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Application of experimental design for extraction of BHA and BHT from edible vegetable oil and their determination using HPLC

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ABSTRACT

The optimal conditions of liquid–liquid extraction of two synthetic phenolic antioxidants, BHA and BHT were investigated in five Iranian edible vegetable oil samples using the central composite design. Stepwise multiple linear regression method was used for construction of different models based on the experimental data. Optimum conditions for BHA and BHT were achieved using 3 ml of ethanolic solution containing (0.25% v/v) of glacial acetic acid, three extractions and a mixing time of 10 minutes. Analytes were separated using HPLC with a C_{18} column using methanol:water:glacial acetic acid (75:24:1, v/v/v) as the mobile phase. The limit of detections, linear ranges and relative standard deviation ($n = 7$) were 0.04 $\mu g/g^{-1}$, 0.5–200 $\mu g/g^{-1}$ and 2.6% for BHA and 0.30 $\mu g/g^{-1}$, 1.0–200 $\mu g/g^{-1}$ and 4.20% for BHT, ($r^2 > 0.99$), respectively. Amounts of BHA and BHT in analyzed oil samples were in the ranges of 29.8–54.5 $\mu g/g^{-1}$ and 0.0–6.8 $\mu g/g^{-1}$ respectively.

Keywords: synthetic phenolic antioxidants, edible vegetable oil, liquid–liquid extraction, HPLC, central composite design

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INTRODUCTION

Lipid oxidation not only lowers the quality and nutritional value of food it is also associated with aging, membrane damage, heart disease and cancer [1]. In food systems naturally occurring antioxidants such as tocopherol and ascorbic acid protect against oxidation by either quenching free radical reactions or by scavenging oxygen [2]. However, natural antioxidants often lost during processing and storage, necessitate the addition of exogenous antioxidants that will effectively retard the lipid oxidation [3]. Two synthetic phenolic antioxidants (SPAs), t-butyl-4-hydroxyanisol (BHA) and 2, 6-di-t-butyl-hydroxytoluene (BHT) are widely used in the food, drug and cosmetic industries as direct or indirect additives through diffusion from plastic packaging [4]. SPAs improve the stability and durability of pharmaceuticals, fat-soluble vitamins, cosmetics, rubber and plastics [3–5]. Studies have noted that use of BHA and BHT at a high dosage however, not only enhances liver and lung damages [6], but also causes stomach and urinary bladder carcinogenesis in animals [7,8]. Due to the potentially harmful effects of SPAs, their content in foodstuffs are regulated by law in many countries. The permissible substances and corresponding legal levels however are variable in different countries [9], for example in the European Union SPAs are forbidden in milk and cosmetics, but allowed in edible oils at a level not exceeding 50–200 μg $g^{-1}$of fat [10]. In Iran, usually 50–175 μg of several antioxidants per gram of fat either alone or in combination are allowed. The determination of BHA and BHT in food products is necessary to ensure fulfillment of the legal requirements. Various analytical methods have been reported for the determination of BHA and BHT in oils such as: thin-layer chromatography (TLC) [11], gas chromatography (GC) [12], gas chromatography mass spectrometry (GC-MS) [13], high performance liquid chromatography (HPLC) [7,10,14] and micellar electrokinetic capillary chromatography (MECC) [15]. The extraction methods that are carried out in the preliminary steps include solid phase extraction (SPE) [14], supercritical fluid extraction (SFE) [16] and liquid–liquid extraction (LLE) [7,13]. Many factors can significantly influence the extraction efficiency such as the extraction solvent, extraction time, number of extractions and extraction temperature, amongst other factors. The traditional one-factor-at-a-time approach for process optimization is time consuming and the interactions between various factors may be ignored. Recent experimental efforts have focused on selecting the best conditions for extraction, instead of using a traditional strategy of optimizing each factor separately [17,18]. The purpose of this study was to investigate the optimization of factors in the liquid–liquid extraction of BHA and BHT from Iranian edible vegetable oil. The factors that were studied for optimization included the effect of extraction solvent, extraction solvent volume, number of extractions, percentage of glacial acetic acid in the extraction solvent, and mixing time. Separation and determination of BHA and BHT was performed by HPLC-UV. The experiments for both modeling and optimization of LLE were conducted according to the central composite design (CCD) [19]. A stepwise multiple linear regression (MLR) method was used for the construction of different models based on the experimental data. The optimized conditions were applied to the extraction of BHA and BHT from five different types of Iranian edible vegetable oils. Separation and determination of these antioxidants were performed using the HPLC method.

MATERIALS AND METHODS

Reagents and materials

Standards of BHA (>98%) and BHT (99%) were purchased from Merck (Darmstadt, Germany) and Alfa Aesar (Karlsruhe, Germany), respectively. All of the solvents including methanol, anhydrous ethanol and acetonitril were obtained from Merck (Darmstadt, Germany). Glacial acetic acid was supplied by Riedel-de Haen, (Seezle-Germany). Water used was deionized, double distilled and filtered through a 0.45 μm filter (Millipore membranes Bedford MA, USA) and degassed under vacuum. Oil not containing any SPAs were prepared by the Salej Syrup manufacture (Babolsar, Iran). Five different edible vegetable oils samples (one type of sunflower oil, three types of soybean oils and one type of olive oil) were purchased from local supermarkets in northern Iran.

Preparation and storage of SPAs standards

A stock solution containing 500 mg L^{-1} of both of BHA and BHT were prepared in methanol and stored at 4°C. Standard working solutions of different concentrations were prepared by diluting an appropriate volume of the stock solution in methanol. These solutions were stable at least for three months.

Apparatus and chromatographic conditions

The sample preparation was performed using an ultrasonic bath, (Dawe 6444A, UK), magnetic stirrer (Heidolph, MR 2002, Germany) and centrifuge (Denley BS400, England). The purpose of this was to increase the interaction between the two phases (oil and organic solvent) and to improve the recovery of the extraction method. The chromatographic measurements were carried out by a HPLC system from Perkin-Elmer (Norwalk, CT, USA), equipped with a series 10 LC pump, UV detector model LC-95 set at 285 nm and model 7125 manual injector with a 10 μL sample loop. Analytes were separated on a C_{18}reversed phase column (250 mm × 4.5 mm, 10 μm) from Waters (Milford, Massachusetts, USA) using a methanol:water:glacial acetic acid mixture (75:24:1, v/v/v) as the mobile phase. The mobile phase was delivered with a flow rate of 1 mL/min^{-1} at room temperature. All statistical calculations were performed on a Pentium IV PC using the SPSS 11.5 software (SPSS, Bologna, Chicago).

Extraction procedure

To one gram of edible vegetable oil sample, 1 ml anhydrous ethanolic solution containing 0.25% of glacial acetic acid was added and placed in an ultrasonic bath for 5 minutes. The mixture was then homogenized for 10 minutes using a magnetic stirrer (750 rpm) and centrifuged for 5 min at 3000 rpm. The ethanolic phase was collected and the oil phase subsequently extracted two more times. All of the extracted phases were combined and dried passing a N_2 gas stream. The dried residue was then dissolved in 1 mL of methanol and used for HPLC analysis.

RESULTS

Selection of extraction solvent

To select the best type of extraction solvent for BHA and BHT from the oil samples, three organic solvents (anhydrous ethanol, methanol and acetonitril) were tested. The average recoveries for BHA and BHT extracted by ethanol, methanol and acetonitril were (91.8%, 91.0%, 88.2%) and (90.6%, 87.0%, 87.0%) respectively. Ethanol was chosen as the extraction solvent because of higher recoveries and less toxicity compared to other tested solvents.

Optimization of extraction conditions

Preliminary experiments showed the factors that affected the liquid–liquid extraction were: volume of extraction solvent (x_1), percentage of glacial acetic acid in extraction solvent (x_2), number of extraction (x_3), extraction time (x_4) and extraction temperature (x_5). Selected factors and their corresponding boundaries are shown in Table 1.

The exploration of the experimental domain was examined by a factorial design. A full factorial design of the five factors and two levels (for BHA and BHT) required 32 experiments. To reduce the number of experiments, a two-level half fractional factorial design consisting of $2^{5-1}(16)$ experiments were used. The experiments 1–16 (Table 2) show the fractional factorial design (FFD) and their corresponding responses (sum of recoveries of BHA and BHT). The fractional factorial design allowed first estimation of the effects of the main factors and their second order interactions which are presented in Table 3.The results of the investigation (Table 3) and their normal plot (Fig. 1) show the

Table 1. Selected experimental factors for LLE of BHA and BHT and their corresponding boundaries.

Level	Factors				
	X_1	X_2	X_3	X_4	X_5
+1	3	1.0	3	30	45
0	2	0.5	2	20	35
−1	1	0.0	1	10	25

X_1: Volume of extraction solvent (ml)
X_2: Percentage of glacial acetic acid (v/v)
X_3: Number of extractions
X_4: Extraction time (min)
X_5: Extraction temperature (°C)

Table 2. First estimation effects of the main factors and their second order interactions calculated from fractional factorial design for extraction of BHA and BHT from edible vegetable oil.

Run	Factors					Response (Sum of recoveries)
	X_1	X_2	X_3	X_4	X_5	
1	−1	−1	−1	−1	+1	147.7
2	+1	−1	−1	−1	−1	173.7
3	−1	+1	−1	−1	−1	118.6
4	+1	+1	−1	−1	+1	168.6
5	−1	−1	+1	−1	−1	130.8
6	+1	−1	+1	−1	+1	163.5
7	−1	+1	+1	−1	+1	125.5
8	+1	+1	+1	−1	−1	192.0
9	−1	−1	−1	+1	−1	149.9
10	+1	−1	−1	+1	+1	119.7
11	−1	+1	−1	+1	+1	130.0
12	+1	+1	−1	+1	−1	171.0
13	−1	−1	+1	+1	+1	100.9
14	+1	−1	+1	+1	−1	118.8
15	−1	+1	+1	+1	−1	98.5
16	+1	+1	+1	+1	+1	165.0

HPLC conditions: mobile phase: methanol: water: glacial acetic acid (75: 24: 1, v/v/v); flow rate = 1 mL min^{-1}; column C_{18} (250 mm × 4.6 mm, 10 μm); injection volume = 10 μL; λ = 285 nm; room temperature.

Table 3. Effects of factors and their interactions calculated from fractional factorial design (Experiments 1–16 in Table 2) for extraction of BHA and BHT from edible vegetable oil.

Term	Effect
X_1	31.506
X_2	5.731
X_3	−12.806
X_4	−23.118
X_5	−6.34
$X_1 \times X_2$	19.918
$X_1 \times X_3$	9.806
$X_1 \times X_4$	−12.281
$X_1 \times X_5$	−7.900
$X_2 \times X_3$	6.430
$X_2 \times X_4$	8.494
$X_2 \times X_5$	4.000
$X_3 \times X_4$	−13.618
$X_3 \times X_5$	5.580
$X_4 \times X_5$	−3.750

effect of extraction temperature (x_5) on the recovery was not very significant and therefore four variables (x_1, x_2, x_3 and x_4) were selected as the essential factors for extraction of the two antioxidants. The normal plot shows that the most important effect on the extraction recovery was due to the volume of extraction solvent (x_1).

The experiments for both modeling and optimization of LLE for BHA and BHT were performed according to the central composite design [19]. This design permitted the response surface to be modeled by fitting a second-order polynomial with the number of experiments equal to ($2^f + 2f + n$) where f and n are the number of factors and the number of center runs ($f = 4$ and $n = 2$), respectively. Factor levels and the corresponding matrix design included twenty-six experiments and their responses are shown in Table 4. A stepwise multiple linear regression (MLR) method was used for the construction of different models based on the experimental data. The model with the best-fitting statistical parameters including: higher Fisher-ratio (F), correlation coefficient values (r) and lower standard error (SE) were then selected as the satisfactory response surface model.

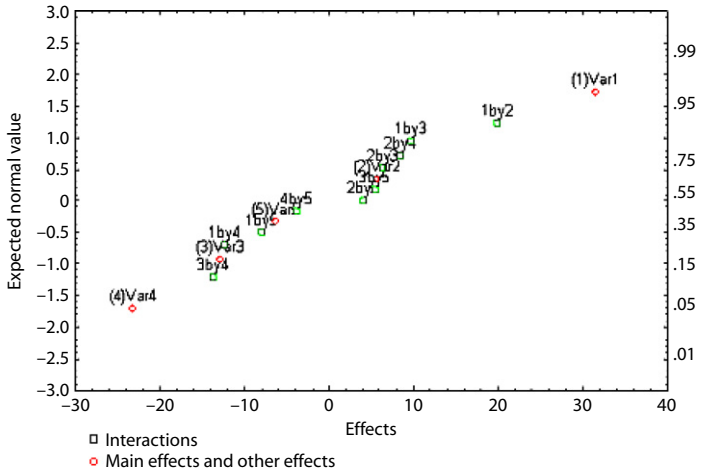

Figure 1. Normal plot of main effects and their interaction.

Table 4. Coded factor levels and responses (sum of recoveries) of CCD for four factors in LLE.

Run Fractional points	Factors				Response (Sum of recoveries)
	X_1	X_2	X_3	X_4	
1	−1	−1	−1	−1	147.7
2	+1	−1	−1	−1	173.7
3	−1	+1	−1	−1	118.6
4	+1	+1	−1	−1	168.6
5	−1	−1	+1	−1	130.8
6	+1	−1	+1	−1	163.5
7	−1	+1	+1	−1	125.5
8	+1	+1	+1	−1	192.0
9	−1	−1	−1	+1	149.9
10	+1	−1	−1	+1	119.7
11	−1	+1	−1	+1	130.0
12	+1	+1	−1	+1	171.0
13	−1	−1	+1	+1	100.9
14	+1	−1	+1	+1	118.8
15	−1	+1	+1	+1	98.5
16	+1	+1	+1	+1	146.7
Central points					
17	0	0	0	0	115.7
18	0	0	0	0	110.4
Star points					
19	−1	0	0	0	92.4
20	+1	0	0	0	154.3
21	0	−1	0	0	130.0
22	0	+1	0	0	157.0
23	0	0	0	0	142.5
24	0	0	−1	0	141.0
25	0	0	+1	−1	120.9

The obtained model and its coefficients are shown in Eq. (1):

$$Y = a_0 + a_1x_1 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{14}x_1x_4 + a_{113}x_1^2x_3 + a_{114}x_1^2x_4 + a_{133}x_1x_3^2 + a_{22}x_2^2 + a_{34}x_3x_4 + a_4x_4 \quad (1)$$

$a_0 = 128.744$ $a_{22} = 12.531$ $a_4 = 15.830$
$a_1 = 30.980$ $a_{34} = -6.809$ $a_{133} = -15.227$
$a_{114} = -27.389$ $a_{113} = -6.403$ $a_{13} = 4.903$
$a_{12} = 9.959$ $a_{14} = -6.141$

In the above equation Y is response, where F, r and SE values for the model are 7.577, 0.914 and 13.28, respectively. The plot of the residual distribution (difference between the experimental and

predicted recoveries against experimental recovery, Fig. 2) and *r* value show that there is no systematic error and the model is satisfactory.

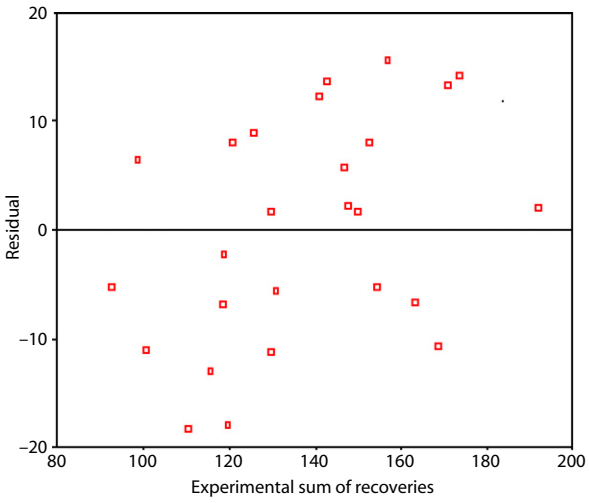

Figure 2. Residual plot for predicted sum of recoveries of BHA and BHT according to the regression model in Eq. (2).

In order to determine the optimum conditions for LLE, a grid search method was used. The responses were calculated by implementing a grid search method for the obtained equation (Eq. (1)), with varied factor levels from −1 to +1. In this method the dimension of each point in the grid framework (in the form of coded values) were applied to the equation and the corresponding responses were obtained. All the obtained responses were then compared and the response with the highest value was considered the optimum condition.

To evaluate the accuracy of the results obtained by this model, three experiments were carried out under the optimum condition and the experimental results corresponding to this condition were found to be satisfactory. The maximum recovery under the optimum conditions (90.2–101.3% for BHA; 90.0–94.6% for BHT) were achieved by using 3 ml of ethanolic solution containing 0.25% v/v of glacial acetic acid, three extractions and a mixing time of 10 minutes. To evaluate the accuracy of the results obtained by the response surface model, triplicate experiments were then carried out under optimum conditions. The predicted and average experimental responses under optimum conditions were 193.2 and 190.3 ± 4.2, respectively. The results showed that there was good agreement between the predicted and experimental responses.

Recovery studies

For further evaluation of the accuracy of the method, a blank oil sample (containing no antioxidants) was spiked with known amounts of standards of BHA and BHT at three concentration levels (20, 50 and 100 μg g^{-1}). Extraction was then performed under optimum conditions in triplicate experiments for each concentration. The percentage recovery, %*R*, of each analyte was calculated using Eq. (2):

$$\%R = [A_1/A_2] \times 100 \quad (2)$$

Where A_1 and A_2 are peak area of the extracted analyte from the spiked blank oil sample and standard (with the same amount of injection into the HPLC system) respectively. Accuracy of the method was determined by comparing spiked and extracted amounts of antioxidants in each concentration (Table 5).

Table 5. Recoveries of the spiked blank oil sample by addition of known amounts of standard BHA and BHT under the optimum condition.

Analyte	Amount added ($\mu g\ g^{-1}$)	Average recovery (%)
BHA	20.0	94.8 ± 4.2
	50.0	98.5 ± 2.6
	100.0	98.0 ± 3.3
BHT	20.0	92.5 ± 5.1
	50.0	94.0 ± 4.2
	100.0	95.6 ± 3.7

Limit of detection, linearity and precision

The limit of detection (LOD) was calculated on the basis of the equation $3S_b/m$, where S_b is the standard deviation of the blank equal to the peak noise when only the mobile phase was passing through the column for 45 minutes and m is the slope of calibration curve. Calibration curves were obtained by triplicate extraction of the spiked blank oil sample and their injection into the HPLC with subsequent plotting of the peak area against concentration.

The linearity studies were carried out with the blank oil samples spiked with BHA and BHT in the range of 0.5–100 mg/kg^{-1} (ten different concentrations). Extraction and separation were performed at optimum conditions with triplicate measurements ($n = 3$). The linear range (LR) of each analyte was carried out by plotting the peak area against concentration.

Precision (RSD) was determined by analyzing a spiked sample containing 20 $\mu g/g^{-1}$ of BHA and BHT in seven replicates. Limit of detections, linear ranges, precisions and squared correlation coefficient are listed in Table 6.

Table 6. Limit of detection (LOD), linear range (LR), repeatability, squared correlation coefficient (r^2) at the optimum condition for the determination of BHA and BHT in an edible vegetable oil sample.

Analyte	LOD ($\mu g\ g^{-1}$)	Linear range ($\mu g\ g^{-1}$)	Repeatability R.S.D [a]. (%)	Squared correlation coefficient (r^2)
BHA	0.04	0.5–200.0	2.6	0.9986
BHT	0.30	1.0–200.0	4.2	0.9980

[a] Relative standard deviation for seven replicate extraction and determination of antioxidants ($n = 7$)

Determination of BHA and BHT in oil samples

The optimum extraction conditions were applied for the determination of BHA and BHT in five edible vegetable oil samples from local supermarkets in Iran. Recoveries of each antioxidant were obtained by adding known amounts of standard antioxidants at two concentration levels (20 and 50 $\mu g/g^{-1}$) to oil samples. The percentage recovery of BHA and BHT were calculated using Eq. (3):

$$\%R = \left[\frac{A_1 - A_2}{A_3}\right] \times 100 \tag{3}$$

Where A_1, A_2 and A_3 are peak area of spiked, non-spiked and standard oil samples respectively. BHA and BHT contents of different edible vegetable oil samples (sunflower oil, soybean oil and olive oil samples) and their recoveries are listed in Table 7. Typical chromatograms of (a) extract of non-spiked soybean oil sample, (b) extract of spiked soybean oil sample and (c) standards of BHA and BHT are shown in Fig. 3.

Table 7. BHA and BHT contents in commercial Iranian edible vegetable oil samples and their extraction recoveries

Sample	Added amounts (μg) of BHA and BHT	Content (μg g^{-1}) [a]		Recovery (%) ± RSD	
		BHA	BHT	BHA	BHT
Sunflower oil	–	45.9 ± 1.6	ND [b]	–	–
	20.0	65.3 ± 1.5	18.4 ± 2.2	97.2 ± 1.5	92.5 ± 3.0
	50.0	94.7 ± 2.3	46.2 ± 2.5	98.0 ± 1.7	92.2 ± 2.5
Soybean oil 1	–	29.9 ± 1.7	ND	–	–
	20.0	49.2 ± 2.1	18.2 ± 3.0	97.3 ± 2.1	91.5 ± 3.7
	50.0	78.9 ± 3.2	49.0 ± 2.3	98.6 ± 4.2	98.5 ± 2.3
Soybean oil 2	–	54.5 ± 1.8	6.8 ± 0.3	–	–
	20.0	73.7 ± 3.1	24.7 ± 1.6	96.0 ± 3.7	90.4 ± 1.1
	50.0	102.2 ± 1.7	53.3 ± 2.1	95.3 ± 1.0	93.6 ± 2.6
Soybean oil 3	–	43.9 ± 1.5	ND	–	–
	20.0	62.7 ± 3.0	18.5 ± 3.4	95.0 ± 3.0	91.7 ± 4.6
	50.0	92.4 ± 2.3	46.8 ± 2.4	96.3 ± 2.6	94.0 ± 3.2
Olive oil	–	ND	ND	–	–
	20.0	19.4 ± 1.6	18.5 ± 2.6	97.0 ± 1.8	92.3 ± 2.6
	50.0	49.3 ± 4.0	47.9 ± 3.8	98.6 ± 2.5	95.4 ± 5.0

[a] Data were shown as mean ±SD ($n = 3$).
[b] Not detected.

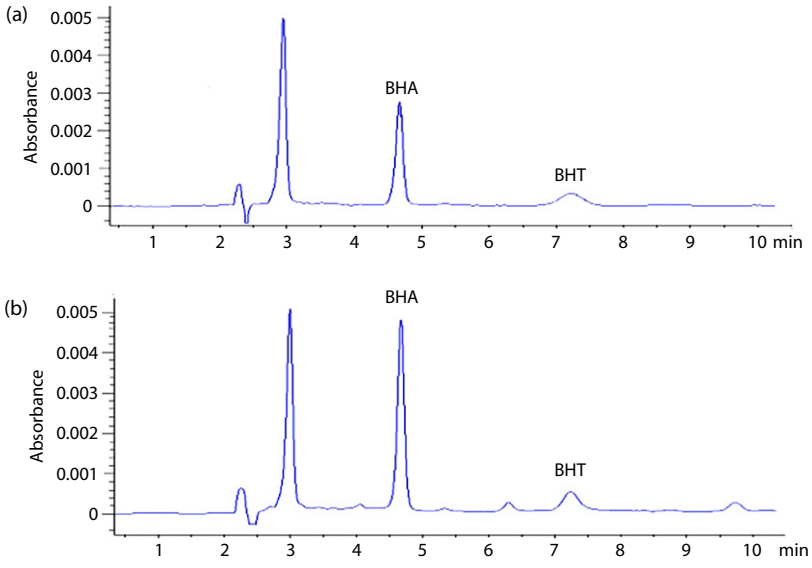

Figure 3. Typical chromatograms of BHA and BHT: (a) extract of non-spiked soybean oil sample, (b) extract of soybean oil sample spiked with 20 μg/g of two antioxidants and conditions mobile phase: methanol: water: glacial acetic acid (75: 24: 1, v/v/v); flow rate = 1 mL/min; column C_{18} (250 mm × 4.6 mm, 10 μm); injection volume = 10 μL; λ = 285 nm; room temperature.

DISCUSSION

A simple analytical method for the extraction of two SPAs (BHA and BHT) from five Iranian edible vegetable oils was found to be preferable in comparison to other extraction methods such as LLE and SPE as these are tedious methods and with high consumption of organic solvents which decrease the extraction efficiency. In this method less organic solvent was used and the effects of several factors were able to be studied simultaneously by chemometrics method in a reduced number of extractions. The analytical determination of SPAs in foods is a continuous activity for law enforcement agencies to ensure the safety of food produced and consumed in Iran. In order to improve the efficiency of liquid–liquid extraction methods and to enable the evaluation of the effects of different factors simultaneously, extraction conditions such as the: volume of ethanol, number of extractions, percentage of glacial acetic acid in ethanol and mixing time were optimized in this study. The

extraction solvent volume was found to be very low in comparison to previous studies [5,7,10], with also a reduced number of extractions performed. Analysis of the five oil samples studied show that the total antioxidant contents of analyzed oil samples are in the permissible range for oils produced in Iran (50–175 μg/g). The present method has not previously been performed for the determination of SPAs in Iran, or by comparing this to other applied extraction methods [5,7,10,12]. The advantages of using such a method include the simultaneous evaluation of several factors on extraction conditions, decreasing the number of experiments, using ultrasonic extraction for increased effective contact two phases and studying the methods validity and considerable diminution of organic solvent volume. Although the statistical parameters that were applied to assess the method were found to be valid, the study however was not without its limitations. In this method an ultraviolet detector was used which is not as sensitive as a fluorescence detector. This extraction method may also be limited to the use of substances with more simple matrices and not as effective for the extraction of SPAs in solid food and in other more complex matrices. This limitation may be overcome by further studies of the optimum conditions of extraction in such substances.

CONCLUSION

Due to the increasing use of synthetic chemical compounds in foodstuffs and their associated effects on human health, accurately determining the amounts of BHA and BHT is of great importance in order to detect products with high dosages that can cause disease.

The proposed method is simple, rapid and reliable and the most important advantage of this experimental design in this study is the evaluation of the effects of several factors on extraction conditions simultaneously. Microextraction procedures as an alternative method can be applied to achieve more efficient pre-concentration of SPAs from different types of foods. Also, it is possible to test this method for extraction of metabolites of SPAs from different matrices.

AUTHOR STATEMENTS

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