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Optimizing the Extraction of Antioxidant Components from Grape (*Vitis vinifera L.*) Skin by Ultrasonic Pre-treatment

Saadat Azizi[1], Seyyed Moein Nazari[2*], Leila MoradiHaghgou[3]

1,2- Department of food science and technology, Faculty of engineering, Hamedan Branch, Islamic Azad university, Hamedan, Iran.

3-Department of Microbiology, Faculty of Basic Science, Hamedan Branch, Islamic Azad university, Hamedan, Iran.

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*Corresponding Author E-Mail:
seyyedmoeinnzr525@gmail.com

ABSTRACT

Grapes are one of the most important agricultural products which could be used either as a fresh fruit or a processed food. In Iran, grapes are very crucial agricultural and commercial products. Every year, a massive volume of grapes waste is made by the units of grape Doshab producers, so the producers are engaging with many problems of waste disposal. Grapes waste is a valuable source of natural pigment and antioxidant compounds specifically polyphenols compounds. Extracting antioxidant compounds from cheap primary materials like grapes waste is a suitable method for various food and pharmaceutical industries. In this research and aim to finding optimum state for extracting phenolic compounds from Shani grapes skin, response surface method (RSM) and central composite design (CCD) with 3 independent variables (extraction time using ultrasonic bath, liquid to solid phase ratio and ethanol solvent concentration) were applied. The optimum condition for extraction of this compound was 59.06% concentration of ethanol, 16.08 ml/g liquid to solid phase ratio and 24.12 min extraction time. The research results have shown that the extract of Shani grapes skin contains 8 different phenolic compounds, which among them epigallocatechin and catechin had highest concentration. Consequently, by finding optimum condition of phenolic compounds extraction, these compounds could be produced in pilot scale for utilizing in food and pharmaceutical industries.

1. Introduction

Grapes are one of the most important agricultural products in the world. They could be used as a fresh fruit or as a processed food such as fruit juice, raisins, jelly and jam. Grapes are rich in phenolic compounds and 85% of these compounds exist inside grapes seed and skin. Increasing industrial food production has significantly raised the rate of food waste. Fruits and vegetables processing makes massive amounts of by-products like stems, leaves, skins, and seeds which are discarded as waste [1; 2; 3]. Different factors such as grape type, planting, harvesting conditions and type of processing can affect the chemical compounds of grape by-products [4].

During some processes like Doshab (Doshab is a concentrated and shelf-life extended form of grape juice, a traditional concentrated grape juice), grapes are compressed, but the chemical structure of bioactive compounds does not change significantly, and a significant amount of these compounds is remained in grape pomace [4; 5]. In a short period of harvest and product processing, large amounts of grape pomace are produced and accumulated in each region. Consequently, there are various compounds such as phenolic compounds in grape pomace; Burning or even burying waste could damage the environment. These compounds can reduce the pH of the environment and increase the resistance biological degradation. Another problem of the accumulation of these wastes is the pollution of surface and underground water. If accumulated piles of grape pomace do not properly treated and processed, they can attract flies and pests and spread diseases. The effluents produced from these wastes are responsible for the reduction of soil oxygen, penetration to the surface, soil and underground water [4].

Grape wastes are rich in phenolic compounds and are applied for enrich animal food or producing fertilizer in order to increase soil fertility or producing ethanol for alcoholic beverages [2]. Phenolic compounds are one of the most widespread natural compounds found in plants. One of the most important health effects of fruits and vegetables is because of phenolic compounds. Phenolic compounds have a wide range of physiological effects such as anti-allergic, anti-inflammatory, antimicrobial, antioxidant and heart protection effects. Due to economic and environmental reasons, a large amount of by-products have been studied to recover valuable compounds [4]. In addition, the raise in universe population and the limitation in natural resources have turned the attentions to renewable resources and the designing extractive processes, like the recovery of valuable compounds of grapes [6]. Around the world, about 5-9 million tons of grapes waste are made and discarded, while they could be used in the food industry, cosmetics, pharmaceutical industries, and bio pesticides [2]. Besides, antioxidant phenolic compounds could be utilized for production food preservatives or food supplements [6]. Feasibility of industrial processes of extraction needs to investigation, research and study. Some factors are effective in selecting industrial processes which include the optimizing the quantities that have a direct effect on the process, the correct selection of the raw materials that are extracted and exposing these raw materials to appropriate pretreatments [6]. One of the effective pre-treatments in the extraction process is applying ultrasonic technology, which reduces extraction time and solvent consumption, increases extraction efficiency and improves the quality of the extract. Due to simplicity and low cost, ultrasound extraction technique is considered an attractive technique. The basis of ultrasound method is using ultrasound energy (with a frequency of 20

KHZ) to facilitate the extraction of analytes from solid samples by solvent, and the choice of solvent depends on the nature of the compound that is extracted. This technique is used to extract organic compounds from different matrices, and by using this technique, the extraction time is reduced by 3 to 10 times [7]. By causing cavitation, ultrasound waves destroy cell membranes and facilitate the penetration of fluid into the cell. Also, by increasing the contact surface of solid particles with the solvent, they reduce the extraction time of phenolic compounds [8].

Novak et al (2008) utilized ultrasound to extract flavonoid compounds from grape skin. The extraction time was 15-30 minutes depending on the type of extracted flavonoid compound. Ghafoor et al (2009) used ultrasound waves to extract phenolic compounds and anthocyanin compounds from grape seed, and the extraction time in this method was 50 minutes. Ghassempour et al (2008) used ultrasonic method to extract anthocyanin compounds from black grape skin; the results of their research showed that the ultrasonic method is a more effective extraction method than the maceration method, and it requires less time and less extracting solvent. Mirbagheri et al (2017) investigated the phenolic content and antioxidant properties of the seeds extract from 3 different types of grapes grown in Iran. The results of their research showed that the highest and lowest amount of phenolic compounds were related to black grape seed extract and green grape seed extract, respectively. In addition, Farhadi et al (2016) investigated the phenolic compounds and antioxidant activity of different types of grapes in West Azerbaijan region of Iran. The results of their research showed that black grape skin (Ghara Shani) has the highest antioxidant activity, phenolic and flavonoid compounds. Caldas et al (2018) investigated the effect of parameters (ethanol concentration, extraction time and solid-to-liquid phase ratio) and different extraction methods such as traditional extraction methods and extraction by ultrasonic and microwave waves on the extraction of phenolic compounds from grape skin. The results of their research demonstrated that extraction by ultrasound had the best performance in extracting phenolic compounds from grape skin and more extract was obtained in a shorter time than traditional extraction methods such as stirring.

Li et al (2019) optimized the conditions of extracting antioxidant compounds from black grape skin. The extraction parameters included the concentration of ethanol as the extracting solvent, temperature and extraction time. The highest yield of phenolic compounds, anthocyanin content and total phenolic compounds happened at 50.79°C, ethanol concentration 48.8% and extraction time 14.82 min.

The extraction efficiency depends on the process conditions. Previous studies have shown that parameters such as temperature, contact time, ratio of solvent to solid materials are effective on the efficiency of extracting phenolic compounds from natural sources [6]. In plant sources, the choice of extraction solvent is very important because the solvent determines the type and amount of extracted phenolic compounds. Liquid solvents such as Acetone, Ethanol and Methanol have been widely used to extract phenolic compounds from plant sources. Ethanol is the best solvent for extracting phenolic compounds used in the food industry due to its low toxicity, high safety, environmental friendliness, and the ability to recycle and reuse it [2].

Grapes are considered as one of the most important horticultural and commercial products of Iran and Hamedan region. In addition and over the economic value, this product is very attending in terms of nutrition and functional compound. Phenolic compounds in grapes are bioactive compounds that

have positive effects on human health. These phenolic compounds are widely applied in food and pharmaceutical industries. Annually, large amounts of grape pomace waste are created due to the production of grape Doshab, and the traditional and industrial producers of grape Doshab are engaged with many problems to dispose of the waste. Since grapes are not chemically altered and are not fermented in the process of preparing grape Doshab, therefore remained grape pomace from Doshab making is a valuable source of phenolic compounds. Extracting valuable antioxidant compounds from cheap raw materials such as waste and grape residue is a suitable method for producing these products. So far, there has not been a comprehensive study on the antioxidant compounds of produced Shani grape pomace in Hamedan province. The main goal of this study is finding the optimal conditions for extracting phenolic compounds from Shani grapes skin, which produced in Doshab processing, in Hamadan province. In this research, to optimize the extraction conditions the response surface method (RSM) and central compound design (CCD) with three independent variables (extraction time using ultrasonic bath, liquid to solid phase ratio and ethanol solvent concentration) , in 5 levels and 6 repetitions, around The central point of the design were applied. By investigating the optimal conditions of extracting phenolic compounds and identifying their exact components, these compounds can be prepared on a Pilot scale and provided to various food and pharmaceutical industries.

2-Material & methods

All chemicals, standards and reagents used in this research were obtained from Sigma and Merck Company with the highest quality.

2-1 Raw Material Preparing

Shani grapes, from *Vitis vinifera L.* variety, were obtained from the vineyards of Hamadan province. First, the stems and leaves were separated from the grapes. After washing the grapes by water, they were placed in strainers until their water was drained, then they were placed in cloth bags and the grape juice was extracted by pressing. The remained pomace was dried in the shade and exposed to air flow. The grape seeds were separated from other components of the pomace. The resulting pomace, which mainly contained Shani grapes skin, was ground and passed through a sieve with 1 mm mesh and kept at -20 C until final use.

2-2 Experiment design

The optimal conditions for extracting Shani grape pomace were designed by using Design Expert 13 (DOE) software. The investigated method was the response surface (RSM) using a central composite design (CCD) with three independent variables. Extraction time was indicated by using an ultrasonic bath (1-30 min), liquid to solid phase ratio (40-10 ml/g) and the solvent concentration of ethanol (0-96%)) was done in 5 levels and 6 repetitions around the central point of the design. The total number of treatments was 20 and the dependent variables included the total amount of phenolic compounds, DPPH free radical inhibition power, the amount of flavonoid compounds and the amount of monomeric anthocyanin compounds (table 1).

2-3 Extraction method of Shani grapes skin extract

Extraction was done based on the variables of solvent concentration, liquid to solid phase ratio and extraction time by ultrasonic waves, (ultrasonic device SOLTEC, EP2200, Italy) at a fixed frequency of 20 kHz according to the experiment plan of this research (Table 2). Then, by a refrigerated centrifuge (Universal, PIT320 R, Iran) with a speed

of 3000 g and at a temperature of 4°C, solid particles were separated from the liquid extracts, and the separated clear liquid was passed through a filter paper and used for the next tests.

2-4 Measurement of total Phenolic compounds

The amount of Phenolic compounds in Shani grapes skin was determined by Folin–Ciocalteu method and according to the modified method [13]. In this method, 1 ml of Folin reagent (v/v) 10% was added to 40 µl of extract and mixed well, then it was incubated at room temperature for 6 min. In the next step, 0.5 ml of 7.5% Sodium carbonate was added to it and vortexed well. Then it was incubated at room temperature and in a dark place for 30 min, then absorbance of the samples was read by a spectrophotometer (Jenway™ 6305 UV/Visible Spectrophotometer, England) at a wavelength of 760 nm. The spectrophotometer was calibrated by water. The amount of total Phenolic compounds was calculated according to the Gallic acid standard curve. The standard curve was drawn based on different concentrations of Gallic acid (0, 20, 40, 60, 80 and 100 µg/ml). The standard equation for Gallic acid was $y = 0.0069x + 0.0135$ and $R^2 = 0.998$.

2-5 Measurement of monomeric Anthocyanin compounds

The amount of monomeric Anthocyanin compounds was determined according to the modified method [15]. This method is based on changing the structure of Anthocyanin compounds at different pH. In this method, 4 ml of buffer with pH = 1 was added to 0.2 ml of extract and after complete vortexing, it was kept at room temperature and in a dark place for 60 min. The mentioned steps were repeated again for buffer pH = 4.5. Then, the absorbance of the samples was read by a spectrophotometer that was calibrated by water at wavelengths of 530 nm and 700 nm. The concentration of monomeric anthocyanin compounds was calculated based on the difference in absorption at different pHs and wavelengths and using the following formula in terms of grams of cyanidin-3-glucoside equivalent per liter (g eq cy-3-glu/L) of the extract:

Formula 1- The formula of calculating monomeric Anthocyanin compounds

$$A = (A_{530nm} - A_{700nm})_{pH1.0} - (A_{530nm} - A_{700nm})_{pH4.5}$$

$$TMAC = \frac{A * MW * DF * 1000}{\varepsilon * l}$$

In this formula, A is the light absorption, MW is the molecular weight of Cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor, ε is the molar absorptivity of Cyanidin-3-glucoside (26900 L cm^{-1} mol^{-1}), and l is the light transmission length(which l is for a standard 1 cm path length).

To prepare a buffer of pH = 1, 1.86 grams of Potassium chloride (Kcl) was added to distilled water until the solution volume became equal to 1000 cc which resulted Kcl solution with concentration of 0.025 M. Then, the pH of the buffer reached 1 by adding dropwise hydrochloric acid (Hcl).

To prepare a buffer with pH = 4.5 for this experiment, first 54.43 grams of Sodium acetate (C2H3NaO2) was added to distilled water, totally up to volume of 1000 cc until 0.4 M solution of Sodium acetate (C2H3NaO2) was maintained. Then, the pH of the buffer reached 4.5 by adding dropwise hydrochloric acid (HCl).

2-6 Measurement of Flavonoid compounds

The amount of Flavonoid compounds was measured according to the method [16]. In this method, 0.5 ml of the extract was mixed with 1.5 ml of 96% Ethanol, 0.1 ml of Aluminum chloride 10% (AlCl3.6H2o), 0.1 ml of Sodium acetate 1M (C2H3NaO2) and 250 ml of distilled water

respectively, and vortexed well. Then, the absorbance of the samples was measured at 415 nm by a spectrophotometer. To calculate the concentration of Flavonoid compounds, the standard curve of Quercetin was used and the amount of Flavonoid compounds was expressed as mg Quercetin per L of the extract. All absorbance were read in triplicate. The standard Quercetin equation was $y = 0.0081x + 0.0341$ and $R^2 = 0.990$.

2-7 Calculation of antioxidant capacity (according to DPPH free radical inhibition power)

Total antioxidant activity was measured by inhibiting DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals according to the method [4]. In this method, 0.2 ml of the extracted extract was combined with 3.8 ml of 0.1 mM DPPH ethanolic solution and vortexed for 1 min. Then the solution was kept at room temperature and in a dark place for 30 minutes and the absorbance of the sample was read at the wavelength of 517 nm.

The inhibition percentage of the compounds was calculated using the following formula.

Formula 2- The formula for calculating the inhibitory percentage of antioxidant compounds

$$\%Inhibition = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} * 100$$

The absorption value of the control sample was based on the absorption value of the extraction solvent. To prepare DPPH solution, 4 mg of DPPH powder was dissolved in 100 ml of 99% ethanol and an ultrasonic bath was applied for completing the dissolution of DPPH powder. Then the container of DPPH solution was covered with aluminum foil and kept in a cool and dark place until usage time. It is better to prepare the DPPH solution fresh every day. Pure DPPH solution is purple in color. As the concentration of antioxidant compounds increases; The DPPH solution color becomes fade and turns yellow.

2-8 Determining type and amount of phenolic compounds

The phenolic compounds of grape skin extract, which extracted under optimal conditions, were studied by HPLC (HP 1100, Agilent, Waldbronn, Germany) based on the modified method of [17]. The column which used was type C18, the length of the column was 25 cm, the size of the base particles was 5 µm, and the experiment was performed at a temperature of 35 °C. In this regard, the extract was evaporated by a rotary evaporator at lower than 35 °C temperature, then dissolved in 20 ml of the mobile phase and became a solution. The analyzed compounds included Quercetin, Hesperidin, Gallic acid, Catechin, Epigallocatechin, Epicatechin, Epigallocatechin gallate and Epicatechin gallate. The flow rate was equal to 1 ml/min, the composition of the mobile phase included Water, Acetic acid and Acetonitrile. Solvent A included (water/acetic acid, 97.5/2.5) and solvent B included (acetonitrile/solvent A, 80/20). The washing gradient at the time of 0-50 min was solvent B (0-44) %, at the time of 50 - 55 min, it included solvent B (100-44) % and B 100% as isocratic for 5 minutes.

3- Results and discussion

3-1 Total phenolic compounds

The amount of phenolic compounds of extracts obtained from different extraction conditions was from 200.217 µg/ml to 649.493 µg/ml. This research analysis results have shown that the amount of phenolic compounds was increased by raise in the amount of ethanol up to 48% and then was decreased (Figure 1-a), which is consistent with the results of the research [2]. In the research Li et al (2019), raise

in ethanol concentration from 20% to about 50% has heightened first and then lowered the amount of extracted phenolic compounds. Increase in liquid to solid phase reduced the amount of extracted phenolic compounds which was probably due to the decrease in the concentration of extracted phenolic compounds per unit volume of solvent. Another reason for this decrease is because of destructive effects of cavitation on phenolic compounds in high ratios of liquid to solid phase [18]. Longer extraction time, the amount of extracted phenolic compounds has been heightened. Caldas et al (2018) have stated that ethanol with a concentration of 50% is the most effective solvent for extracting phenolic compounds from grape skins.

At the constant time of extraction (15. 5 min), the amount of extracted phenolic compounds at first has been increased by raise in concentration of ethanol up to 48%, but then by increase in ethanol concentration, the amount of extracted phenolic compounds has been lowered (Figure1-b), which is in line with the research results of [19]. More concentration of ethanol in the extracting solvent, the polarity of the solvent is probably reduced and less phenolic compounds are extracted [20]. Besides, probably in high concentrations of ethanol, plant cells are dehydrated and destroyed and cell wall proteins are destroyed, and this issue has reduced the release of polyphenolic compounds from plant materials to the extracting solvent [21]. In the constant time of extraction (min 15. 5) by increase of the ratio of liquid to solid phase from 16 ml/g to 33.91 ml/g, the amount of phenolic compounds extracted has been decreased (Figure1-b). In the research [22], the concentration of phenolic compounds extracted from ground berries was increased by raise in the ratio of liquid to solid phase.

At the ratio of liquid to solid phase of 25 ml/g, the amount of extracted phenolic compounds was not changed much due to longer extraction time from 6.87 min to 24.12 min. The research result [22] has shown that the amount of phenolic compounds was not changed by the increase of extraction time in the presence of ultrasonic waves (Figure 1-c). Also at the constant ratios of liquid to solid phase of extraction (25 ml/g), the rate of extracted phenolic compounds has been heightened by more concentration of ethanol up to 48%, and after that, the amount of extracted phenolic compounds has been reduced due to increase of ethanol concentration (Figure 1-c). The result is consistent with the research result [22].

At a constant concentration of ethanol (48%), higher extraction time has raised the rate of extracted phenolic compounds, while increase in the ratio of liquid to solid phase has reduced extracted phenolic compounds amount (Figure 1-d). The final model resulting from the coded factors for total phenolic compounds is based on the following formula (Table 3):

Formula 3 - Final model resulting from coded factors for total phenolic compounds

$$Total\ phenolic\ compound = 525 + 5.42A - 73.77B + 62.44C + 43.21AB + 62.29AC + 27.27BC - 109.17A^2 - 19.21C^2$$

A = solvent concentration, B = ratio of liquid to solid phase, C = extraction time

The amount of monomeric Anthocyanin compounds in the extracts from different extraction conditions was from 42.78 g/l to 264.76 g/l. By the increase of ethanol concentration, the amount of extracted

3-2 Monomeric Anthocyanin compounds

anthocyanin compounds first was increased and then was decreased. Raise in the liquid-to-solid phase ratio, the extracted monomeric anthocyanin compounds was reduced (Figure 2-a), which is probably due to the decrease in the concentration of the extracted anthocyanin compounds per unit volume of the solvent or the destructive effects of cavitation caused in the high liquid-to-solid phase ratio[18]. Longer extraction time did not change much the amount of these compounds (Figure 2-a). At the constant time of extraction (15.5 min) the rate of monomeric anthocyanin compounds was not differed much by increase in the ratio of liquid to solid phase (Figure 2-b). He et al (2016) stated that the amount of anthocyanin compounds extracted from blueberry pomace was lowered due to higher ratio of liquid to solid phase during the constant extraction time. At the constant time of extraction (*15.5* min) by raise in ethanol concentration up to 62.27%, anthocyanin compounds first was increased and then was decreased (Figure 2-b). Probably, by increasing the concentration of ethanol in the extracting solvent, the polarity of the solvent is reduced and less anthocyanin compounds are extracted [20]. On the other hand, probably in high concentrations of ethanol, plant cells are dehydrated and cell wall proteins are destroyed, and this causes a decrease in the release of anthocyanin compounds from plant materials to the extraction solvent [21]. The final model resulting from the coded factors for monomeric anthocyanin compounds is based on the following formula (Table 3).

Formula 4 - The final model resulting from the coded factors for monomeric anthocyanin compounds

$$Total\ monomeric\ anthocyanin = 4.85 + 0.4082A - 0.3152B + 0.0499C - 0.4127A^2 + 0.0789B^2$$

A = solvent concentration, B = ratio of liquid to solid phase, C = extraction time

3-3 Flavonoid compounds

The amount of flavonoid compounds of the extracts from different extraction conditions was from 22.20 mg Quercetin/l to 62.58 mg Quercetin/l. The rate of extracted flavonoid compounds was lowered by increase of liquid to solid phase ratio. By increase of ethanol concentration and extraction time, more flavonoid compounds were extracted (Figure 3-a). Higher ethanol concentration at constant extraction time (15.5 min) heightened the extracted flavonoid compounds rate from 19.45% to 76.54%; However, more ratio of liquid to solid phase from 16.08 ml/g to 33.91 ml/g, the amount of extracted flavonoid compounds was reduced (Figure 3-b), which is probably due to the decrease in the concentration of flavonoid compounds per unit volume of the solvent or the destructive effects of cavitation which is occurred in the high ratio of liquid to solid phase [18]. At constant ethanol concentration (48%), the amount of extracted flavonoid compounds was increased from 6.78 min to 24.12 min due to longer extraction time (Figure 3-c). It is probably due to the fact that at higher extraction times, more flavonoid compounds are contaminated with extraction solvent. Higher liquid to solid phase ratio from 16.08 ml/g to 33.91 ml/g, the amount of extracted flavonoid compounds was decreased (Figure 3-c). The final model resulting from the coded factors for flavonoid compounds is based on the following formula (Table 3):

Formula 5 - The final model resulting from the coded factors for flavonoid compounds

$$Total\ Flavonoids = 28.12 + 4.30A - 8.83B + 4.38C - 3.67AB - 2.56BC + 6.27B^2$$

A = solvent concentration, B = ratio of liquid to solid phase, C = extraction time

3-4 Antioxidant capacity (according to DPPH free radical inhibition power)

The inhibition percentage of DPPH free radicals of extracts from different extraction conditions varied from 96.3% to 97.5%. By increasing the concentration of ethanol up to 48%, the inhibition of DPPH free radicals first was raised and then was decreased. More ratio of liquid to solid phase, the inhibition percentage of DPPH free radicals was lowered (Figure 4-a). It is probably due to the decrease in the concentration of extracted phenolic compounds per unit volume of solvent, or the cavitation created in high liquid-to-solid phase ratios had destructive effects on phenolic compounds and caused the decomposition of these compounds [18]. By increase of extraction time, the DPPH free radical inhibition rate increased (Figure 4-a), which is probably due to the release of more antioxidant compounds into the extraction solvent at higher contact times. The result of the research [23] has also shown that the amount of free radical inhibition has been increased due to longer extraction time. In the constant ratio of liquid to solid phase (25 ml/g), by raise in extraction time, the amount of free radical inhibition was not changed much; However, higher ethanol concentration up to 48%, the percentage of free radical inhibition was first heightened and then was reduced (Figure 4-b), which is consistent with the results of research [21& 2]. The final model resulting from the coded factors for DPPH free radical inhibition power is based on the following formula (Table 3):

Formula 6 - The final model resulting from the coded factors for DPPH free radical inhibition power

$$\text{Radical scavenger activity (DPPH)} = 97.06 - 0.0532\,A - 0.2372B + 0.1029C + 0.1938AC - 0.2733A^2$$

A = solvent concentration, B = ratio of liquid to solid phase, C = extraction time

3-5 Fitting Model

According to the results of the variance analysis of the data obtained from the dependent variables (total phenolic compounds, monomeric anthocyanin compounds, flavonoid compounds and DPPH free radical inhibition power), the Quadratic polynomial equation was the best proposed model for investigating the influence of independent variables (Ethanol concentration, liquid to solid phase ratio and extraction time). The necessary condition for fitting the selected model is that the model is significant (p value<0.05) and lack of fit is not significant (p value>0.05). The Predicted R^2 and Adjusted $R^2$are closer to each other and their difference is less than 0.2. Although Adeq Precision measures the signal to noise ratio. For a model to have an accurate prediction of a process, it must be (Adeq Precision>4) (Table 4).

3-6 Finding and validating optimum extraction condition

Maximum phenolic compounds (649.49 µg Gallic acid/ml), maximum flavonoid compounds (62.58 mg Quercetin/l) and maximum antioxidant capacity (97.5%) in extracts with 48% extracted ethanol concentration, liquid to solid phase ratio 10 ml/g and 15.5 min extraction time were observed. The maximum amount of flavonoid compounds (213.21 g eq cy-3-glu/L) was occurred in extracts with an ethanol concentration of 76.54%, the ratio of liquid

to solid phase of 16.08 ml/g and 24.12 minutes extraction time. The optimal conditions for extracting beneficial compounds from Shani grape skin were obtained in according to the maximum amount of phenolic, flavonoid and anthocyanin compounds, as well as the maximum antioxidant capacity calculated in according to (power to inhibit DPPH free radicals). The best concentration of ethanol for extracting phenolic, flavonoid and anthocyanin compounds was 59.06%, the ratio of liquid to solid phase was 16.08 ml/g, and the optimal extraction time was 24.12 min, and the desirability of these extraction conditions was 0.88. Due to validation of model formulas, additive experiments in optimum conditions were taken which admitted the model. The optimum conditions were amended to include 59% ethanol concentration, 16 ml/g liquid to solid phase, and extraction time of 24 min. this was done to account for operability in actual production. In this regard, relative error percentage between obtained and predicted values and relative standard deviation percentage of obtained values were calculated. The results have shown that the model could predict optimum extraction conditions properly (table 5)

3-7 Determination of the type and amount of phenolic compounds using HPLC

High performance liquid chromatography (HPLC) was used to identify and quantify phenolic compounds in grape skin extract. The evaluation results showed that Shani grapes skin extract contains 8 different phenolic compounds (Quercetin, Hesperidin, Gallic acid, Catechin, Epigallocatechin, Epicatechin, Epigallocatechin gallate and Epicatechin gallate). The highest concentration of phenolic compounds were related to Epigallocatechin (21.8 mg/kg) and then Catechin (13.4 mg/kg) respectively, while Epigallocatechin gallate with a concentration of 0.3 had the lowest amount of phenolic compound (Table 6). The results of [13] research has shown that the phenolic compounds of Catechin, Epicatechin and quercetin are existed in the skin of black grapes of the West Azerbaijan region of Iran, and the highest amount of phenolic compounds was related to Catechin and Epicatechin. The results of research [14; 25] have shown that grape skin extract contained some compounds such as quercetin, Catechin, and Epicatechin.

4-Conclusions

Massive amount of grape waste is being produced and discarded all over the world, which causes many environmental and economic problems. Therefore, the use of grape pomace waste into the food, cosmetics& pharmaceutical and bio pesticide industries is very important. In this research, the CCD design was applied to optimize the extraction of phenolic compounds from the skin of Shani grapes, moreover the effect of the parameters of the extraction time using an ultrasonic bath, the ratio of liquid to solid phase and the concentration of the ethanol solvent on the amount of phenolic, flavonoid and anthocyanin compounds and the antioxidant capacity (calculated in according to power to inhibit DPPH free radicals) were studied. The result of ANOVA analysis showed that the effects of all variables (extraction time using ultrasonic bath, ratio of liquid to solid phase and concentration of ethanol solvent) on the studied parameters were significant. Also, the Quadratic polynomial model was the best proposed a model for predicting the results of this research. The optimal extraction conditions for phenolic, flavonoid and anthocyanin compounds happened in ethanol concentration of 59.06%, liquid to solid phase ratio of 16.08, extraction time of 24.12 min, while the desirability of these extraction conditions was 0.88. The results of HPLC analysis showed that Shani grapes skin extract contains

various phenolic compounds, such as epigallocatechin and Catechin which have the highest concentration among phenolic compounds. Finally, this research showed that the skin and pomace of Hamadan Shani grapes are a good source of antioxidant compounds that can be extracted in optimal conditions and used in various food and pharmaceutical industries.

5- References

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Table 1: Real and coded parameters value in CCD extraction method

Factor	Name	Units	Type	Subtype	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Ethanol	%	Numeric	Continuous	0.0000	96.00	-1 ↔ 19.46	+1 ↔ 76.54	48.00	24.20

B	Liquid phase/Solid phase	ml/g	Numeric	Continuous	10.00	40.00	-1 ↔ 16.08	+1 ↔ 33.92	2 5.00	7 .56
C	time	min	Numeric	Continuous	0.0000	30.00	-1 ↔ 6.88	+1 ↔ 24.12	1 5.45	7 .42

Table 2: Experiment design matrix in CCD method and experiment results

Run	Ethanol (%)	Liquid phase/Solid phase (ml/g)	Time (min)	phenolic compound (µg/ml)	Total Monomeric Anthocyanin content(mg/l)	Flavonoids(mg/ml)	Radical scavenger activity(DPPH) %
1	48	40	15.5	444.42	86.6171	29.493	96.4
2	48	10	15.5	649.493	264.761	62.5802	97.5
3	96	25	15.5	200.217	75.0448	34.679	96.2
4	48	25	15.5	529.203	131.153	29.98	96.92
5	48	25	15.5	516.884	119.931	28.8765	97.11
6	76.541	16.0809	24.1218	560.362	213.211	56.5309	97.01
7	48	25	15.5	498.043	130.101	24.4321	97.05
8	48	25	15.5	536.449	104.852	28.3827	97.02
9	76.541	33.9191	24.1218	520.02	93.28	26.7778	96.9
10	48	25	15.5	534.275	143.076	29.123	97.4
11	19.459	16.0809	6.87825	532.101	83.1103	29	97.4
12	76.541	33.9191	6.87825	224.13	111.866	27.642	96.3
13	0	25	15.5	243.696	18.9365	22.2099	96.4
14	48	25	0	363.986	104.537	18.5062	96.77
15	76.541	16.0809	6.87825	342.246	182.352	45.0494	96.7
16	19.459	33.9191	6.87825	209.87	42.7825	24.1852	96.57
17	19.459	33.9191	24.1218	287.899	52.9522	26.037	96.64
18	48	25	15.5	505.29	140.271	24.0617	97.01
19	19.459	16.0809	24.1218	469.783	82.7597	39	96.69
20	48	25	30	571.957	140.271	41.0988	97.45

Table 3: ANOVA statistics analysis and statistics parameters results

Source	Sum of Squares	df	Mean Square	F-value	p-value	
			Total phenolic compound			
Model	3.570E+05	8	44624.26	60.18	< 0.0001	significant
A-Ethanol	400.81	1	400.81	0.5405	0.4776	
B-Liquid phase/Solid phase	74320.34	1	74320.34	100.22	< 0.0001	
C-time	54596.66	1	54596.66	73.63	< 0.0001	
AB	14934.87	1	14934.87	20.14	0.0009	
AC	31037.34	1	31037.34	41.86	< 0.0001	

BC	5947.10	1	5947.10	8.02	0.0163	
A^2	1.730E+05	1	1.730E+05	233.35	< 0.0001	
C^2	6023.90	1	6023.90	8.12	0.0158	
Residual	8156.94	11	741.54			
Lack of Fit	6889.70	6	1148.28	4.53	0.0593	not significant
Pure Error	1267.24	5	253.45			
Cor Total	3.652E+05	19				
			Monomeric Anthocyanin content			
Model	6.34	5	1.27	112.34	< 0.0001	significant
A-Ethanol	2.28	1	2.28	201.56	< 0.0001	
B-Liquid phase/Solid phase	1.36	1	1.36	120.18	< 0.0001	
C-time	0.0349	1	0.0349	3.09	0.1004	
A^2	2.48	1	2.48	219.53	< 0.0001	
B^2	0.0907	1	0.0907	8.03	0.0133	
Residual	0.1581	14	0.0113			
Lack of Fit	0.0925	9	0.0103	0.7835	0.6472	not significant
Pure Error	0.0656	5	0.0131			
Cor Total	6.50					
			Flavonoids			
Model	2329.40	6	388.23	47.87	< 0.0001	significant
A-Ethanol	252.72	1	252.72	31.16	< 0.0001	
B-Liquid phase/Solid phase	1064.71	1	1064.71	131.27	< 0.0001	
C-time	269.69	1	269.69	33.25	< 0.0001	
AB	107.92	1	107.92	13.31	0.0030	
BC	52.50	1	52.50	6.47	0.0245	
B^2	577.49	1	577.49	71.20	< 0.0001	
Residual	105.44	13	8.11			
Lack of Fit	72.75	8	9.09	1.39	0.3727	not significant
Pure Error	32.69	5	6.54			
Cor Total	2434.84	19				
			Radical scavenger activity(DPPH)			
Model	2.35	5	0.4696	12.35	0.0001	significant
A-Ethanol	0.0386	1	0.0386	1.02	0.3305	
B-Liquid phase/Solid phase	0.7687	1	0.7687	20.22	0.0005	
C-time	0.1489	1	0.1489	3.92	0.0678	

AC	**0.3003**	**1**	**0.3003**	**7.90**	**0.0139**	
A^2	1.10	1	1.10	28.83	< 0.0001	
Residual	0.5321	14	0.0380			
Lack of Fit	0.3940	9	0.0438	1.58	0.3185	not significant
Pure Error	0.1382	5	0.0276			
Cor Total	2.88	19				

Table 4: Statistics table for selected models of evaluated parameters

Source	Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
Total phenolic compound	27.23	437.02	6.23	0.97	o.96	0.88	24.19
Monomeric Anthocyanin content	0.1063	4.62	2.30	0.97	0.96	0.95	44.79
Flavonoids	2.85	32.38	8.79	0.95	0.94	0.89	24.02
Radical scavenger activity(DPPH)	0.195	96.87	0.20	0.82	0.74	0.60	11.81

Source	Std. Dev.	Mean	C. V. %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
Total phenolic compound	27.23	437.02	6.23	0.97	o.96	0.88	24.19
Monomeric Anthocyanin content	0.1063	4.62	2.30	0.97	0.96	0.95	44.79
Flavonoids	2.85	32.38	8.79	0.95	0.94	0.89	24.02
Radical scavenger activity(DPPH)	0.195	96.87	0.20	0.82	0.74	0.60	11.81

Source	Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
Total phenolic compound	27.23	437.02	6.23	0.97	o.96	0.88	24.19
Monomeric Anthocyanin content	0.1063	4.62	2.30	0.97	0.96	0.95	44.79
Flavonoids	2.85	32.38	8.79	0.95	0.94	0.89	24.02
Radical scavenger activity(DPPH)	0.195	96.87	0.20	0.82	0.74	0.60	11.81

Table 5: Calculation of relative error percentage and relative standard deviation percentage for obtained and predicted values

Source	Predicted Value	Obtained Value	Relative error (%)	Relative Standard Deviation (%)
phenolic compound	608.31	592.43±13.48	2.61	2.28
Anthocyanin	219.72	229.03±6.65	4.24	2.17
Flavonoids	53.25	50.10±0.94	5.92	1.88
(DPPH) %	97.41	96.93±0.93	0.49	0.96

Table 6: Type and concentration of phenol components in Shani grapes skin

NO.	Type of phenolic compound	Content(µg/g)
1	Quercetin	7.8
2	Hesperidin	2.3
3	Gallic acid	2.5

4	**Catechin**	**13.4**
5	Epigallocatechin	21.8
6	Epicatechin	5.6
7	Epigallocatechin gallate	4.8
8	Epicatechin gallate	0.3

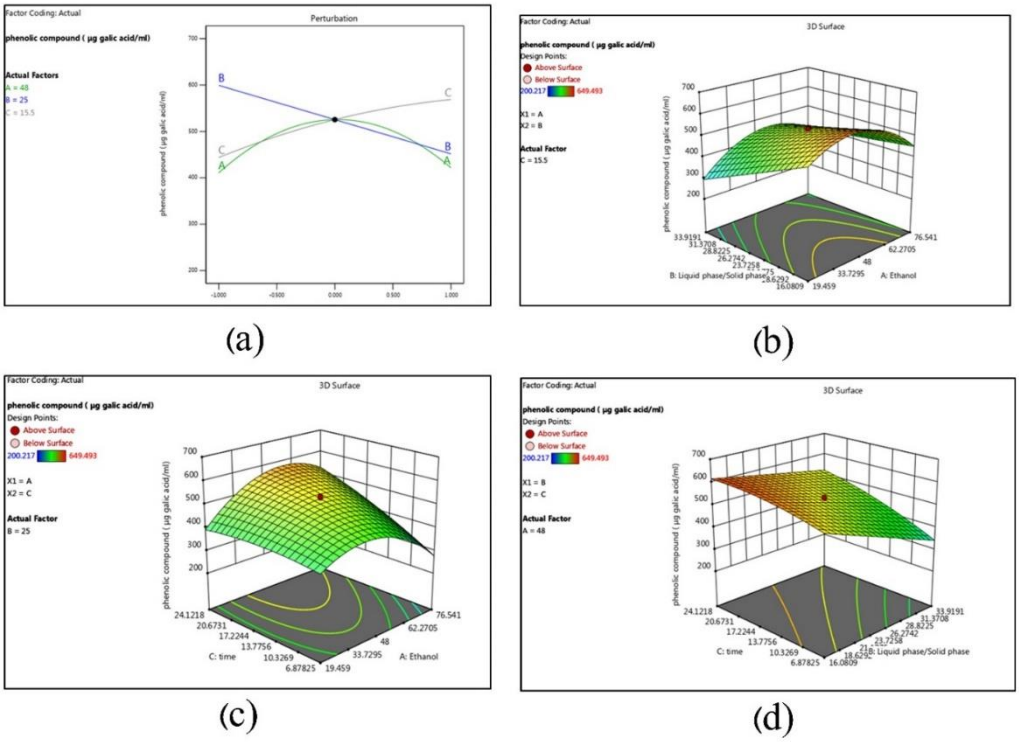


Fig 1

(a) Interaction plot for independent parameters (ethanol concentration, Liquid phase/Solid phase and Extraction time) on the content of extracted phenol components

(b) Response surface plot of phenolic compound for various binary combination (Ethanol and Liquid phase/Solid phase)

(c) Response surface plot of phenolic compound for various binary combination (time and Ethanol)

(d) Response surface plot of phenolic compound for various binary combination (time and Liquid phase/Solid phase)

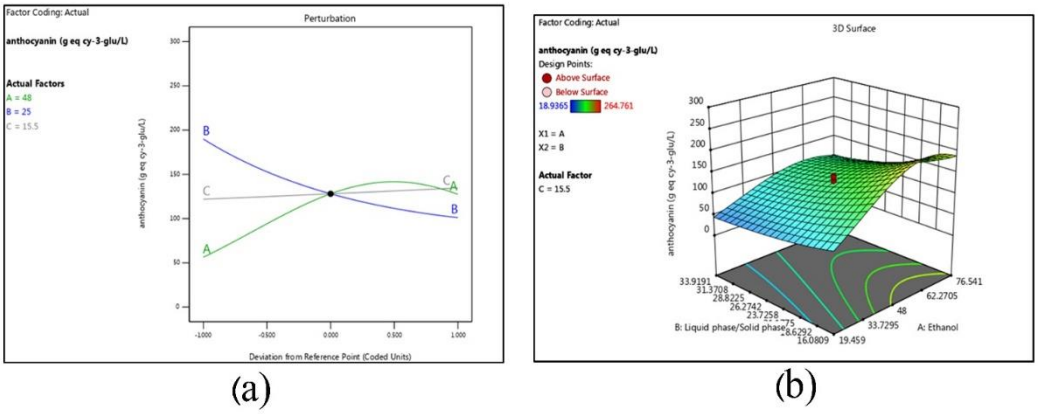


Fig 2

(a) Interaction plot for independent parameters (ethanol concentration, Liquid phase/Solid phase and Extraction time) on the content of monomeric Anthocyanin compounds

(b) Response surface plot of monomeric Anthocyanin compounds for various binary combination (Liquid phase/Solid phase and Ethanol)

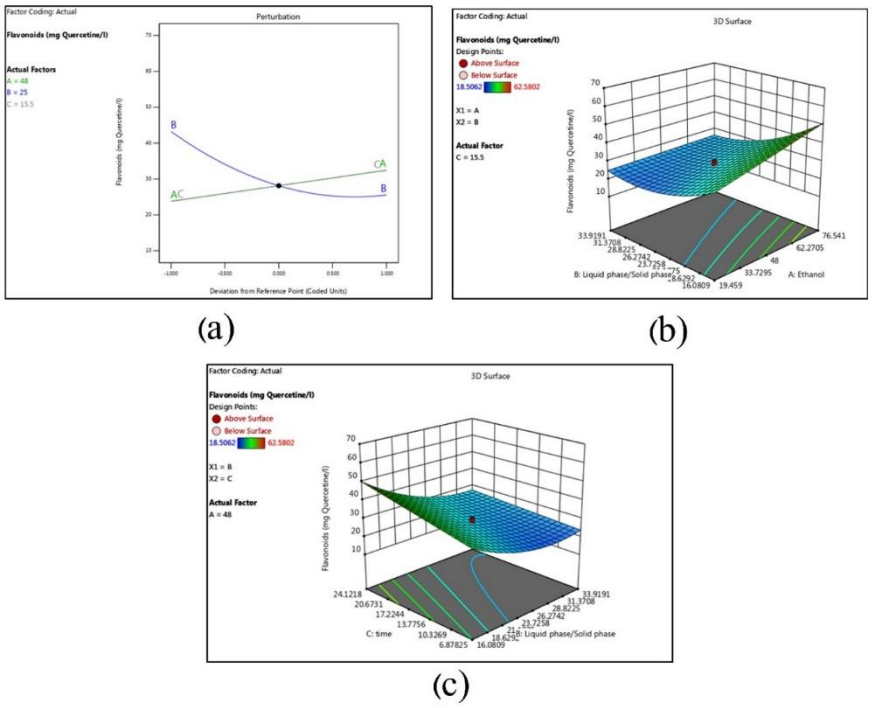
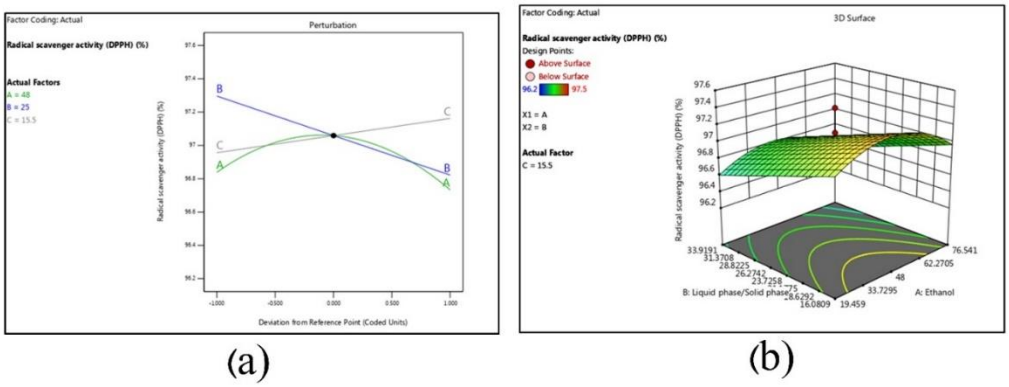


Fig 3

(a) Interaction plot for independent parameters (Ethanol concentration, Liquid phase/Solid phase and Extraction time) on the content of extracted Flavonoid compounds

(b) Response surface plot of Flavonoid compounds for various binary combination (Ethanol and Liquid phase/Solid phase)

(c) Response surface plot of Flavonoid compounds for various binary combination (Time and Liquid phase/Solid phase)



(a) (b)

Fig 4

(a) Interaction plot for independent parameters (Ethanol concentration, Liquid phase/Solid phase and extraction time) on the Radical scavenger activity (DPPH)

(b) Response surface plot of Radical scavenger activity (DPPH) for various binary combination (Ethanol and Liquid phase/Solid phase)

مجله علوم و صنایع غذایی ایران

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مقاله علمی_پژوهشی

بهینه سازی استخراج ترکیبات آنتی اکسیدانی از پوست انگور(*Vitis vinifera L.*) با کمک پیش تیمار اولتراسونیک

سعادت عزیزی[۱]، سید معین نظری[۲*]، لیلا مرادی حق گو[۳]

۱و۲- گروه علوم و صنایع غذایی، واحد همدان، دانشگاه آزاد اسلامی، همدان، ایران

۳- گروه میکروبیولوژی، واحد همدان، دانشگاه آزاد اسلامی، همدان، ایران.

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* مسئول مکاتبات:

seyyedmoeinnzr525@gmail.com

چکیده

انگور یکی از مهمترین محصولات کشاورزی است که می تواند به صورت میوه تازه یا فرآیند شده مصرف گردد. انگور یکی از مهمترین محصولات باغی و تجاری ایران به شمار می آید. سالیانه مقادیر زیادی پسماند انگور در واحدهای تولید کننده ی شیره انگور تولید می شود و تولید کنندگان این محصول را با مشکلات زیادی برای دفع پسماند رو به رو می کند. ضایعات و پسماندهای انگور منبع ارزشمندی از رنگدانه ها و ترکیبات آنتی اکسیدانی طبیعی خصوصاً ترکیبات پلی فنولی هستند. استخراج ترکیبات آنتی اکسیدانی از مواد اولیه ارزان قیمتی مانند ضایعات انگور روش مناسبی برای استفاده از آنها در صنایع غذایی و دارویی است. در این تحقیق برای یافتن شرایط بهینه استخراج ترکیبات فنولی از پوست انگورشانی استان همدان از روش سطح پاسخ (RSM) و طرح مرکب مرکزی (CCD) با سه متغییرمستقل (زمان استخراج با استفاده از حمام اولتراسونیک، نسبت فاز مایع به جامد وغلظت حلال اتانول)استفاده شد. شرایط بهینه استخراج ترکیبات فراسودمند از پوست انگور شانی برحسب ماکزیمم مقدار ترکیبات فنولی، ترکیبات فلاونوئیدی، ترکیبات آنتوسیانینی وهمچنین ماکزیمم ظرفیت آنتی اکسیدانی (بر حسب DPPH) به دست آمد. شرایط بهینه استخراج ترکیبات فنولی، فلاونوئیدی و آنتوسیانینی شامل غلظت اتانول ۵۹.۰٦٪، نسبت فاز مایع به جامد ۱٦.۰۸ml/g و زمان بهینه استخراج ۲٤.۱۲ min بود. نتایج تحقیق نشان داد عصاره پوست انگور شانی حاوی ۸ نوع ترکیب فنولی مختلف است که درمیان آنها اپی گالوکاتچین و کاتچین بالاترین غلظت را دارند. بنابراین با یافتن شرایط بهینه استخراج ترکیبات فنولی و شناسایی اجزای دقیق آنها، میتوان این ترکیبات را در مقیاس کارگاهی تهیه کرد و در اختیار صنایع مختلف غذایی و دارویی قرار داد.