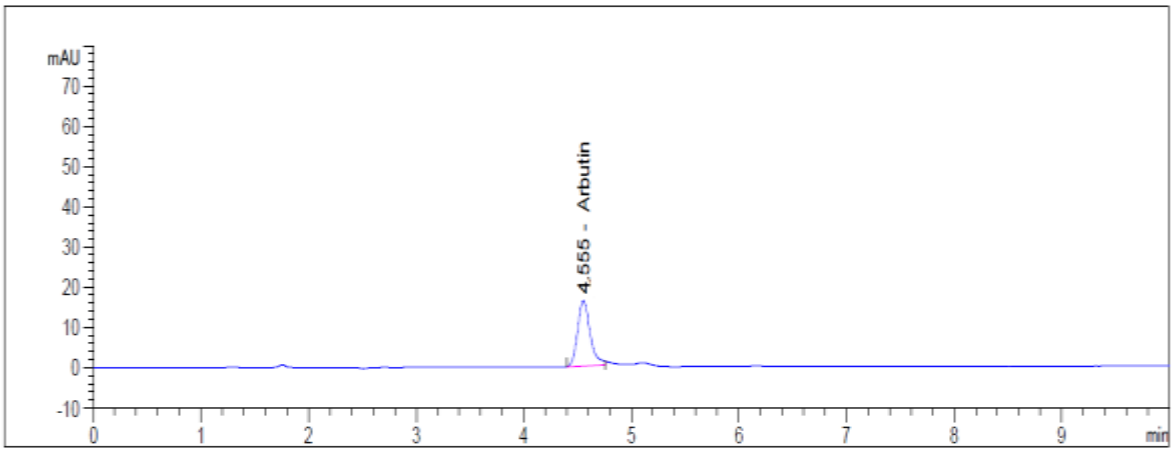


**Fig. 9** Chromatogram of arbutin standard solution (Concentration: 150 ppm)

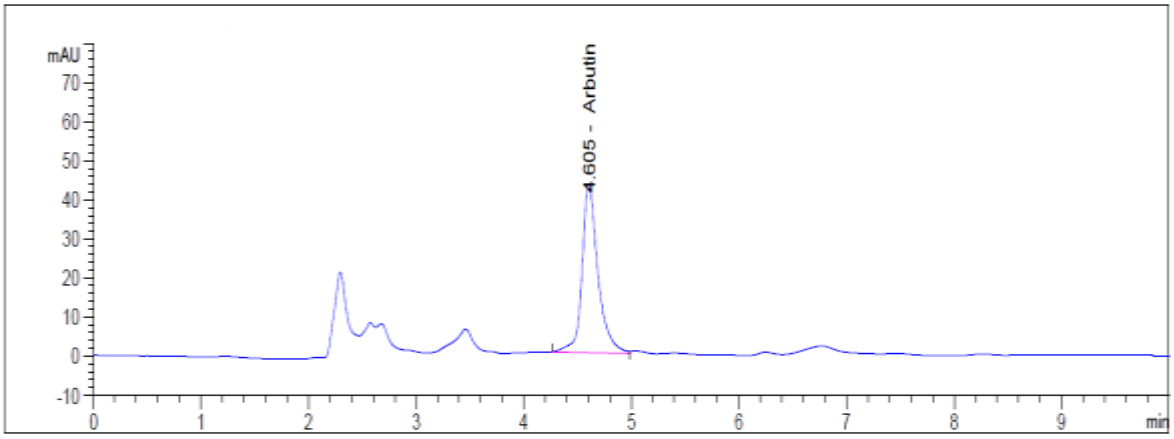


**Fig. 10** Chromatogram of *Pyrus elaeagnifolia* ssp. *elaeagnifolia* leaves extract.

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

**Table 5.** The results of arbutin analyses of leaves, fruit and branches of *Pyrus elaeagnifolia* ssp. *elaeagnifolia*.

| Source | Arbutin % |
|---|---|
| Leaves | 5.37 |
| Branches | 4.29 |
| Fruits | 0.055 |

## IV. Conclusions

Response surface methodology was successfully used to investigate the optimum extraction parameters for extraction of arbutin from *Pyrus elaeagnifolia* ssp. *elaeagnifolia leaves*. To optimize various parameters for extraction of arbutin from *Pyrus elaeagnifolia* ssp. *elaeagnifolia 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 elaeagnifolia* ssp. *elaeagnifolia leaves* was found to be extraction time (39.32 minutes), temperature (43.71 °C) and solvent composition (48.54 % 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 elaeagnifolia* ssp. *elaeagnifolia leaves* was 5.37 % 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 (%): 5.37 ± 0.04. Results is appropriate for the statistical evaluation.

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**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**REFERENCES**

[1.] Grin. "*Pyrus elaeagnifolia ssp. elaeagnifolia* Pall.". *Taxonomy for Plants*. National Germplasm Resources Laboratory, Beltsville, Maryland: USDA, ARS, National Genetic Resources Program. Retrieved January 29, 2014. (May 10, 2012).(https://en.wikipedia.org/wiki/Pyrus_elaeagrifolia.15.01.2016)

[2.] Zargari, A. Medicinal plants (6th ed.). Tehran: Tehran University Publications. (1996).

[3.] Azadbakht, M., Marston, A., Hostettmann, K., Ramezani, M., & Jahromi, MBiological activity of leaf extract and phenolglycoside arbutin of Pyrus boissieriana Buhse. Journal of Medicinal Plants, 3, 9–14. (2004).

[4.] Rychlinska,İ., Nowak, S. 'Quantitative Determination of Arbutin and Hydroquinone in Different Plant Materials by HPLC' *Notulae Botanicae Horti AgrobotaniciCluj-Napoca*, Not Bot Horti Agrobo, 40(2): 109-113. 2012.

[5.] Funayama M, Arakawa H, Yamamoto R, Nishino H, Shin T, Murao S. Effects of alpha- and beta-arbutin on activiety of tyrosinases from mushroom and mouse melanoma. Biosci. Biotechnol. Biochem. 1995;59:143–144.

[6.] Nihei K, Kubo I. Identification of oxidation product of arbutin in mushroom tyrosinase assay system. Bioorg. Med. Chem. Lett. 2003;13:2409–2412.

[7.] Nishimura T, Kometani T, Okada S, Ueno N, Yamamoto T. Inhibitory effects of hydroquinone-alpha-glucoside on melanin synthesis. Yakugaku Zasshi (in Japanese). 1995;115:626–632.

[8.] Sugimoto K, Nishimura T, Nomura K, Sugimoto K, Kuriki T. Inhibitory effects of alpha-arbutin on melanin synthesis in cultured human melanoma cells and a three-dimensional human skin model. Biol. Pharm. Bull. 2004;27:510–514.

[9.] Tomita K, Fukuda M, Kawasai K. Mechanism of arbutin inhibitory effect on melanogenesis and effect on the human skin with cosmetic use. Fragrance J. 1990;18:72–77.

[10.] Petkou, D., Diamantidis, G., & Vasilakakis, M. Arbutin oxidation by pear (Pyrus elaeagnifolia ssp. elaeagnifolia L.) peroxidases. Plant Science, 162(1), 115–119. (2002).

[11.] Myagmar, B. E., Shinno, E., Ichiba, T., & Aniya, Y. Antioxidant activity of medicinal herb Rhodococcum vitis-idaea on galactosamine-induced liver injury in rats. Phytomedicine, 11(5), 416–423.(2004)

[12.] Cho JY, Park KY, Lee KH, Lee HJ, Lee SH, Cho JA, Kim WS, Shin SC, Park KH, Moon JH. Recovery of arbutin in high purity from fuit peels of pear (Pyrus pyrifolia Nakai). Food Sci. Biotechnol. 2011;20:801–807.

[13.] Lee BD, Eun JB. Optimum extraction conditions for arbutin from asian pear peel by supercritical fluid extraction (SFE) using Box-Behnken design. J. Med. Plants Res. 2012;6:2348–2364.

[14.] Azadbakht M, Marstonm A, Hostettmann K, Ramezani M, Jahromi M. Biological activity of leaf extract and phenolglycoside arbutin of Pyrus boissieriana Buhse. J. Med. Plants.2004;3:9–14.

[15.] Shahaboddin ME, Pouramir M, Moghadamnia AA, Parsian H, Lakzaei M, Mir H. Pyrus biossieriana Buhse leaf extract: An antioxidant, antihyperglycaemic and antihyperlipidemic agent. Food Chem. 2011;126:1730–1733.

[16.] Cui T, Nakamura K, Ma L, Li JZ, Kayahara H. Analyses of arbutin and chlorogenic acid, the major phenolic constituents in oriental pear. J. Agric. Food Chem. 2005;53:3882–3887.

[17.] Pavlović R.D, Lakušić B, Došlov-Kokoruš Z, Kovačević N. Arbutin content and antioxidant activity of some *Ericaceae* species. Pharmazie 64:656-659.2009

[18.] Glöckl I, Blaschke G, Veit M . 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):261-266.2001.

[19.] Pyka A, Bober K, Stolarczyk A Densitometric determination of arbutinin cowberry leaves (*Vaccinium Vitis-idaeae*). Acta Pol Pharm 63(5):395-400.2007.

[20.] Lamien-Meda A, Lukas B, Schmiderer C, Franz Ch, Novak J. Validation of a quantitative assay of arbutin using gas chromatography in *Origanum Majorana* and *Arctostaphylos uva-ursi* extracts. Phytochem Anal 20:416-420.2009.

[21.] Asaaf M, Ali A, Makboul M, Beck JP, Anton R . Preliminary study of phenolic glycosides from *Origanum majorana*; quantitative estimation of arbutin; cytotoxic activity of hydroquinone. Planta Med 53:343-345.1986.

[22.] Parejo I, Viladomat F, Bastida J, Codina C . A single extraction step in the quantitative analysis of arbutin in bearberry (*Arctostaphylos uva-ursi*) leaves by HPLC. Phytochem Anal 12(5):336-339.2001.
[23.] Wettasinghe, M., & Shahidi, F. 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. 1999.
[24.] Cacace, J. E., & Mazza, G. Optimization of extraction of anthocyanins from black currants with aqueous ethanol. Journal of Food Science, 68, 240–248.2003a.
[25.] Cacace, J. E., & Mazza, G. Extraction of anthocyanins and other phenolics from black currants with sulfured water. Journal of Agricultural and Food Chemistry, 50, 5939–5946. 2002.
[26.] Haaland, P. O. Experimental design in biotechnology. New York: Marcel Dekker.1989.
[27.] Box, G. E. P., & Wilson, K. B. (1951). On the experimental attainment of optimum conditions. Journal of the Royal Statistical Society, 13,1–45.1951.
[28.] Myers, R. H., & Montgomery, D. C. Response surface methodology: Process and product optimization using designed experiments (2nd ed.). New York: Wiley.2002.
[29.] Cacace, J. E., & Mazza, G. Mass transfer process during extraction of phenolic compounds from milled berries. Journal of Food Engineering, 59, 379–389. (2003b).
[30.] Parajo, J. C., Santos, V., Dominguez, H., & Vazquez, M. NH4OH-based pretreatment for improving the nutritional quality of single-cell protein (SCP). Applied Biochemistry and Biotechnology, 55, 133–150. (1995).
[31.] Senanayake, S. P. J. N., & Shahidi, F. 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. (1999).
[32.] Senanayake, S. P. J. N., & Shahidi, F. Lipase-catalyzed incorporation of docosahexaenoic acid (DMA) into borage oil: optimization using response surface methodology. Food Chemistry, 77, 115–123. (2002).
[33.] Telez-Luis, S. J., Moldes, A. B., Alonso, J. L., & Vazquez, M. Optimization of lactic acid production by Lactobacillus delbrueckii through response surface methodology. Journal of Food Science, 68, 1454–1458. (2003).
[34.] Vasquez, M., & Martin, A. Optimization of Phaffia rhodozyma continuous culture through response surface methodology. Biotechnology and Bioengineering, 57, 314–320. (1998).
[35.] Gao, L., & Mazza, G. Extraction of anthocyanin pigments from purple sunflower hulls. Journal of Food Science, 61, 600–603. (1996).
[36.] Ge, Y., Ni, Y., Yan, H., Chen, Y., & Cai, T. Optimization of the supercritical fluid extraction of natural vitamin E from wheat germ using response surface methodology. Journal of Food Science, 67, 239–243. (2002).