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Extraction of antioxidant compounds from agricultural waste of pomegranate, production of lipid nanocarriers, and assessment of its antioxidant activity in delaying the oxidation of soybean oil

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ABSTRACT

In this study, ultrasound extraction technology was used to extract the active constituents of pomegranate (Punica granatum L. variety SisheKape-Saveh). The effects of independent variables such as ultrasound exposure time and temperature on extraction yield, anthocyanin content, TPC, EC50, and FRAP were examined using the response surface technique. The extraction and measurement of various polyphenols from agricultural pomegranate waste is a precious source. Encapsulation of this compound is also a practical idea to preserve its unique properties during storage. To achieve this goal, the extraction conditions of antioxidants from pomegranate waste were optimized and the physical properties of a nanostructured lipid carrier with wall materials were evaluated. Furthermore, the impact of the resulting nanostructures on the oxidative stability of soybean oil was examined through the measurement of peroxide. According to the approximation of the desired functions, the optimal conditions were 39.8 min and 63.4 °C. Under these conditions, the extraction yield, anthocyanin content, TPC, EC_{50}, and FRAP were measured to be 17.12%, 39.74 mg/L, 41.45 mg GA/mL, 5.55 mg/mL, and 2227 µmol Fe^{2+}/L, respectively. The experimental values agreed well with the predicted values. From the results, the size of the resulting nanoparticles ranged from 82.6 to 196.7 nm. Nanocarriers that contained pomegranate extract exhibited an encapsulation efficiency (EE) of 85.2-92.5 %. The highest EE was related to a sample containing 5% pomegranate extract, 4% glycerol distearate, 3% Tween 80, and 0.6% lecithin. This extract at 1000 ppm compared to BHT at 200 ppm could effectively prevent the formation of peroxides in soybean oil.

KEYWORDS: *Antioxidant, Extraction, Optimization, Pomegranate, Ultrasound.*

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INTRODUCTION

Value-added compounds like natural antioxidants can be produced from agricultural waste through efficient technology [1]. The use of synthetic antioxidants in foods as preservatives is one of the significant challenges of the food industry, and their potential risks to human health have been reported [2]. Various polyphenols, such as ellagitannins, dihydroflavonol, anthocyanins, hydroxybenzoic acids, gallotannins, hydroxycinnamic acids, and gallic esters have been detected in pomegranates. These have antimicrobial, anti-mutagenic, and antioxidant features. Thus, studying natural antioxidant sources is necessary to replace them with synthetic types [2, 3]. Conventional techniques have become obsolete. The need for costly and pure solvents, long extraction times, and damage to heat-sensitive ingredients are some of the limitations [4]. The ultrasonic-assisted extraction (UAE) technique is cheap and easy in practice compared to other methods of extraction, for example, supercritical fluid extraction and microwave-assisted extraction (MAE) [5]. The enhancement in extraction achieved through the utilization of ultrasound is primarily attributed to the impact of acoustic cavitation generated in the solvent as a result of ultrasound wave passage [1]. Ultrasound waves have a mechanical impact, and enabling more penetration of the solvent into the tissue and incrementing the contact surface area between the liquid and solid phases [4]. Recently, different active components have been extracted using UAE, for example, the amount of anthocyanins, flavonoids content, antimicrobial and antioxidant activity of Punica granatum extract [6, 7], antioxidants from black soybean [8], phillyrin from Forsythia suspensa [9], anthocyanins from mulberry [10], pigments of annatto [11], and antioxidants from pomegranate marc [12].

Maleki *et al*. (2023) found that the highest amount of anthocyanin (5.3669 Mmol/g), flavonoids (9.0502 mg/ml) and rate of free radical scavenging (8.0452 mg/ml) in the extract of Punica granatum were reported using methanol at power 300 w for 9 min [6]. Velásquez *et al*. (2021) evaluated anthocyanins from the Chilean Luma chequen Gray berry were extracted using ultrasound and then incorporated into edible carrageenan films. The results indicated that natural deep eutectic solvents were effective at extracting anthocyanin. The inhibition diameter of the extracts against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi* strains was between 5 and 34 mm [13].

In a similar research, Mousavi *et al*. (2022a) showed that the maximum extraction efficiency of total phenols (36.6989 mg/g) from G. lucidum was obtained at 300 W for 15 min using methanol solvent [7]. Mousavi *et al*. (2022c) extracted phenolic compounds from Ferulago angulata by soaking and ultrasonic assisted methods. The highest total phenol content of soaking extract and ultrasound extract was 747.25 mg/100 g and 788.43 mg/100 g in100 % ethanol with 100 rpm in 2 h and 80 rpm in 5 min, respectively The highest antioxidant inhibitory activity was 76.57% for soaking extract and 86.06 % for ultrasound extract [14]. While Masjedi *et al*. (2022 optimized the extraction of antioxidant, flavonoid, antimicrobial and total phenolic compounds from Ganoderma Lucidum by soaking method. The results indicated that the highest concentration of flavonoid (15.19 mg/g) and antioxidant compounds (3.03 mg/g) were obtained at 60 °C for 48 h [15].

Polyphenols are among the compounds that are sensitive to environmental conditions (such as temperature, light, or oxygen), but have nutraceutical properties. Therefore, encapsulation technology is one of the suitable solutions to minimize these drawbacks, such as low stability, undesirable taste, low solubility, non-targeted release and low bioavailability [16]. Poor taste, quality degradation, and loss of nutritional value of oils and fats are caused by the process of oxidation and oxidative destruction. It is considered one of the most important problems of the oil industry [17]. Antioxidants are compounds that delay the development of rancidity and spoilage of oils and

fats by extending shelf life. Considering the problems and undesirable side effects caused by the consumption of synthetic antioxidants, in recent years, plant-based compounds are increasingly used as new sources of natural antioxidants in the food industry [18]. Soybean oil is one of the prominent vegetable oils whose importance is due to its abundance, cheapness and good quality. In addition, due to the relatively large amount of unsaturated fatty acids in this oil, its stability against oxidation is low and it is prone to oxidation. The most widely used chemical antioxidants in the food industry are propyl gallate, BHA, TBHQ and BHT. Several studies have shown the negative and carcinogenic effects of these compounds on human health [19]. Mousavi *et al.* (2022b) evaluated the effects of Chavir ultrasound and soaking extracts (100, 200, and 300 ppm) on the oxidative stability and antioxidant activity of virgin olive oil. The highest antioxidant activity was observed in olive oil containing a concentration of 300 ppm of Chavir ultrasound extract [20]. Salmanian *et al.* (2013) showed that 1000 ppm hawthorn (Crataegus elbursensis) extract inhibited the formation of peroxides in soybean oil and showed strong antioxidant activity compared to 200 ppm BHT [21]. Similar results were obtained regarding subcritical water extracts from Pistachio Hull, Mentha pulegium leaf extract, green Tea and Cinnamon on antioxidant and stability of soybean oil [22, 23, 24]. Therefore, this study was carried out to optimize the extraction conditions of antioxidants from pomegranate agricultural waste, prepare and investigate the physical characteristics of nanostructured lipid carriers (NLC) with wall materials (glycerol distearate), emulsifier (lecithin, Tween 80) and core (pomegranate extract). Also, the effect of the resulting nanostructures on the oxidative stability of soybean oil was investigated by measuring the peroxide value.

EXPERIMENTAL SECTION

Materials

Pomegranate (*Punica granatum*) agricultural waste was prepared from Saveh, Iran and stored at -18 °C until use and further analyses. All the chemicals, solvents, lecithin, glycerol, monostearate, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydrochloric acid, 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ), gallic acid, and folin–ciocalteu reagent used were prepared from Merck (Germany) and Sigma-Aldrich (USA) Companies.

Methods

Ultrasound-Assisted extraction

After defrosting, the samples were dissolved ethanol- hydrochloric acid (0.1 N) in 1:4 ratios and sonicated using an ultrasonic device (Sonicator, VCX750 model, USA) at 40, 55, and 70 °C for 10, 25, and 40 min. The sonication process was done at constant power (20 kHz and 550 W). The liquid was passed through the Whatman filter paper NO.5, followed by drying in a rotary evaporator (RV 10 digital V model, Heidolph company, Germany) and vacuum oven (1410D-2E model, Shel Lab, USA) to remove solvent and produce dye powder. A low temperature (43 °C) was used to prevent the thermal destruction of antioxidants and phenolic compounds [25].

Production of lipid nanocarriers containing pomegranate extract polyphenols

The production of nanocarriers in this research was done through homogenization with high shear pressure and ultrasonic waves. 3% Tween 80 and 0.6% lecithin were used for all formulations. In this study, the effect of lipids glycerol distearate (3, 4 and 5 % w/w) and pomegranate extract (0, 0.25 and 0.5 % w/w) were investigated. The aqueous phase containing emulsifier (Tween 80) and the lipid phase containing lecithin and pomegranate extract were prepared separately and placed at 80 °C for 15 minutes to reach the same temperature. The aqueous phase was mixed with the fat phase and homogenized for 1 min at a speed of 20000 rpm. The

produced pre-emulsion was subjected to ultrasound treatment using the ultrasonic probe (Misonix Sonicator XL2020, USA) with an output power of 25% for 50 cycles (4 seconds on and 1 second off). Then it was homogenized for 1 minute at a speed of 20,000 rpm. Finally, after cooling the manufactured dispersion in an ice bath, lipid nanocarriers were produced.

Evaluation of physicochemical properties

Assessment of extraction efficiency

The produced powder was weighed and the mass ratio of powder to primary weight was taken as the extraction yield [8].

Anthocyanin content of pomegranate extract

200 µL pomegranate extract and 3 mL buffer (potassium chloride at pH= 1 or sodium acetate at pH= 4.5) were mixed. The absorbance was measured at 511 (λ_{max}) and 700 nm and calculated using the following Eq. (1) [10]:

$$\Delta A = (A_{511} - A_{700})_{pH=1} - (A_{511} - A_{700})_{pH=4.5} \quad (1)$$

$$C = \Delta A \times M \times D / \varepsilon \times L$$

Where C, M, D, L, and ε are the anthocyanin content, molecular mass of dominant anthocyanin, dilution factor, length of cell, and molar absorbance, respectively.

Total phenolic content of pomegranate extract

The total phenolic content (TPC) of pomegranate extract was measured by the Folin-Ciocalteu method as described by Sharayei *et al.* (2018). The results were expressed as mg GA/mL of sample weight, where GA stands for Gallic Acid.

Antioxidant activity of pomegranate extract

The antioxidant activities of pomegranate extract were evaluated by radical scavenging activity (EC_{50}) and ferric reducing-antioxidant power (FRAP) according to the method Rahnemoon *et al.* (2017) [26]. Ascorbic acid and α-tocopherol were utilized as positive references. EC50 is the effectiveness of an antioxidant substance to eliminate 50% of initial DPPH radicals. This was calculated by interpolating the percent radical scavenging activity against the sample concentrations plot [27]. FRAP was measured using TPTZ base on Sharayei *et al.* (2018).

Determination of particle size, particle dispersion index and zeta potential

The particle size of all samples and the dispersion index of particles were measured by Particle Size Analyzer (30 HS model, Nanoseries, Germany) at an angle of 90° and 25 °C. Zeta potential, which is actually an indicator of the electric charge on the surface of nanoparticles, is calculated using a zeta sizer (30 HS model, Nanoseries, Germany) and based on the Smoluchowski equation. Zeta potential indicates the same surface charge and repulsion between particles, and as a result, the degree of particle stability, the closer it is to zero, the more unfavorable it is [28].

Encapsulation efficiency (EE)

It is the ratio of pomegranate extract encapsulated in nanoparticles to the total pomegranate extract added to the lipid phase. After dessolving 10 mL of nanocarrier containing pomegranate extract in 10 mL of acetonitrile solvent. It was filtered with a 0.45 µ filter. After 15 min of ultrasound at 25 °C, the effective substance was removed from the lipid nanocarriers. The solution was diluted with 10 mL acetonitrile and analyzed using a UV-Vis at 254 nm. The efficiency of encapsulation was calculated using the following equation [29].

$$\text{Encapsulation efficiency } \% = \frac{\text{Amount of pomegranate extract entraped}}{\text{Theorical total amount of pomegranate extract added}} * 100$$

Stability of nanocarriers

The dispersion of nanocarriers containing pomegranate extract was kept for 60 days at the temperature of the refrigerator (4 °C) and ambient (25 °C) and away from light. Then the particle size and zeta potential of the samples were measured.

Evaluation of the antioxidant activity of the lipid nanocarrier in delaying the oxidation of soybean oil

Lipid nanocarrier containing pomegranate extract (250, 500, 750 and 1000 ppm) and synthetic antioxidant BHT (200 ppm) was added to refined soybean oil without antioxidants. It was placed in an oven at 70 °C for 18 days. During the time intervals of the 0, 3, 6, 9, 12, 15, and 18 days, peroxide, acidity, iodine, p-anisidine, and Totox values of the oil samples were measured [30, 31, 32].

Experimental design and Statistical analysis

A two-variable Central Composite Rotary Design (CCRD) response surface methodology by a Design Expert Software v.10 was used to optimize the extraction process according to two important reaction variables, including Ultrasonic exposure time and applied temperature. The analysis of variance (ANOVA) was then performed to study the statistical significance of the regression coefficients by conducting Duncan's multiple range tests.

RESULT AND DISCUSSION

Choosing the right model

Fitting response surface models based on the design applied in this study, 13 experiments were conducted, and the observed results are shown in Table 1. The extraction yield ranged from 14.95 to 17.82 %, anthocyanin content 1.87 to 3.96 mg/100 mL, TPC from 2067.3 to 4193.2 mg GA/100 mL, EC_{50} from 2.81 to 6.97 mg/mL, and FRAP from 124 to 249 µmol Fe^{2+} /100 mL (Table 1).

Table 1- The CCD matrix, independent variables, and experimental data for the responses

Treatment	*Independent variables*		*Dependent variables*				
	Time (min) (A)	*Temperature (°C) (B)*	*Extraction yield (%)*	*Anthocyanin (mg/100mL)*	*TPC (mg GA/100mL)*	*EC_{50} (mg/ml)*	*FRAP (µmol Fe^{2+}/100mL)*
1	*10*	*70*	*15.26*	*2.6345*	*2923.94*	*3.3519*	*141*
2	*10*	*40*	*14.95*	*1.8692*	*2067.32*	*2.8427*	*124*
3	*25*	*55*	*15.83*	*2.2735*	*2829.83*	*4.3621*	*179*
4	*10*	*55*	*15.21*	*2.1066*	*2129.74*	*2.8225*	*132*
5	*40*	*70*	*17.82*	*3.9601*	*4193.26*	*6.9740*	*249*
6	*25*	*70*	*16.63*	*3.3100*	*3431.90*	*6.3190*	*204*
7	*25*	*40*	*15.45*	*2.1691*	*2185.80*	*4.6237*	*170*
8	*25*	*55*	*15.66*	*2.3612*	*2906.02*	*3.5358*	*198*
9	*25*	*55*	*16.17*	*1.9010*	*2769.22*	*4.2405*	*137*
10	*40*	*55*	*16.71*	*3.4238*	*3109.17*	*5.0371*	*237*
11	*25*	*55*	*15.93*	*2.0690*	*3101.79*	*3.6920*	*165*
12	*40*	*40*	*16.47*	*2.3658*	*2289.71*	*5.4876*	*197*
13	*25*	*55*	*15.78*	*2.3162*	*2881.61*	*4.3091*	*168*

Treatments 5 and 2 had the highest and lowest values for all responses, respectively. The values of R^2, R^2-adj, and R^2-pred showed that the 2FI model was more suitable for extraction yield and TPC than other models. However, a quadratic model was fit for anthocyanin content. The linear model was more accurate in free radical scavenging and iron reduction activities (Table 1 Supplementary). In addition, the misfit values of the selected models were not statistically significant (P>0.05), which indicates the suitability of the selected models for predicting the responses (Table 2 Supplementary). In ANOVA evaluation, a small P-value and a large F-value for each term in the models would show much effect on the response. Therefore, the linear term of temperature (B) had the most effect on anthocyanin content; however, the linear term of sonication time (A) had the most effect on the other responses (Table 2 Supplementary).

Effects of independent variables on responses

Extraction yield

The three-dimensional response surface diagram (Figure 1) shows the relationship between the extraction yield of antioxidant compounds from Pomegranate agricultural waste and experimental variables. The maximum extraction yield was obtained at higher temperatures following relatively longer ultrasonic exposure time. These parameters are synergistic, and therefore it can be expected that the combination of the two will increase the extraction yield. It was related to the phenomenon of cavitation in the ultrasonic process, which increases the permeability of Pomegranate tissues. The results obtained from the supplementary table 1 (R^2 = 0. 90 and R^2-adj = 0.88) indicate a good matching of the computational model with the tested points and the high accuracy of the model. The values of linear regression coefficients for the predictive model (Eq. (2)) of extraction yield are as follows:

Extraction yield = 4.29 - 0.001 A + 0.003 B + 0.001 AB (2)

Yolmeh *et al.* (2014) reported that the extraction yield of natural pigment from annatto seeds was determined as 6.35% at the optimal UAE condition (72.7 °C, 7.25 min, a ratio of seed to solvent of 14%, and a duty cycle of 0.8 s). They noted that extraction yield was increased by increasing temperature and sonication time [11]. Similar results regarding extraction performance were noted for the phenolic compounds of Forsythia suspense and red grape juice [9, 33]. Based on our results, the extraction efficiency of antioxidant compounds from pomegranate using ultrasound at constant power (20 kHz and 550 W), 70 °C for 40 min was 17.12%, which was higher than the extraction yield of phenolic compounds by Falah *et al.,* 2015 (7.7%) [34], Sharayei *et al.,* 2018 (13.1%) [4], Anaya-Esparza *et al.,* 2018 (7.42%) [35], Faizan, & Amardeep, 2018 (14.2%) [36], and Noshad, 2020 (8.8%) [37].

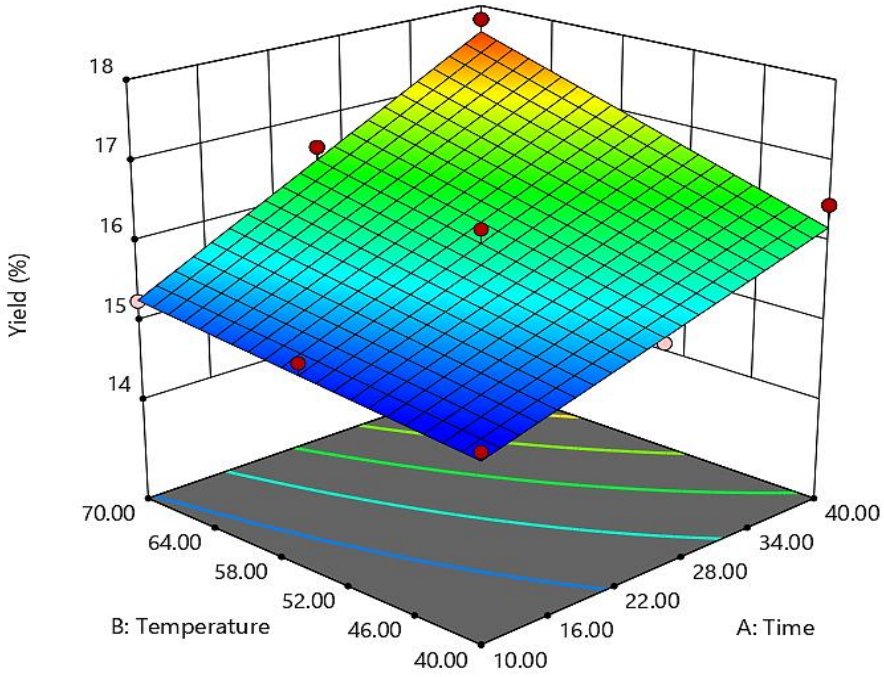

Figure 1- Effects of the independent variables on extraction yield

Anthocyanin content

The anthocyanin content was increased by increasing temperature and sonication time, so that the highest anthocyanin content (39.74 mg/L) was observed at 70 °C for 40 min (Figure 2). The findings indicated that the concentration of anthocyanin exhibited an increase with the increase in sonication duration. The quadratic regression equation (Eq. (3)) for the prediction of anthocyanin content is as follows (Table 1 supplementary):

$$\text{Anthocyanin content} = 4.8 - 0.08\,A - 0.11\,B + 0.001\,AB + 0.001\,A^2 + 0.001\,B^2 \qquad (3)$$

The results of Tang *et al.*'s (2015) investigation into the influence of temperature on the extraction of blackberry anthocyanins by ultrasound revealed that, when temperatures ranged from 20 to 40 °C, the concentration of anthocyanins rose from 54.1 to 62.6 mg/g. However, the amount of anthocyanin decreased with an increase in temperature from 40 - 60 °C. The optimal temperature for extracting higher amounts of blackberry anthocyanin was reported to be 40 °C [38]. The results of Rahnemoon *et al.* (2016) showed that the anthocyanin content was 252.05 mg cyaniding glucoside/100g dried extract and sample produced at ethanol/water ratio 60: 40, 25 °C and 24 h had the strongest antimicrobial activity. Also, Abid *et al.* (2017) recorded the highest value of anthocyanins in the acetone extract of the Acid ecotype with 54.5 mg cy-3-glu/100 g [39].

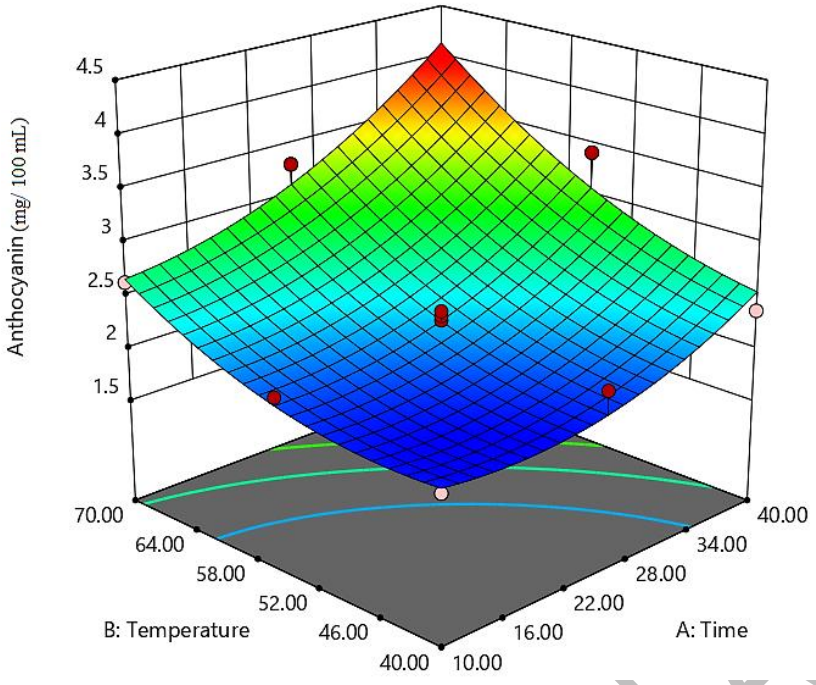

Figure 2- Effects of the independent variables on anthocyanin content

Total phenolic content (TPC)

The total phenolic content of the total extracts produced under the influence of different extraction conditions is presented in Figure 3. At the same temperature, the TPC of the samples increased with increasing ultrasonic exposure time. Also, the anthocyanin content of the samples increased by increasing the temperature applied in the ultrasonic method at a constant time. The maximum amount of phenolic compounds (41.45 mg GA/ml) was obtained at 70 °C for 40 min. As the 2FI fitted model had R^2= 0.95, R^2-adj =0.93, and R^2-pred =0.90, the experimental findings showed a high correlation with the predicted results from the Design Expert software (Table 1 supplementary). The values of 2FI regression equation coefficients for the prediction of TPC are as follows (Eq. (4)):

$$TPC = 1297.03 - 36.52\ A + 15.43\ B + 1.16\ AB \quad (4)$$

As observed, the extraction time has an essential effect on the extraction rate of total phenolic compounds. Due to with the increase of ultrasonic exposure time, the cell wall of the pomegranate fruit matrix is destroyed, and its contents are primarily left in the environment. It was consistent with the results obtained by Anaya-Esparza *et al.* (2018) and Sharayei *et al.* (2018) for phenolic compound extraction from Justicia spicigera leaves and pomegranate (*Punica granatum* L.) peel, respectively.

Chan *et al.* (2009) reported that TPC increased with increasing the mass-to-solvent ratio at short sonication time but decreased at long sonication time. They mentioned that this was due to the extraction of impurity components in a long ultrasonic time. Also, high temperatures can destroy plant components, and plant compounds enter the environment. For this reason, the amount of extracted phenolic compounds increases with increasing temperature [40]. Rahnemoon *et al.* (2016) examined the effects of extraction conditions on the phenolic compounds and antimicrobial properties of pomegranate (Punica Granatum) peels. They mentioned that the maximum amount of TPC was 349.5 mg Gallic Acid/g dried extract at a ratio of 60:40 between ethanol and

water, 25 °C and 24 h. They also mentioned that temperatures higher than 50 cause the destruction of phenolic compounds and flavonoids. In a similar study, Machado *et al.* (2019) has found that that TPC increased up to 50 °C and decreased at 60 °C. They believe that other phenolic compounds that were not found may have been broken down faster than ellagic acid, and punicalagin at 60 °C.

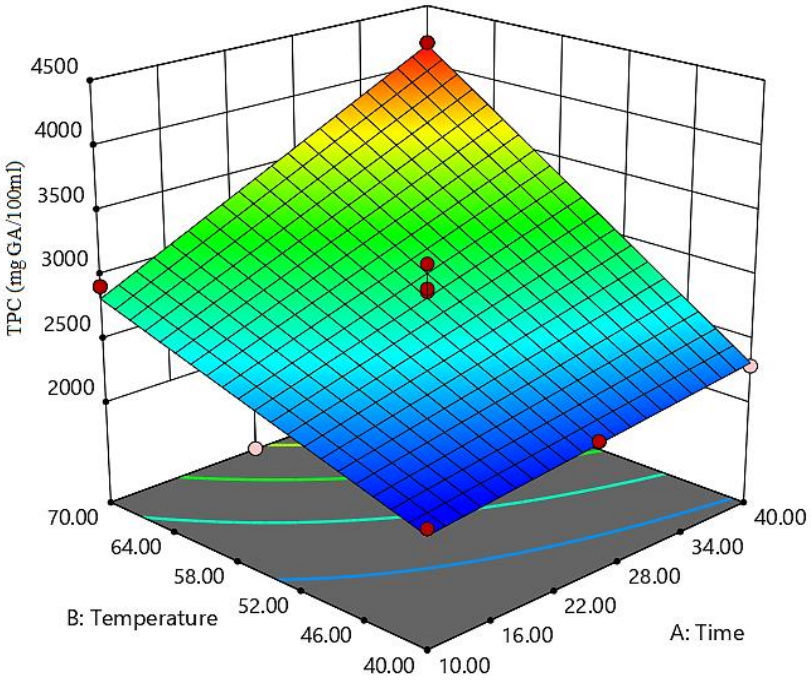

Figure 3- Effects of the independent variables on TPC

Antioxidant capacity

Based on the results, the samples that were produced at 60-70 °C had the highest antioxidant capacity. This effect may be related to the extraction of compounds, such as hydroxycinnamic acids, dihydroflavonols, and hydroxybenzoic acids because these compounds also have antioxidant properties. Polyphenol content in the extracts had a significant correlation with antioxidant capacity. The regression equations (Eq. (5 & 6)) for the prediction of EC_{50} and FRAP are as follows:

$$EC_{50} = 4.16 + 1.35\,A + 0.62\,B + 0.99\,B^2 \quad (5)$$

$$FRAP = 38.71 + 3.29\,A + 1.03\,B \quad (6)$$

The antioxidant activity of extracts based on EC_{50} was 2.81 to 6.97 mg/ml. As the temperature increases, the value of EC_{50} decreases first and then increases, as shown in Figure 4. This means that the free radical inhibitory property of the extract first increases and then decreases. Also, with enhancement time at a constant temperature, the value of EC_{50} increases, and the free radical inhibitory property of pomegranate extract is reduced. In general, the lower the inhibition rate based on EC_{50}, the stronger the antioxidant effect of the target sample. Therefore, the extract obtained at 60 °C and 10 min with an EC_{50} equal to 2.81 mg/mL has higher antioxidant activity and has more roles in neutralizing free radicals. Treatments No. 4, and 2 were placed in the next ranks of iron reduction power, respectively. The antioxidant capacity of plant extracts is attributed to their phenolic constituents. Therefore, the sample obtained under these conditions had more phenolic compounds. Rafiee *et al.* (2011) studied how different types of olive leaf extracts affect their antimicrobial and antioxidant

activity. Their results revealed that the highest phenolic content (244.7±0.1 mg TAE/g extract) and the lowest EC_{50} in DPPH, reducing power, and total antioxidant capacity were 74.2 ±0.15 µg/ml, 148.01±0.05 µg/ml and 160.4±0.02 µg/ml, respectively. These results were related to MAE extract of Cronaiky by microwave-assisted extraction method [41]. The same results have been presented by Sharayei *et al.* (2018), Šavikin *et al.* (2018) and Noshad, (2020).

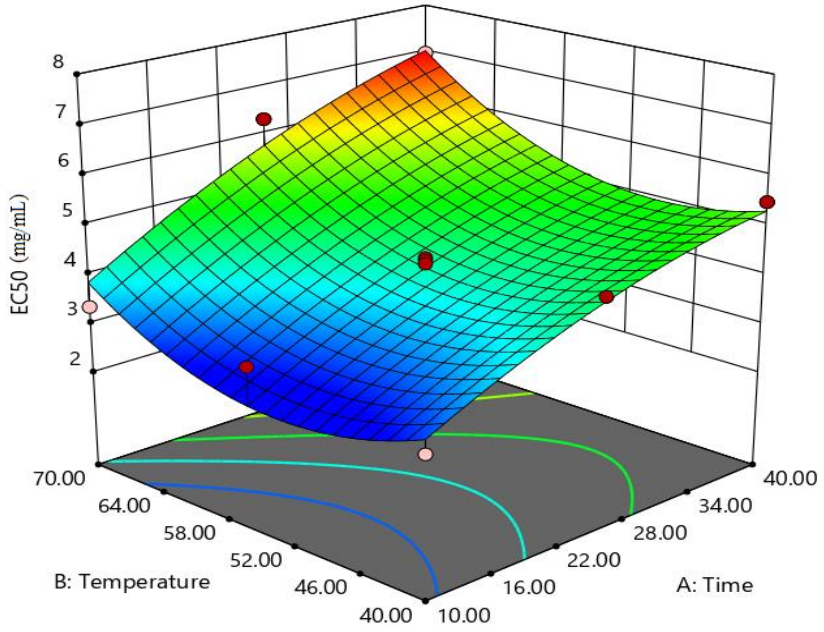

Figure 4- Effects of the independent variables on EC_{50}

Figure 5 shows the effects of the independent variables on ferric reducing-antioxidant power. The amount of FRAP in pomegranate extract has a direct relationship with temperature and time variables. The highest FRAP (2493 µmol Fe^{2+}/L) was related to the pomegranate extract obtained under the conditions 69 °C and 39 min. Free radicals are highly reactive species that react with any type of molecule in biological systems [42]. Free radicals cause the destruction of proteins, the breaking of DNA strands, and the beginning of the peroxidation of various compounds. Most of the vital compounds of cells are susceptible to destruction by free radicals [43]. According to the results of the present research, pomegranate extract can be used as a rich source of phenolic compounds with significant antioxidant activity to control the oxidation of foods containing unsaturated fatty acids and also to neutralize free radicals. Garcia *et al.* (2021) presented that the antioxidant capacity of pomegranate peel extract was 2265.6 µmol TE/g measured by FRAP and 916.4 µmol TE/g by oxygen radical absorbance capacity. Our findings were consistent with the results of Sharayei *et al.* (2018) and Machado *et al.* (2019) on the extraction of antioxidants from Pomegranate fruit and peel.

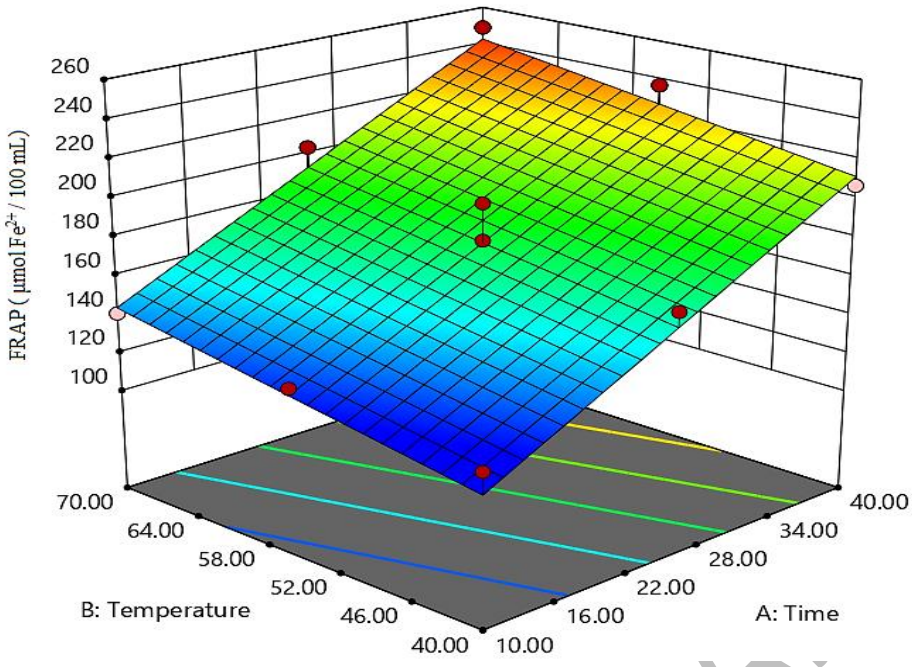

Figure 5- Effects of the independent variables on FRAP

Optimization

The extraction process aims to achieve the highest antioxidant activity and preserve active compounds. Therefore, dependent variables, such as the amount of TPC, antioxidant capacity, extraction yield, and anthocyanin content of the extracts were considered as a maximum. Also, the independent variables were optimized to decrease the adverse effects. The sonication process was optimized through a numerical optimization technique when the weight and importance values were equal for all responses. The values used for the optimization process are shown in Supplementary Table 3. Finally, the results obtained for optimizing the extraction of antioxidant compounds from pomegranate agricultural waste using Design Expert are shown in Table 2.

Table 2- The results obtained from the optimization process

Optimal points	*Time (min)*	*Temperature (°C)*	*Extraction yield (%)*	*Anthocyanin (mg/100mL)*	*TPC (mg GA/100mL)*	*FRAP (µmol Fe^{2+}/100mL)*	*EC_{50} (mg/mL)*
1	*39.82*	*63.41*	*17.3531*	*3.602141*	*3692.608*	*240.7187*	*5.844069*
2	*39.76*	*63.58*	*17.3634*	*3.61429*	*3703.962*	*241.0519*	*5.868091*
3	*39.91*	*62.75*	*17.3124*	*3.554408*	*3647.581*	*239.4095*	*5.751409*

Particle size, particle dispersion index (PDI) and zeta potential

The size of particles and their dispersion affect the physical and chemical stability, solubility, biological function, dissolution rate, turbidity, and darkness of nanodispersions. The dispersion index of nanoparticles theoretically ranges from 0 to 1, and values above 0.5 indicate a wide dispersion of particle size [44]. The PDI value in the samples was less than 0.35, indicating a relatively uniform distribution of the particle size of the

nanocarrier. The stability of particles against adhesion and prevention of sediment formation is due to the presence of electrostatic repulsion between particles [18].

The average diameter of all nanocarriers prepared in this study was less than 200 nm. Nanoparticles with smaller sizes are more stable because they offer more resistance to gravity due to Brownian motion. Also, they result in an increase in the rate of reaction because the surface area of the reactant has been increased. The surface area is important for the interaction of molecules to one another. The fewer interactions such as collisions between molecules, when that the smaller the surface area [45]. Nanocarriers 5 and 8, with average sizes of 75.01 and 251 nm, respectively, were the smallest and largest particle sizes. As can be seen in Table 3, the encapsulation efficiency (EE) and loading efficiency (LE) of the sample 8 (210 nm) was higher than that of sample 9 (225 nm). According to the results, loading anthocyanin into the particles had a significant effect on the particle size of the prepared lipid nanocarriers. As in many other research studies, loading the nanocarriers with anthocyanin particles increased the particle size and the viscosity of the dispersed phase, which made it difficult to reduce the particle size during the homogenization process. Sheybani *et al.* (2023a) found that nanostructured lipid carriers containing α-tocopherol had a particle size of 198 nm and PDI of 0.28 with a spherical shape and a smooth surface. The results of differential scanning calorimetry (DSC) and spectroscopy infrared (FTIR) analyses indicated that there was no interaction between the active compounds and the obtained NLCs.

Zeta potential is also an important parameter for physical stability of nanocarriers, and its higher value indicates greater stability of nanocarriers. Zeta tension or electrokinetic is the difference in potential between the mobile ion layer and the immobile layer. It is the surface electrical condition of dispersion that is the most reliable indicator. This indicates how much charge accumulates in the immobilized layer and how many ions are absorbed by the particle surface [46]. In this study, the absolute value of the zeta potential of the emulsions was less than 30 mV, which indicates the stability of the samples. The zeta potential was negative in all samples and varied from -6.2 mV (nanocarrier 1) to -22 mV (nanocarrier 8). The reason for the negative surface charge in the nanocarriers is related to lecithin, which is an anionic emulsifier. However, the hydroxyl group in Tween 80 (nonionic emulsifier) can also generate a negative charge. For nonionic emulsifiers, ester inhibition is one of the factors affecting nanoparticle stability during storage. The simultaneous use of hydrophilic and lipophilic surfactants can create more stable dispersion systems. Therefore, Tween 80 was used as a hydrophilic emulsifier and lecithin was used as a fat-soluble emulsifier. The zeta potential increased significantly after anthocyanin particle loading ($p<0.05$). Our research findings are consistent with the results of Acevedo-Fani *et al.* (2015) and Erfani *et al.* (2019) [47,16].

The type of emulsifier and oil phase used are effective on the droplet size of nanoemulsion particles produced containing vitamins. Using Tween 80 as a nonionic surfactant and Miglyol 812 as a carrier oil phase produced the smallest droplet size [48]. Hamedi and Razavi (2021) Effect of two surfactants soluble in water (Tween 80) and soluble in oil polyglycerol and polyriciniolate fatty acids (PGPR) in different ratios of 9:1, 8:2, 7:3, 6:4 and 5:5 investigated crostin for the preparation of nanoencapsulation. The results showed that microemulsions with dimensions less than 400 nm can be produced by using two water-soluble Tween 80 surfactants and PGPR with a ratio of 6:4 [49]. HassanFAmyan and Pezeshzi (2017) and Hasani *et al.* (2015) reported similar results in the production of nanoemulsion containing vitamins and Beta-carotene [50, 51].

Encapsulation efficiency and loading efficiency

The encapsulation efficiency depends on parameters such as the properties of the compounds used (e.g., solubility, active ingredient in the lipid phase, crystallinity index of the nanoparticles, and viscosity), the manufacturing process, and the environmental conditions during production [18]. The encapsulation efficiency and anthocyanin loading efficiency in the prepared nanocarriers ranged from 87.8% to 98% and 5.2% to 9.17%, respectively. The highest EE and LE were related to sample 8 containing 5 % pomegranate extract, 4% glycerol distearate, 3% Tween 80 and 0.6% lecithin (Table 3). Encapsulation efficiency of indocyanine gree and Methoxyl-Poly (ethylene glycol 2000)-Block-Poly (L-lactic acid 8000) in Cetuximab-Pt-INPs were 20.85 ± 1.04% and 40.62 ± 3.67%, respectively, while loading efficiency of ICG and MPEG-PLA-Pt in Cetuximab-Pt- INPs were approximately 6.98 ± 0.22% and 6.75 ± 1.08%, respectively [52]. Shigehiro *et al.* (2014) evaluated encapsulation into liposomes coupled with immunoliposomes preparation. Encapsulation of glucosyloxyacetylpaclitaxe by remote loading showed 70.8% of encapsulation efficiency and 8.0% of loading efficiency, whereas direct encapsulation in Cremophor EL/ethanol/PBS (CEP) showed 44.6% of EE and 5.0% of LE [53]. Other researchers have reported the encapsulation efficiency of nanocarriers on chitosan (61.8-63.15 %) [54], chitosan and pectin (66.68%) [55], lutein (81.85- 82.93%) [56], and also encapsulation efficiency of copigmentation for Stabilization of anthocyanins was 62–80% [57]. Hosseinnia *et al.* (2017) found that the amount of microencapsulated efficiency increases with the addition of essential oil to biopolymer. Increasing the amount of encapsulation accelerates the release of anthocyanins. Although more bioactive compounds are released at the desired site with the increase of encapsulation charge, high encapsulation charge efficiency leads to damage of the nanocarrier wall [58].

Table 3- particle size, PDI, zeta potential, EE and LE of nanocarriers

Code number	*particle size (nm)*	*PDI*	*zeta potential (mV)*	*EE (%)*	*LE (%)*
1	***94.9***	***0.23***	***-6.2***	**-**	**-**
2	***84.1***	***0.25***	***-10.25***	**-**	**-**
3	***75.01***	***0.24***	***-10.7***	**-**	**-**
4	***122.02***	***0.27***	***-13.2***	***87.8***	***5.2***
5	***184***	***0.33***	***-21***	***95***	***8.87***
6	***133.4***	***0.29***	***-15.79***	***89.13***	***6.39***
7	***135.6***	***0.28***	***-13.94***	***88.8***	***7.09***
8	***210***	***0.31***	***-22***	***98***	***9.17***
9	***225***	***0.35***	***-18.82***	***92.9***	***8.16***

Antioxidant activity of the lipid nanocarrier in delaying the oxidation of soybean oil

Effect of pomegranate anthocyanin nanocarriers on soybean oil peroxide

Hydroperoxides are the main oxidation products of lipids without off-flavors, while their degradation products are mostly responsible for off-flavors. The primary products of lipid oxidation are peroxides [48]. In general, oil or lipids are more ready for oxidation when their degree of unsaturation is higher. Aldehydic and ketonic volatile substances are made when peroxide levels reach a certain level. These substances are effective at making fatty substances smell and taste unpleasant [19]. Figure 1 shown the degree of oxidation of soybean oil in the presence of natural antioxidant compounds of pomegranate extract with different concentrations and the synthetic antioxidant TBHQ, as well as in the sample without antioxidants (18 days of incubation at 70 °C). The results indicated that the significance of the influence of treatment and time on the peroxide content of the samples was evident ($p<0.05$). The process of changes in the peroxide value of soybean oil started on the second day of

the test in all the samples. There is an increasing slope, which is a function of the storage time. The highest amount of peroxide value on all days is related to the control sample that did not contain any antioxidants (Figure 6). All the samples were significantly different from the control sample. There was no significant difference between the samples containing synthetic and natural antioxidants in the early days; however, with the increase of oxidation reactions, the difference between the samples is in the final days. It was also observed that treatments with 250 ppm and 500 ppm of oil containing nanocarriers of anthocyanin did not have much effect on the reduction of peroxide value, but peroxide value decreased in treatments with higher concentrations of lipid nanocarrier containing pomegranate extract. It is due to the increase of phenolic compounds in higher concentrations of pomegranate extract. Compared to the control sample, all concentrations of natural extracts had low peroxide values and their performance was more effective in reducing the oil oxidation process. The performance of 1000 ppm anthocyanin capsules was more effective than 200 ppm TBHQ. Sheybani *et al.* (2023a) evaluated NLCs containing α-tocopherol to make camelina oil more stable and resistant to oxidation. The results indicated that the addition of NLCs-αt reduced the TPC (228.8–169.0 mg GAE/g) and oxidative stability (4.21–3.30 h), DPPH radical scavenging (83.33–46.25%), but increased anisidine value (0.63–1.50), TOTOX value (2.37–6.68), and peroxide value (0.87–2.59 meqO2.kg−1), after 90 days of storage. Furthermore, the antioxidant activity of camelina oil containing NLCs-αt (46.25%) was comparable to sample containing TBHQ (49.45%).

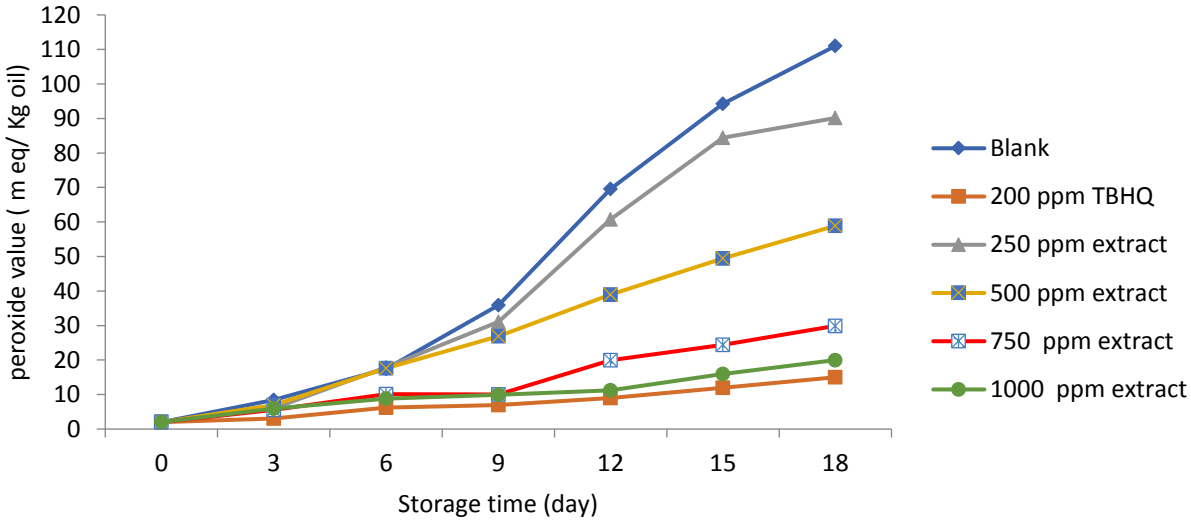

Figure 6- Peroxide values of soybean oil containing different concentrations of lipid nanocarrier containing pomegranate extract (18 days, 70 °C)

Antioxidant properties of ethanolic extract of turmeric powder on soybean oil were investigated. The trend of changes in peroxide value of soybean oil was calculated as a function of storage time. It was seen that the trend slope of these changes is increasing in all samples. The results showed that the oil sample containing 700 ppm ethanolic extract of turmeric had less change than the oil containing 120 ppm synthetic antioxidant TBHQ. In this case, the concentration of 700 ppm of ethanol extract of turmeric is able to compete with 120 ppm of TBHQ antioxidant [59]. Tinello *et al.* (2020) reported that the increase in soybean oil peroxide was slow in the first 14 days, while a sharp increase was seen until the end of storage [60].

Effect of pomegranate anthocyanin nanocarriers on soybean oil acidity

From the breakdown of oil triglycerides during oil storage, free fatty acids are created, and the measurement of the acid number, which shows the amount of free fatty acids, is an important parameter for measuring pungency in foods [61]. The results showed that all the treatments went through an upward trend, and the control treatment had the highest amount. The 1000 ppm treatment of capsules containing anthocyanin and TBHQ 200 ppm showed the lowest amount (Figure 7). The cause of this upward trend can be attributed to the brief breakdown of triglycerides and as a result a slight increase in the free fatty acids of the oils. It was also observed that antioxidant effect increased and the acidity of the oil samples decreased with increasing the concentration of capsules containing pomegranate extract. Also, a significant difference was observed between the control samples compared to the treated samples ($p<0.05$). The samples containing lipid nanocarriers of anthocyanin were more effective in preventing the increase of acidity than the control sample without antioxidants. Vellido-Perez *et al.* (2021) found that temperature significantly affected the formation of free fatty acids and increased the acidity of soybean oil samples with curcumin. Also, the acidity changes during storage showed that the formation of free fatty acids decreased with increasing pomegranate anthocyanin concentration [62]. The research results of Sneha *et al.* (2021) are also in agreement with our results.

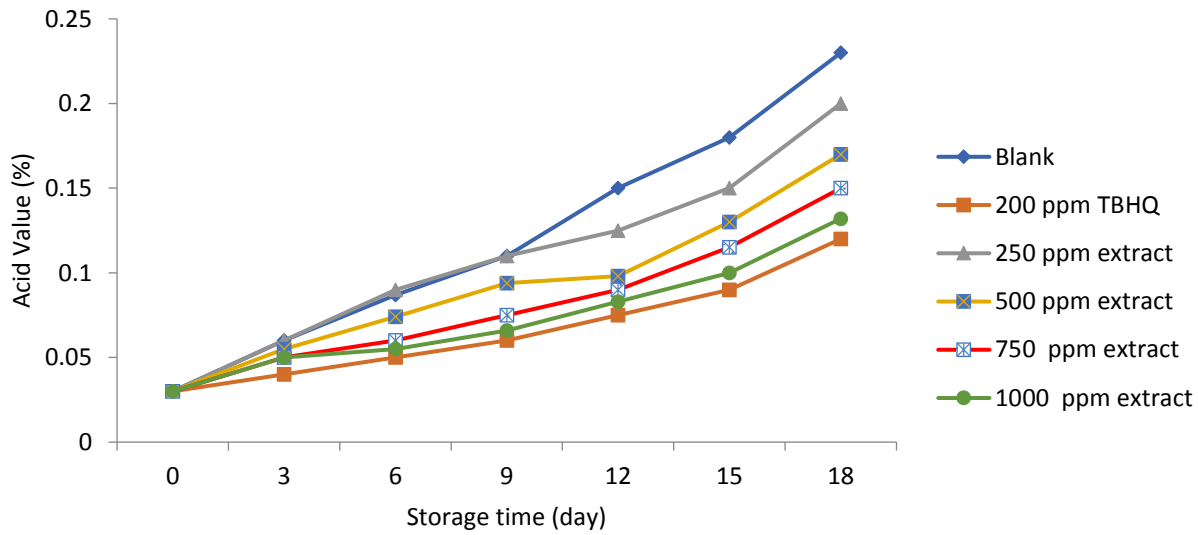

Figure 7- Acid values of soybean oil containing different concentrations of lipid nanocarrier containing pomegranate extract

Effect of pomegranate anthocyanin nanocarriers on the iodine value of soybean oil

Iodine number estimates the degree of unsaturation of fats and oils and is used to estimate the oxidative stability of oils. A decrease in iodine number is consistent with an increase in fat oxidation [63]. The effect of the treatments on the iodine number of the oil samples was significant (Figure 8). The treatment with 1000 ppm of pomegranate anthocyanin nanocarriers had the highest and the sample without antioxidants had the lowest iodine number. By increasing the concentration of pomegranate anthocyanin nanocarriers, the amount of iodine value increased, which indicates the effect of phenolic and bioactive compounds of pomegranate in preventing the loss of double bonds of triglyceride fatty acids. Lodh *et al.* (2108) reported that buffalo oil samples containing curcumin had a lower decrease in the iodine value compared to oil samples without curcumin [64]. Natural phenolic compounds in plants delay the formation of conjugated dienes and prevent the decomposition of linoleic

acid. These compounds carry hydrogen atoms to peroxyl radicals.Therefore, aryl oxyl is formed and then it is coupled with other radicals to quench the radical process [63]. Romola *et al.* (2021) reported that the very small decrease in iodine content in samples containing natural antioxidants is due to the effect of this antioxidant on soybean biodiesel [65]. The addition of synthetic antioxidant and potato peel extract has delayed the process of decreasing iodine value in soybean oil during storage [66, 67, 68].

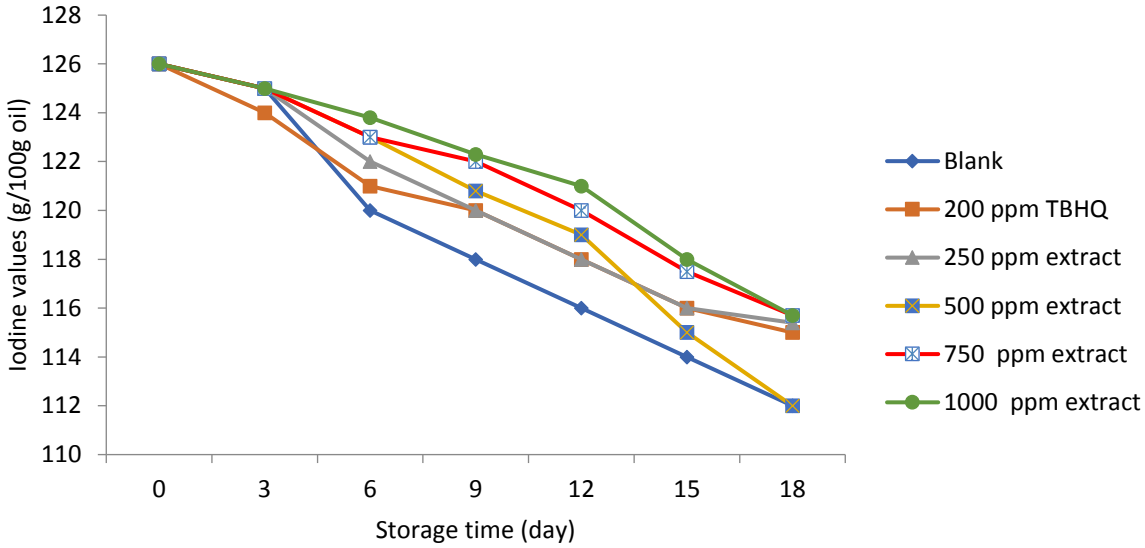

Figure 8- Iodine values of different soybean oil treatments containing lipid nanocarrier of pomegranate extract

Effect of pomegranate anthocyanin nanocarriers on the para-anisidine of soybean oil

The para-anisidine test is for the detection of secondary oxidation products, which measures the content of aldehydes that are formed during the decomposition of hydroperoxides [69]. With time, the number of anisidines increased in all treatments (Figure 9). The average anisidine value in the control sample with all the investigated treatments has a significant statistical difference at the level of 5%. The finding indicates that if the pomegranate anthocyanin nanocarriers is more, it can have more antioxidant activity. It increased significantly and among all the treatments, the concentration of 1000 ppm of nanocarriers had the greatest effect in reducing the value of anisidine. The presence of nanocarriers in low concentrations results in lower concentrations of compounds with anti-radical properties, such as phenolic compounds. Oil oxidation continues with increasing storage time.

Therefore, anisidine levels in the samples increase and reaches its maximum at the end of the storage period. An increase in the amount of anisidine indicates a spontaneous oxidation reaction and an increase in secondary products arising from the breakdown of hydroperoxides and carbonyl Combinations during the time. Antony *et al.* (2019) evaluated the effects of dried zanthoxylum rhetsa DC antioxidants in controlling the rancidity of peanut oil. The results showed that 1 g of dried Zanthoxylum rhetsa powder and 1 g of Zanthoxylum rhetsa methanol extract in peanut oil have peroxide and para-anisidine values 16.8meqO^2/kg, 16.5 meq O^2/kg and 17.4, 17.9 respectively. However, blank oil has 19.4 meqO^2/kg as peroxide and 20.3 para anisidine value [19]. Research showed that Research on grape skin extracts as a source of antioxidants in an oil-in-water emulsion showed that they have thiobarbituric acid reactive species (6.08 to 11.15 mg MDA/L) and p-anisidine (4.30 to 20.71) during

20 days of storage have thiobarbituric acid reactive species (6.08 to 11.15 mg MDA/L) and p-anisidine (4.30 to 20.71) during 20 days of storage [70]. Liu *et al.* (2019) reported that emulsions with Kyoho skin extracts had para-anisidine value similar to ascorbic acid and propyl gallate (standards) [71]. Sheybani *et al.* (2023b) developed ascorbyl palmitate (AP) incorporated in NLCs to make camelina oil more stable. The highest antioxidant activity, oxidative protection, and TPC were obtained in camelina oil containing NLCs-AP with rosemary essential oil. TOTOX, peroxide and anisidine values of camelina oil with optimized NLCs-AP were slightly higher than oil with TBHQ [72]. Abd El-aal, and Halaweish (2010) and Singh (2018) reported similar results about antioxidant efficacy of meal extracts and orange peel extracts (Citrus sinensis L.) [73, 69].

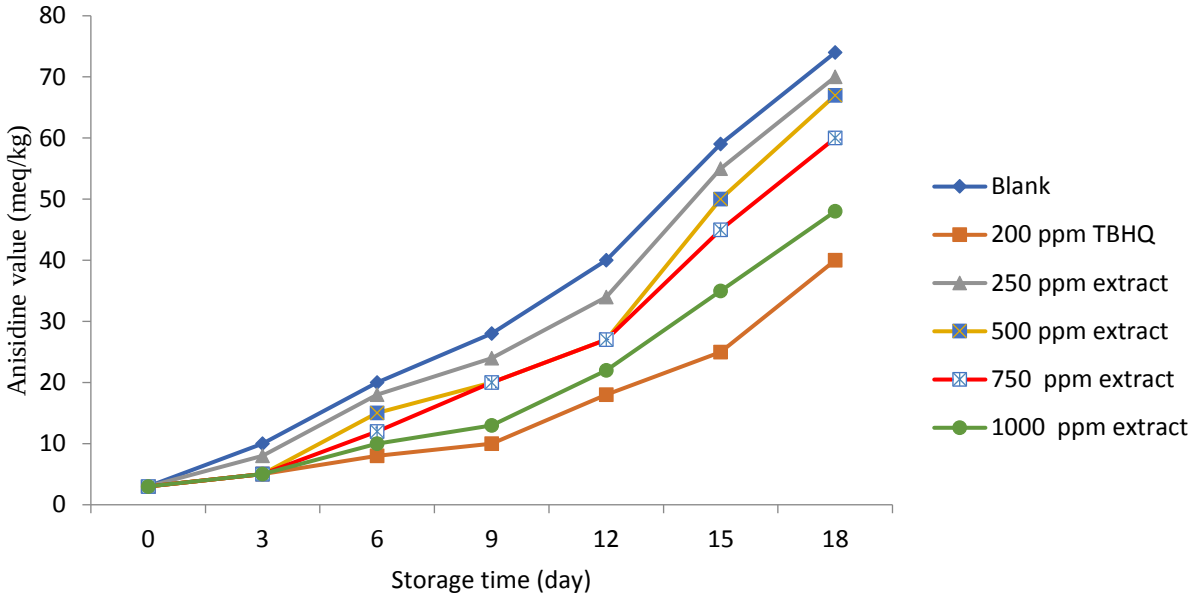

Figure 9- Anisidine values of different soybean oil treatments containing lipid nanocarrier of pomegranate extract

Effect of pomegranate extract nanocarriers on the Totox value of soybean oil

The Totox value is an index that depends on two peroxide and anisidine values. As the value of these two increases, the Totox value also increases [74]. The results of the analysis of variance of the effect of pomegranate extract nanocarriers on Totox value were significant at the 5% level. As shown in Figure 10, the sample without any antioxidants had the highest production of oxidation products. Oil treatment containing 1000 ppm nanocarriers like TBHQ 200 ppm was effective in reducing the increasing trend of Totox value. In oil treatments containing nanocarriers, the amount of Totox value decreases significantly with increasing concentration, which can be due to the increased oxidative effects of phenolic compounds. These results showed that pomegranate extract nanocarriers effectively improved the thermal stability and shelf-life of soybean oil. Neves *et al.* (2020) showed that the highest level of prickled broom extract (1000 ppm) had the best inhibitory effect on oil oxidation during heating. Quality parameters of the oil improved with a reduction of 17.2%, 22.4%, and 45.6% in the values of peroxide, acidity, and p-anisidine values, respectively [75]. The research results of Okhli *et al.* (2020) investigated the antioxidant activity of essential oil and citrus peel extract on the stabilization of sunflower oil. The findings indicated that Totox, anisidine, and peroxide indices have an enhancement trend over the course of time.

The results of this research are consistent with the results of the studies of Sarabi *et al.* (2017), Singh *et al.* (2018), and Tonfack Djikeng *et al.* (2022) [76, 69, 77].

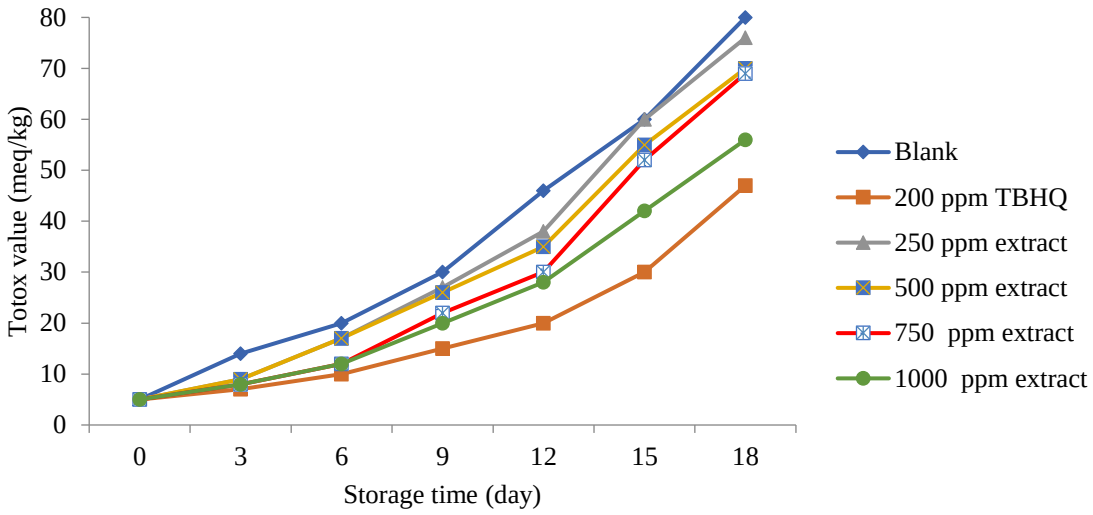

Figure 10- Totox values of different soybean oil treatments containing lipid nanocarrier of pomegranate extract

CONCLUSIONS

With the increase in ultrasound time, the extraction rate of phenolic compounds and DPPH increased. The impact of 40 min was the most significant compared to 10 and 30 min. Therefore, the time using ultrasound and temperature is very effective in the extraction process. By optimizing the extraction conditions by ultrasound, the most extraction of antioxidant compounds can be obtained along with reducing energy consumption, solvent, cost, and time. The resulting extract is a value-added product in the food and pharmaceutical industries. The antioxidant activity of pomegranate anthocyanin lipid nanocarriers in soybean oil was dependent on the concentration. In the investigated treatments, this feature increases with increasing concentration. In general, pomegranate anthocyanin lipid nanocarriers have good antioxidant activity in concentrations 1000 ppm in soybean oil. In suitable concentrations, they can be used as a natural alternative to the synthetic antioxidant TBHQ in soybean oil.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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