Dicle Üniversitesi Veteriner Fakültesi Dergisi
https://dergipark.org.tr/tr/pub/duvetfd
Araştırma Makalesi/Research Article
ISSN:1307-9972 Dicle Üniv Vet Fak Derg 2024;17(Özel Sayı):45-50 e-ISSN:1308-0679
DOI: 10.47027/duvetfd.1486962

Application of Box-Behnken Experimental Design Method in Licorice Extraction

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Geliş Tarihi/Received	Kabul Tarihi/Accepted	Yayın Tarihi/Published
21.05.2024	05.12.2024	17.12.2024

Abstract

Licorice root is a traditional food substance used for various purposes and has diverse applications in the food industry. It is most commonly consumed as a sherbet. This study aimed to improve the efficiency of extraction in licorice root. For this purpose, the Box-Behnken Experimental Design was used. Temperature, time, and concentration were used as independent variables, while the total phenolic content was employed as the dependent variable. For solid-liquid extraction, heat treatment was carried out at 30-60°C for 10-30 min. The licorice concentration was in the range of 1-5% (w/v). A quadratic model was developed for the total phenolic content results. It was found that temperature and licorice concentration had a significant effect on the total phenolic content ($p<0.05$), while time had no significant effect. The optimum extraction conditions were determined as 60°C 10 min and 56°C 10 min heat treatment to extracts containing 5% licorice root.

Anahtar Kelimeler: Box-behnken, licorice, solid-liquid extraction, total phenolic compound

Meyan Kökü Ekstraksiyonunda Box-Behnken Deney Tasarım Yönteminin Uygulanması

Öz

Meyan kökü, çeşitli amaçlarla kullanılan geleneksel bir gıda maddesidir ve gıda endüstrisinde çeşitli kullanım alanlarına sahiptir. En çok şerbet olarak tüketilmektedir. Yapılan bu çalışmada meyan kökünde ekstraksiyon verimliliği arttırılmaya çalışılmıştır. Bu amaçla Box Benhken deney tasarımı kullanılmıştır. Bağımsız değişkenler olarak sıcaklık, süre ve konsantrasyon; bağımlı değişken olarak toplam fenolik madde miktarı kullanılmıştır. Katı-sıvı ekstrasyonu için 30-60°C aralığında 10-30 dk ısıl muamele yapılmıştır. Meyan konsantrasyonu ise %1-5 (w/v) aralığındadır. Toplam fenolik madde miktarı sonuçları için kuadretik model geliştirilmiştir. Sıcaklık ve meyan konsantrasyonunun toplam fenolik madde miktarı üzerinde anlamlı bir etkisi olduğu ($p<0.05$), sürenin ise anlamlı bir etkisi olmadığı bulunmuştur. Optimum ekstraksiyon koşulları %5 meyan kökü içeren ekstratlara 60°C 10 dk ve 56°C 10 dk ısıl uygulaması şeklinde tespit edilmiştir.

Key Words: Box-behnken, katı-sıvı ekstraksiyon, meyan kökü, toplam fenolik bileşik

INTRODUCTION

Oxidative stress in cellular components such as amino acids, DNA, lipids and proteins has been linked to many chronic and terminal diseases (1). Although there are systems that protect biological cells against free radical damage, in some cases these systems are insufficient. Oxidation caused by free radicals in foods is an important factor affecting the quality of foods. This reactivity and the products formed oxidize lipids, nucleic acids and proteins, causing negative consequences in metabolism. Antioxidant substances are effective in reducing the effects of these oxidized products (2).

Interest in antioxidants has increased due to their ability to protect foods against oxidation as well as their positive effects on human health. Various plant-based foodstuffs are reported to be natural sources of antioxidants (3). Consequently, studies involving phenolic compounds, which are a natural source of antioxidants, have increased in the food and pharmaceutical industries (4,5).

Phenolic compounds are phytochemicals naturally found in the structure of fruits, vegetables, grains, and various plant-based products, which influence various characteristic features such as color, taste, and smell of the foods they are found in. Based on their chemical structures, phenolic compounds are referred to as compounds containing an aromatic ring bound to one or more hydroxyl groups (6,7).

Licorice is a perennial plant belonging to the legume family (*Fabaceae*) and growing wild in the wet and humid areas of Türkiye, Mediterranean countries, Ukraine, Russia and Turkestan. The licorice plant, which has been used in the field of medicine from ancient times to the present day, is also known as the "grandfather of plants". There are about 30 varieties of licorice. Among the hundreds of bioactive components identified in the structure of licorice root are triterpenoids, saponins and flavonoids, isoflavones, coumarins and stilbenoids (8,9).

Licorice root (*Glycyrrhiza glabra*) is widely used in many countries for its antiviral, antiallergic, antioxidant and anticancer properties. It is mainly used as a flavoring agent in food, confectionery, pharmaceuticals and tobacco products (10). Glycyrrhizin, which is 4-20% in the dry matter of licorice root, is sweeter than sucrose. Licorice has high antioxidant activity. Due to its high flavonoid content, it shows a more important antioxidant property compared to vitamin E (11).

Microwave assisted extraction was applied in a study in which response surface methodology was used to optimise the extraction conditions of phenolic compounds from liquorice. According to this study, it is reported that 80% ethanol as solvent, 5-6 min extraction time and 12.7/1 liquid/solid ratio yielded the best results. It is also stated that extraction conditions have a significant effect on the extraction efficiency and antioxidant capacity of phenolic compounds (12). In a study, it was reported that there was no statistically significant difference in total phenol and total flavonoid values in licorice root samples extracted at room temperature compared to those processed at 40℃. In the same study, it was stated that extraction at 75℃ increased phenol and total flavonoid transfers at a statistically significant level (13).

Solid-liquid extraction is a multi-faceted process. It depends on many different factors such as temperature, time, solid concentration. Box-behnken design is an experimental planning method designed to understand the interactions of various factors by reducing the number of experiments in such multi-factorial cases.

The Box-behnken Design Method is a response surface method that requires relatively few experiments compared to the widely used orthogonal design (14). That is, with this design, the independent variables have an empirical relationship on the response function, unlike classical statistics, where all parameters can be estimated independently of the others. The response function affected by several independent variables is optimized to obtain the quadratic polynomial given in Formula 1.

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1;\, i<j}^{k=1} \sum_{j=2}^{k} b_{ij}\, x_j + \sum_{i=2}^{k} b_{ii}\, x_{ii}^2 \qquad (1)$$

Given in Formula 1 b_0, b_i, b_{ij} and b_{ii} are the regression coefficients; x_i and x_j are the independent variables affecting the dependent response function (Y), and k is the number of parameters.

This method can examine experimental conditions with 3-7 factors and three levels (high, medium and low) to produce comprehensive data sets (15). These three levels of control are given in Table 1. It can allocate the correlation between response outcomes and relevant factors through a series of experiments to obtain the best process conditions (16).

Table 1. Box-behnken experimental design levels

Variables	Low Level	Medium Level	High Level
A	-1	0	+1
B	-1	0	+1
C	-1	0	+1

The aim of this study was to determine the effect of different temperature, concentration and time on the amount of phenolic compounds in the extraction process and to determine the optimum range. The temperature, concentration and time ranges to be used in the study were determined using Box-behnken experimental design method. The extraction of licorice root was carried out by solid-liquid extraction method. Solid-liquid extraction method was preferred due to its low cost, simple equipment usage and easy applicability (17).

MATERIALS AND METHODS

Material

Licorice root used in the study was obtained from a local market. Gallic acid (Merck), Folin Ciocalteu (Merck), sodium carbonate (Merck), centrifuge (Nüve NF 1200R) and analytical balance (And GX-600), shaking water bath (Wisebath), thermometer (Thermo Orion Star), spectrophotometer (PG Instruments T80 UV/VIS) were used. Box-behnken experimental design was established using Minitab Version 18.1.

Method

Determination of solid-liquid extraction parameters

The temperature, time and concentration ranges to be studied during the extraction process were determined using the Minitab Box-behnken experimental design. Licorice root was extracted without grinding. The experimental design was implemented in two replicates and two parallels. Box-benhken experimental design is given in Table 2 and extraction parameters are given in Table 3. The concentration of the licorice in the solvent was adjusted between 1% and 5%, based on weight/volume.

Table 2. Box-behnken design of experiment

Independent variables	Symbol	Coded Levels		
		1	0	-1
Temperature (°C)	X_1	60	45	30
Time (min)	X_2	30	20	10
Concentration (%w/v)	X_3	5	3	1

Table 3. Parameters used for solid-liquid extraction process

Experiment No	Temperature	Time	Concentration
1	30	10	3
2	60	10	3
3	30	30	3
4	60	30	3
5	30	20	1
6	60	20	1
7	30	20	5
8	60	20	5
9	45	10	1
10	45	30	1
11	45	10	5
12	45	30	5
13	45	20	3
14	45	20	3
15	45	20	3

Solid-liquid extraction and total phenolic content

Licorice was extracted in a shaking mixer at the specified temperatures (30°C, 45°C and 60°C) and times (10, 20 and 30 minute). The supernatant was separated by centrifugation at 4500g for 10 min. Total phenolic compound amount Folin-Ciocalteu method was used with some modifications (18). First, 0.1 mL licorice and 0.2 mL Folin Ciocalteu reagent were mixed. Purified water (0.2 mL) was added and kept at room temperature for 3 minutes. Then 1 mL of sodium carbonate (20% w/v) was added and vortexed. The resulting mixture was kept in a 50°C water bath for 25 min. Absorbance was measured at 765 nm in a spectrophotometer. Purified water was used as a blind. The calibration curve was plotted in terms of gallic acid in the range of 50 to 250 ppm.

RESULTS

The data of the samples were determined by applying the surface response method with quadretic model. The statistical significance of model terms is assumed by their respective P-value. Also, "The Lack of Fit F-value" is a special diagnostic test for adequacy of a model. Considering the parameters studied, the effect of extraction temperature and licorice concentration on total phenolic content was found to be significant ($p<0.05$). The effect of extraction time had not been significant. Lack of alignment was not found to be significant ($p>0.05$), which indicates that the model is suitable for interpreting the data. The outputs of the quadretic model applied for phenolic extraction in the samples are given in Table 4.

Table 4. Box-benhken guadretic model analysis of variance results for total phenolic content

Source	DF	SS	MS	F-Value	P-Value
Model	9	43526.2	4836.2	80.36	0.000
Linear	3	42925.8	14308.6	237.75	0.000
Temperature	1	375.9	375.9	6.25	0.021
Time	1	261.5	261.5	4.35	0.050
Concentration	1	42288.4	42288.4	702.66	0.000
Square	3	523.8	174.6	2.90	0.060
Temperature*Temperature	1	23.5	23.5	0.39	0.539
Time*Time	1	27.5	27.5	0.46	0.507
Concentration*Concentration	1	503.0	503.0	8.36	0.009
2-Way Interaction	3	76.7	25.6	0.42	0.737
Temperature*Time	1	15.8	15.8	0.26	0.614
Temperature*Concentration	1	57.6	57.6	0.96	0.340
Time*Concentration	1	3.3	3.3	0.05	0.818
Error	20	1203.7	60.2		
Lack-of-Fit	3	410.6	136.9	2.93	0.063
Pure Error	17	793.1	46.7		
Total	29	44729.9			

DF: Degrees of freedom, SD: Mean squares, MS: squares of means

The 5 samples with the highest total phenolic content among the phenolic data optimized by the surface response method are given in Table 5 together with the analysis parameters.

In the study, the model R^2 value was found to be 0.973. In the study, the estimated and calculated R^2 values were 0.961 and 0.943, respectively. The values of R^2 imply a correlation between the experimental results and predicted values. Statistically, these two values are adequately consistent. The response surface function showing the effect of independent variables is given in mathematical model.

$$\text{Total phenolic content} = -23{,}2 + 0{,}96\,X_1 + 0{,}89\,X_2 + 34{,}70\,X_3 - 0{,}0079\,X_1{}^*\,X_1 - 0{,}0193\,X_2{}^*\,X_2 - 2{,}063\,X_3{}^*\,X_3 - 0{,}0094\,X_1{}^*\,X_2 + 0{,}0895\,X_1{}^*\,X_3 - 0{,}032\,X_2{}^*\,X_3$$

In our study, extraction temperature and sample concentration were found to have an effect on the total amount of extractable phenolic matter. The effect of independent variables on the total phenolic content of licorice extract is shown in Figure 1.

Table 5. Optimized total phenolic content

Solution	Temperature X_1	Time X_2	Concentration X_3	Total Phenolic Content (mg GAE/100 g)	Composite Desirability
1	60.00	10.00	5.00	154.93	1.00
2	56.43	10.00	5.00	153.78	1.00
3	60.00	10.00	4.90	152.27	0.99
4	60.00	29.90	5.00	146.89	0.94
5	32.73	10.03	4.97	145.33	0.93

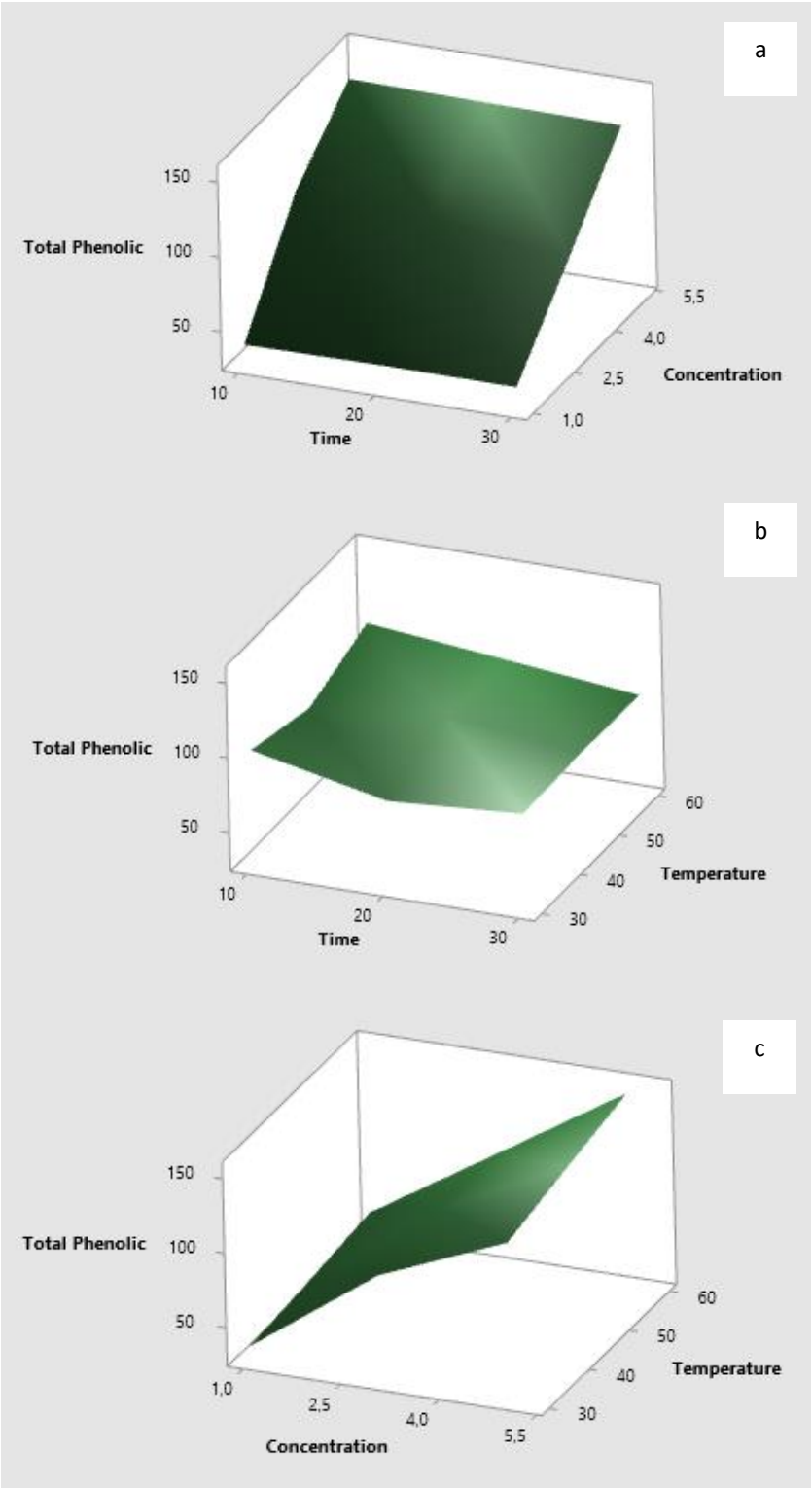

Figure 1. Effect of independent variables on total phenolic content of licorice extract a. Time and Concentration b. Temperature and Time c. Concentration and Temperature

DISCUSSION AND CONCLUSION

Bioactive components found in foods are defined as food ingredients or components with physiological effects on human health, beyond providing energy. The aqueous extract from the roots of the licorice plant contains many phenolic compounds. Bioactive components are substances that significantly contribute to the antioxidant properties of products (13,19). In a study performed by Özcan (8), the amount of total phenolic matter in licorice root extract was investigated. It was reported that the amount of total phenolic matter in the aqueous extract was 37.96 mg GA / g. In a different study conducted by Pala (13), the total phenolic content was found to be 521.2 mg GA / l in the aqueous extract at room temperature and 623.3 mg GA / l at 75°C. In our study, however, the total phenolic content was determined to be 154.93 mg GA / g. The variations observed in these studies may be due to different parameters such as the extraction time and the concentration of licorice root used in the preparation of the extract.

It has been determined that the extraction temperature and liquid-solid ratio have a statistically significant effect on the total phenolic content in the aqueous extraction of licorice root ($p<0.05$). Our results revealed that the liquid/solid ratio and extraction temperature have a linear effect ($p<0.05$). No significant relationship was found between extraction time and the total phenolic content measured in the licorice root extract ($p>0.05$). Similarly, in a study conducted by Ünalan, (20) and Yeler (21), it was reported that the effect of extraction temperature was significant. In a study by Yazıcı (22), it was reported that increasing the extraction temperature and time increased the phenolic content to some degree. It is stated that the total phenolic content decreased with further increase in temperature and time. In their study, Daştan et al., (23) examined the quantity of phenolic compounds obtained from fenugreek extraction at different temperatures, durations, and stirring speeds. At the end of the study, it was observed that the amount of phenolic compounds obtained from the extraction process was better at 55°C compared to 85°C. It is suggested that this is due to the adverse effect of high temperatures on certain phenolic compounds beyond a certain level.

R^2 values are important in terms of showing that there is a correlation between experimental results and predicted values (24). These two statistically estimated and calculated values should be close. Palamutoğlu and Kasnak (25) reports that experimental and predicted values are consistent when the difference between estimated and adjusted R^2 is less than 0.2. In this respect, the results obtained in our study are consistent. In different studies, it has been determined that there is consistency between the predictive values and experimental values in Box-behnken experimental design (20,26). Box-benhken experimental design has been proven to give statistically reliable results in the determination of phenolic extraction parameters from licorice. More studies can be conducted and more detailed data can be obtained by using different parameters.

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