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## Effect of grape pomace antioxidants extracted with subcritical water on oxidative parameters of soybean oil

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### Abstract

*Application of bioactive compounds and addition of natural antioxidants are two methods of increasing the oxidative stability of oil during processing and storage. In this study, the antioxidant extract of grape pomace was used to raise the oxidative stability of soybean oil under accelerated oxidation conditions. For this purpose, first, the subcritical water extraction (SWE) of the grape pomace antioxidants was conducted at different temperatures (130-170°C) and times (15-45 min). After optimizing the process using response surface methodology (RSM), the major phenolic compounds of the optimal extract were identified using HPLC. The results showed that with increases in the SWE temperature and time, the ferric-reducing antioxidant power (FRAP) was first elevated and then decreased. However, the ascorbic acid content of the samples continually lowered as the process temperature and time rose. The optimum SWE conditions were found to be the temperature of 135.46°C and the time of 24.23 min with a desirability of 0.807. Gallic acid was the major phenolic compound of both the SWE and conventional extracts. The optimal extract was added to antioxidant-free deodorized soybean oil at 500 ppm, which was further compared with the butylated hydroxytoluene (BHT)-containing and antioxidant-free samples in terms of oxidative stability under accelerated oxidation conditions (63°C for 7 days). Our findings revealed that the anisidine value (AV) and oxidative stability of the oil increased and diminished during storage, respectively. At the same time, the peroxide value (PV) of the samples increased until the 5th day of storage and then declined. It was also realized that the grape pomace antioxidants were able to compete with BHT in reducing these changes. In conclusion, it can be declared that the grape pomace antioxidants extracted through SWE are suitable alternatives to synthetic antioxidants available in the market.*

Keywords: Grape pomace, Subcritical water, Oxidative stability, Soybean oil.

## Introduction

Oxidative reactions are among the sequential reactions that can occur during the processing, storage, and preparation of oil and some fat-containing foods. These reactions are regularly initiated immediately after the oil extraction and lead to off-flavor, off-odor, and color change in the oil or the food, as well as influencing the quality and shelf-life of the food. Auto-oxidation, photo-oxidation, keto-oxidation, and enzymatic oxidation are the different types of these harmful reactions, with auto-oxidation being the most common one (Kariminejad et al., 2023). Soybean oil is a vegetable oil extracted from soybeans (*Glycinemax*). It is known as the most common and healthiest vegetable oil, as it contains large amounts of polyunsaturated fatty acids (PUFA) including linoleic and linolenic acids (Prabakaran et al., 2018). At the same time, soybean oil has low oxidative stability during frying and storage, due to its high PUFA content. These oxidative changes result in off-flavor, degradation of valuable nutrients, and production of toxic substances in the oil, leading to economic losses for producers and consumers (Thorat et al., 2012). Various methods can be employed to raise the oxidative stability of oils during processing and storage, including keeping them away from light, oxygen, and high temperatures, changing the extraction conditions to elevate the content of bioactive components, and adding antioxidants (Grosshagauer et al., 2019). In the edible oil industry, synthetic antioxidants such as BHT are incorporated at a concentration of 200 µg/ml to lessen the oil oxidation. On the other hand, many concerns have arisen about synthetic antioxidants. For instance, BHT is unstable during frying and has shown a toxic effect on the liver in short-term studies. Therefore, finding alternative natural antioxidant sources seems vital (Abdo et al., 2023). Antioxidants should protect the unsaturated fatty acids of edible oils against thermal degradation, as well as exhibiting good thermal stability. The extracts of plants such as thyme, rosemary, sage, marjoram, oregano, and grape pomace are rich sources of natural antioxidants (Kozłowska and Gruczyńska, 2018; Meini et al., 2021). Grape pomace extract has anti-obesity, anti-diabetes, and anti-inflammatory activities, in addition to the antioxidant one (Meini et al., 2019). Moreover, some polyphenols found in grape pomace, such as resveratrol and epigallocatechin gallate, have antiviral and immune system-boosting properties and are suggested as candidates for the prevention of SARS-CoV-2 (Mehany et al.,

2021). Some researchers including Hegazy and Abdel-Maksoud (2016), Freitas et al. (2017), and Shaker (2006) reported that the oxidative stability of vegetable oils was improved by using grape extract or pomace. Conventional extraction methods, such as Soxhlet and hydro-distillation, which have been applied for years, have various drawbacks and technical disadvantages. These methods are very time-consuming and cause the thermal degradation of heat-sensitive compounds, in addition to consuming a large amount of solvent. As a result, there is a great demand for novel environment-friendly extraction methods with shorter times, lower temperatures, and less solvent consumption. Researchers have always been seeking alternatives to conventional methods in the food and pharmaceutical industries (Rodrigues and Pinto, 2007). SWE is a simple, acid-free, and enzyme-free extraction method in which water is heated in the temperature range of 100-374°C and at the corresponding pressure (1.1-22.0 MPa) to remain in the liquid phase and change into a subcritical fluid. Under these conditions, hydrogen bonds are weakened, water polarity is significantly reduced, and the ionization constant of water increases (Das and Arora, 2021). Hosseini et al. (2021) employed RSM to optimize the extraction of cornmeal antioxidants using subcritical water and pulsed electric field (PEF). They concluded that the use of subcritical water led to an increase in the extract total phenolic content (TPC). Park et al. (2022) and Cvetanović et al. (2018) also utilized subcritical water to extract antioxidants from a type of seaweed and chamomile, respectively. The objective of this study is to optimize the SWE of antioxidants from grape pomace to produce an extract with higher added value, as well as using it to improve the oxidative stability of soybean oil.

## **Materials and methods**

### **Materials**

Grape pomace and antioxidant-free refined soybean oil were acquired from Khorasan Cotton and Oilseeds Co., Iran. Other reagents including hydrochloric acid, iron solution, 2,6-dichlorophenol indophenol, syringic acid, vanillic acid, acetic acid, chloroform, isooctane, anisidine solution, and dichloromethane were all supplied by reputable companies.

### Extraction of antioxidants

In this study, SWE and conventional soaking were used for the extraction of antioxidants from grape pomace. The applied SWE device was designed in Research Institute of Food Science and Technology (Mashhad, Iran), which was equipped with an adjustable pressure pump operating at 30 bar in this study. The balance tank of the device, 145 ml in capacity, was filled with distilled water. To extract the antioxidants, the samples were firstly mixed with distilled water at a ratio of 1:10. Then, it was placed in the device chamber. In the SWE, the effects of different temperatures (130, 150, and 170°C) and times (15, 30, and 45 min) were investigated on the extraction yield of the antioxidants. The control sample was prepared using the soaking method at a sample: water ratio of 1:10 for 8h. The obtained extracts were finally dried using a smooth filter and a rotary evaporator (Bakhshabadi et al., 2018; Ozel et al., 2003; Hosseini et al., 2021). The treatments used in this section are given in Table 1.

**Table 1. Treatments used in extract extraction**

Number	Process temperatures(°C)	Process time(min)
1	130	15
2	170	15
3	130	45
4	170	45
5	130	30
6	170	30
7	150	15
8	150	45
9	150	30
10	150	30
11	150	30
12	150	30
13	150	30

## **FRAP**

The method previously presented by Fu, et al. (2011) was used with some modifications to measure FRAP. Accordingly, the FRAP reagent was prepared by mixing sodium acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution (40 mM HCl as solvent), 20 mM iron (III) chloride solution, and distilled water together at a volume ratio of 10:1:1:1.2. Next, 1.8 ml of this reagent was thoroughly combined with 0.2 ml of the diluted sample. After 10 min of incubation at 37°C, the absorbance value of the solution was measured at 593 nm. FRAP was expressed as mM Fe (II)/100 g dry weight. The standard curve was drawn using FeSO<sub>4</sub> solution.

## **Measurement of ascorbic acid content**

The ascorbic acid content of the samples was determined using a spectroscopic method in the presence of oxalic acid or meta-phosphoric acid solutions with acetic acid (Rahman et al., 2007). The solution of 2,6-dichlorophenol indophenol was reduced by ascorbic acid. Xylene was utilized in the extra extraction of the dye solution. The absorbance value of the extra amount of the dye solution was read at 500nm.

## **Identification of major phenolic compounds of antioxidant extracts**

HPLC was employed to identify the major phenolics of the extracts. The standards used in this research included gallic acid, syringic acid, and vanillic acid. First, 20 ml of methanol was added to each sample, and the mixtures were centrifuged at 3000g for 10 min at room temperature. Afterwards, the methanol of the samples was evaporated at room temperature, and ethyl acetate was subsequently added to them in three steps. Then, the supernatant containing ethyl acetate was taken out and dried. After that, 10 mg of each sample was dissolved in 2 ml of acetonitrile. A mixture of water, acetonitrile, and acetic acid (67, 32, and 1%, respectively) was used as the mobile phase at a flow rate of 1 ml/min. The volume of the injected sample was 30 µl, and each test lasted 10 min (Tomaino et al., 2010).

## **Incorporation of grape pomace extract into soybean oil**

After preparing the grape pomace extract, it was directly added to the antioxidant-free soybean oil at 500 ppm which was selected according to the literature and pre-tests. Furthermore, a sample containing 200 ppm BHT and an antioxidant-free sample were kept in a laboratory oven

(Memmert, Germany) at 63°C for 7 days. After sampling on the 0th, 1st, 3rd, 5th, and 7th days, the PV, AV, and oxidative stability of the samples were quantified (Przybylski et al., 2013; Mansour et al., 2022).

### **Determination of PV**

The PV of the samples was measured according to AOCS Cd 8-53 (1993). 5 g of the oil sample was weighed in a 250-ml Erlenmeyer flask. Then, 300 ml of acetic acid-chloroform solvent was added at a ratio of 3:2. After stirring, 0.5 ml of saturated potassium iodide solution was added, and the mixture was kept in the dark for 1 min. 30 ml of distilled water was added to the resulting solution which was further titrated with 0.1 M sodium thiosulfate. The titration continued until the yellow color disappeared. Eventually, 0.5 ml of starch reagent was added, and the titration continued until the disappearance of the blue color. PV was calculated using Equation 1 (AOCS, 1993):

$$1) \quad P = \frac{S \times M \times 100}{W}$$

where S and M respectively stand for the volume (ml) and molarity of the sodium thiosulfate solution, W is the oil weight (g), and P denotes the oil PV (meqO<sub>2</sub>/kg oil).

### **Quantification of AV**

The AV of the oil samples was measured according to the method described by Metzner Ungureanu et al. (2020). For this purpose, 1 mg of each oil sample was weighed in a 25-ml flask that had previously been heated by 10°C above the oil melting point. The sample was then made to the volume with isooctane. Next, 5 ml of this solution was mixed with 1 ml of 0.25% glacial acetic acid solution of anisidine and kept in a dark place at 23°C for 8 min. At 2-min intervals, the absorbance values of the samples were measured using a spectrophotometer at 350 nm. AV was computed using Equation 2:

$$2) \quad A = \frac{25 \times (1.2A_s - A_b)}{m}$$

Where A<sub>s</sub> and A<sub>b</sub> respectively indicate the absorbance values of the solution before and after the reaction with the anisidine solution, and m represents the sample weight (g).

### **Oxidative stability**

In order to determine the oxidative stability of the oil samples, the Rancimat device was employed according to AOCS Cd 12b-92 (1993) which is based on the change in the electrical conductivity of the water in the device chamber by the compounds resulting from the oxidation of the oil in the device cell. The test was carried out at 110°C and an inlet air flowrate of 20 L/h on the 1st day of production (AOCS, 1993).

### **Statistical analysis**

In the first phase of the study (SWE optimization), a rotatable central composite response surface design was used to evaluate the effects of the independent variables on the dependent ones. All the coefficients of the quadratic regression model and the interactive effects of the factors can be estimated with the help of this design. The most important aim of this research was to examine the interaction between the factors and to optimize the SWE of the grape pomace antioxidants. As a result, RSM was employed. In order to assess the behavior of the response surfaces, the quadratic polynomial model was fitted to the experimental data. The adequacy of the regression model and the goodness of the fit were determined by the model lack-of-fit and determination coefficients. All the analyses were conducted with Design Expert version 6.0.2. In the second phase of the research, for three repetitions of a general factorial design was created using the SAS software, and Duncan's multiple-range test was utilized for mean comparison. Excel 2007 was applied to draw the curves.

## **Results and discussion**

### **FRAP**

FRAP demonstrates the ability to reduce trivalent iron and convert it to the divalent one. The presence of antioxidants (reducing agents/electron donors) in extracts leads to the reduction of ferric cyanide complexes and their conversion to the ferrous form. The color of the solution alters from yellow to green or blue, depending on the reducing properties of the extract (Do et al., 2013). The results of the statistical analysis showed that the SWE time and temperature had significant effects ( $p < 0.05$ ) on this response (Table 2). The suggested model indicated that the second power of the temperature had the greatest effect on FRAP (Table 3). Fig.1 illustrates that FRAP firstly increased and then declined with a rise in the process temperature and time. These

alterations can be related to the changes in the TPC of the extracts during processing. The effect of the temperature was more profound than that of the time. Phenolic compounds can donate electrons or hydrogen atoms to break down free radicals and diminish oxidation. Reducing agents, the main factor of FRAP, do their antioxidant activity by breaking the free radical reactions. Esmaeliani et al. (2020) optimized the SWE of saffron bioactive compounds and attributed the increase and decrease in the FRAP of the samples to rises in the process temperature and time, respectively.

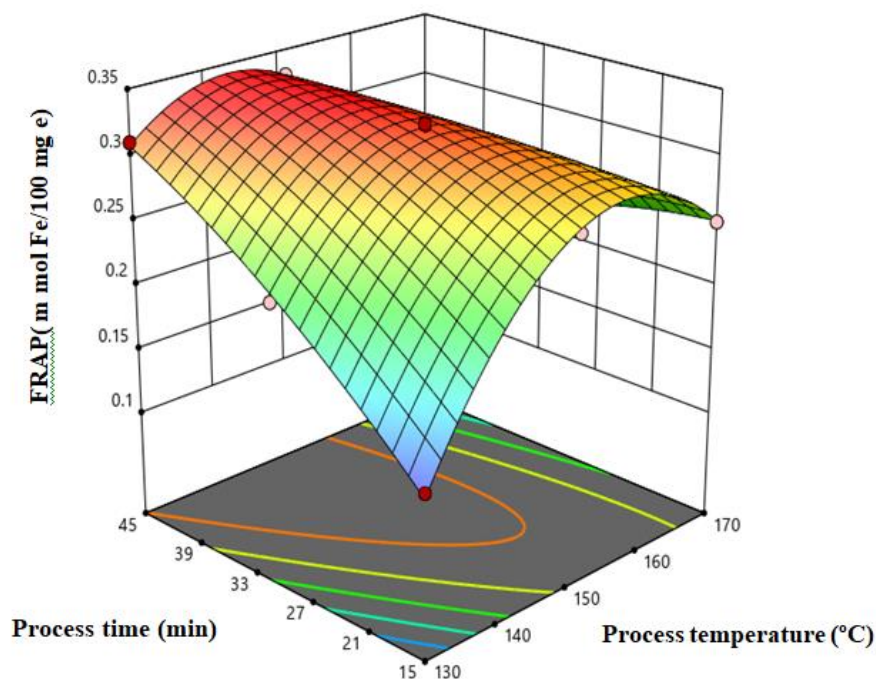
Zhang et al. (2019) claimed that water properties such as dielectric constant, viscosity, and diffusion coefficient change under subcritical conditions. Therefore, owing to its solubility at high temperatures, water can be applied as a solvent in the recycling of value-added compounds, because it acts like organic solvents such as methanol and ethanol under these conditions.

**Table 2. Analysis of variance for dependent variables**

Source	FRAP			Ascorbic acid content		
	SS	F-Value	Pb>F	SS	F-Value	Pb>F
<b>Model</b>	0.049	589.37	< 0.0001	345.05	620.58	< 0.0001
<b>X<sub>1</sub></b>	0.0015	8.97	0.0201	84.38	758.75	< 0.0001
<b>X<sub>2</sub></b>	0.003	195.29	< 0.0001	235.63	2118.89	< 0.0001
<b>X<sub>1</sub><sup>2</sup></b>	0.022	1338.44	< 0.0001	0.17	11.89	0.247
<b>X<sub>2</sub><sup>2</sup></b>	0.006	37.22	0.0005	18.72	1.60	< 0.0001
<b>X<sub>1</sub>X<sub>2</sub></b>	0.016	934.09	< 0.0001	1.32	168.34	0.011
<b>Residual</b>	0.0001			0.77		
<b>Lack of fit</b>	0.00009	7.74	0.0684	0.406	1.46	0.352
<b>Pure Error</b>	0.00001			0.372		
<b>Total</b>	0.049			345.83		

X<sub>1</sub>: temperature and X<sub>2</sub>: time





**Fig. 1. Variations in ferric-reducing antioxidant power during SWE at different times and temperatures**

### Ascorbic acid content

The statistical analysis (Table 2) revealed that only the quadratic effect of the SWE temperature had no significant effect on the ascorbic acid content of the extracts ( $p > 0.05$ ). The quadratic model of this response is presented in Table 3, showing the more dramatic effect of the linear term of the process time on it. Figure 2 depicts that as the SWE time and temperature were elevated, the ascorbic acid content of the samples decreased. Hosseini et al. (2021) also stated that with a rise in subcritical water temperature, the ascorbic acid content of the samples was reduced, due to the destruction of this substance. Ascorbic acid is a very susceptible water-soluble compound which is easily decomposed at high temperatures and converts into other products because of hydrolysis or polymerization. Consequently, its content lowers (Okmen and Bayindirli, 1999; Perez-Conesa et al., 2009).

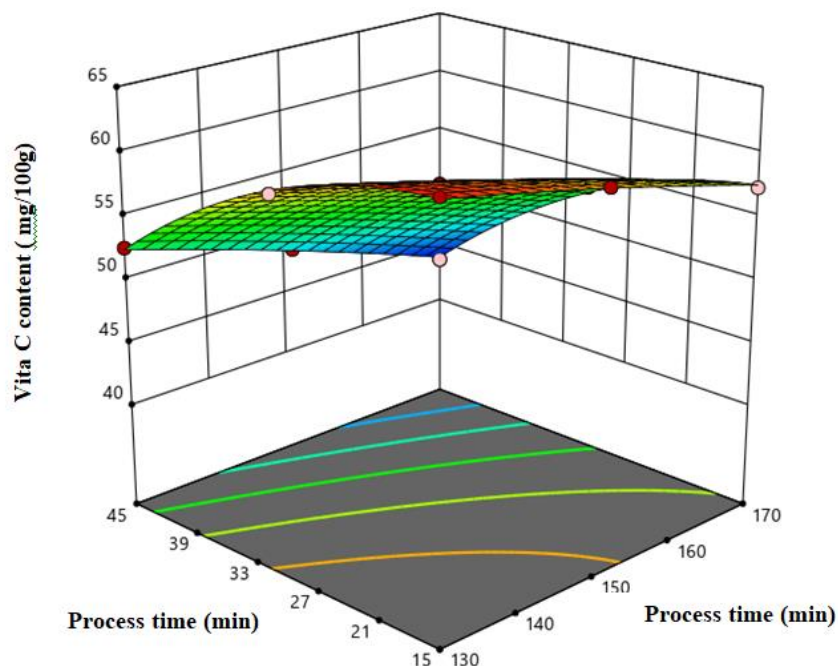


Fig. 2. Variations in ascorbic acid content during SWE at different times and temperatures

Table 3. Quadratic model equations for dependent variable

NO.	Dependent variable	Equation	R2	R2-adj	CV
1	Ferric-reducing antioxidant power	$y = +0.32 - 0.005X_1 - 0.023 X_2 - 0.09 X_1^2 - 0.015 X_2^2 - 0.062X_1X_2$	0.998	0.995	1.50
2	Ascorbic acid content	$y = +57.27 - 3.75 X_1 - 6.27 X_2 - 0.253 X_1^2 - 2.60 X_2^2 - 0.57X_1X_2$	0.997	0.996	1.59

### SWE optimization

Given that the SWE of antioxidants from grape pomace was conducted at 130-170°C for 15-45 min, the process was optimized so as to achieve the maximum FRAP and ascorbic acid content. The optimal conditions were found to be the temperature of 135.46°C and the time of 24.23 min with a desirability of 0.807. In order to assess the effects of the SWE under the optimal conditions, the properties of the optimal sample were compared with those of the control one (Table 4). Based on the statistical analysis, it can be maintained that the application of SWE in the optimal conditions raised the antioxidant activity of the grape pomace extract compared to

the control sample ( $p < 0.05$ ), which demonstrates the efficiency of this method in the processing of food products.

**Table 4. Comparison between properties of optimal and control samples.**

Properties	Control	SWE
FRAP (mmol Fe /100g )	0.13 <sup>b</sup>	0.33 <sup>a</sup>
Ascorbic acid content (ppm)	61.00 <sup>b</sup>	63.07 <sup>a</sup>

The data are mean and lowercase letters in each row indicate significance at the 5% level.

#### Major phenolic compounds of antioxidant extracts

As summarized in Table 4, the application of the different extraction methods caused significant differences in the concentration of the phenolic compounds ( $p < 0.05$ ). The grape pomace extract obtained through SWE contained a higher phenolic compounds (Table 5), owing to the decrease in the solvent polarity at higher temperatures, resulting in the weakening of the hydrogen bonds and further dissolution of medium-polar phenolic compounds in the solvent (Matshediso, Cukrowska, & Chimuka, 2015). It was observed that syringic acid was only detected in the SWE extract. In both methods, gallic acid had the highest content. The same result has been accomplished for the SWE of wine and grape pomace (Casagrande, et al., 2019). Gallic acid was the major phenolic compound, followed by catechins in grape seed pomace (Aybastier, Dawbaa, & Demir, 2018).

**Table 5. Concentrations of phenolic compounds of control and SWE extracts**

Phenolic compound	SWE	Control
Gallic acid (ppm)	42.11±2.23 <sup>aA</sup>	31.04±0.08 <sup>bA</sup>
Syringic acid (ppm)	22.17±1.09 <sup>aB</sup>	ND <sup>bC</sup>
Vanillic acid (ppm)	8.91±1.65 <sup>aC</sup>	5.82±0.50 <sup>bB</sup>

The similar capital and small letters respectively demonstrate non-significant difference in each column and row at  $p < 0.05$ . ND: not detected

#### PV

Peroxides are considered the primary indicators of lipid reactions and with an increase in these compounds, the secondary products of lipid oxidation, including carbonyl, aldehyde, and conjugated DN compounds, also increase. Therefore, quantification of PV can be necessary for oxidation (Dana et al., 2003). Mean comparison (Figure 3) indicated that PV rose in all the

samples until the 5th day of storage, but diminished thereafter. It was also realized that the PV less increased by using the antioxidant extracts and BHT. The phenolic compounds present in an antioxidant extract are able to give a hydrogen atom to free radicals and thus restrain the progression of the reaction chain of lipid oxidation (Barros et al., 2009). Table 6 showed that all investigated parameters (antioxidant type, storage time and the interaction effect of antioxidant type with storage time) had a significant effect on the amount of PV in the samples ( $p < 0.001$ ). The reduction in the PV of the samples with increasing the storage time from 5 to 7 days can be attributed to the breakdown of hydroperoxides, secondary oxidation reactions, and the production of carbonyls and volatile compounds (Vidya and Srikar, 1996). On the other hand, it was found out that the highest PV (8.72 meqO<sub>2</sub>/kg oil) belonged to the control sample on the 5th day of storage. Tinello and Lante (2020) studied the accelerated oxidation of soybean oil containing the antioxidant extracts of ginger and turmeric and mentioned that with the prolongation of the storage period up to 21 days in an oven, the PV of soybean oil increased up to 80 meq O<sub>2</sub>/kg oil, and the addition of these extracts to the oil made this increase smaller. Hussain et al. (2018) investigated the effect of sesame seed extract on sunflower oil and stated that the PV of the samples was elevated during storage, and the incorporation of the extract into the oil mitigated this increase. In line with these results, Iqbal and Bhanger (2007) also observed that the effect of garlic methanolic extract on sunflower oil stability was similar to that of BHT.

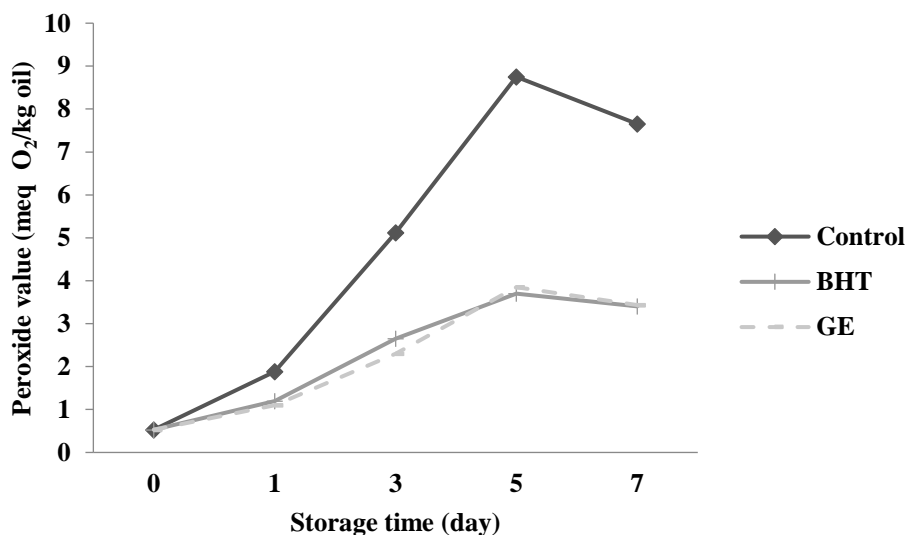


Figure 3- Effects of antioxidant type and storage time on peroxide value of soybean oil

Table 6- ANOVA test results

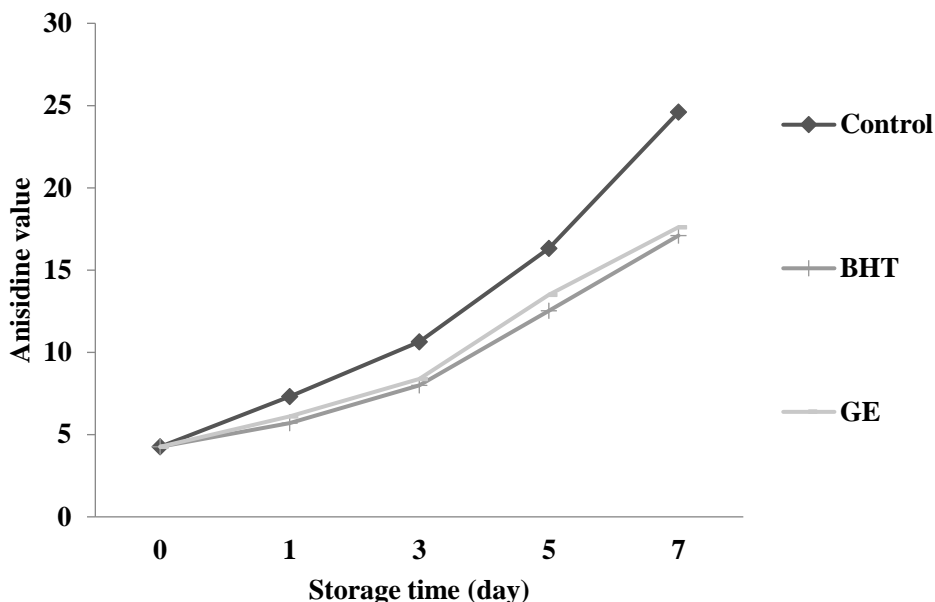
Characteristics	PV	AV	Oxidative stability
Source	Mean Square		
Type of sample	26.78**	40.51**	37.41**
Storage time	41.52**	343.64**	12.71**
Type of sample ×Storage time	4.59**	6.91**	0.439**

\*\* Significance at the 1% level

## AV

During lipid oxidation, hydroperoxides, as the primary oxidation products, are decomposed into the secondary ones which are very stable and responsible for the off-odor and off-flavor of edible oils (Poiana, 2012). AV is a reliable measure to determine the amount of the secondary products (Zhang et al., 2010). PV increases in the initial stage of oxidation; however, when the peroxides are degraded into aldehydes and ketones, AV is elevated, and ultimately, PV starts to decrease (Chandrasekara and Shahidi, 2011). Consequently, the increase in AV is simultaneous with the change in the increase in PV (Guillen and Cabo, 2002). Mean comparison using the Duncan's test (Figure 4) revealed that the AV of the samples rose during storage ( $p < 0.05$ ), which was more pronounced in the control (antioxidant-free) sample than in the other ones. Additionally, our findings showed that the lowest AV was related to the BHT-containing sample on the 7th day of storage, which did not have statistically significant differences with the ones containing the grape pomace extracts.

An elevated AV demonstrates the progression of auto-oxidation and an increase in the secondary products, resulting from the decomposition of hydroperoxides and carbonyl compounds, during storage. Ling et al. (2015) cited that banana antioxidant extract diminished the AV of sunflower oil under accelerated oxidation conditions, due to possessing phenolic compounds. The reduction in AV with the use of antioxidant extracts can be ascribed to the presence of phenolic compounds in such extracts. Alsufiani and Ashour (2021) investigated the effect of 2,4,4-Trihydroxychalcone on the oxidative stability of sunflower oil and claimed that AV increased during storage; nonetheless, this increase was dampened by employing the antioxidant, which was consistent with the results of the present research.



**Figure 4- Effects of antioxidant type and storage time on anisidine value of soybean oil**

### **Oxidative stability**

Oxidative stability is the time required to reach a point where one of the oxidation quantities such as PV or carbonyl value suddenly increases after being gradually elevated and causes off-odor and off-flavor in the oil. Oxidation causes spoilage, resulting in the food unpleasant smell and declined quality. There are several indices for evaluating compounds resulting from thermal processes that have dramatic effects on the chemical, physical, and nutritional properties of oil. One of the most important indices is the oxidative stability index (Holser, 2003). Measuring only this index during the thermal treatment of an oil sample is not enough to examine its quality. However, it provides information about the initial status of the oil sample (Matthaus, 2006). Figure 5 illustrates that the oxidative stability of the samples decreased during storage in the oven, and the highest oxidative stability (7.49 h) was associated with the BHT-containing one which did not have a statistically significant difference ( $p > 0.05$ ) with the one containing 500 ppm of the grape pomace extract on the same day. Casagrande et al. (2019) and Aybastier et al. (2018) demonstrated that gallic acid and catechins were the major phenolic compounds of grape pomace extract, which brought about a reduction in the rate of oxidation. Hosseini et al. (2021) optimized the PEF-SWE of antioxidants from cornmeal and incorporated the resulting extract into soybean oil. They declared that by increasing the antioxidant concentration to 750 ppm in the oil,

its oxidative stability was raised due to the increased TPC of the oil. Salta et al. (2007) indicated that when olive leaf extract containing polyphenols was added to commercial oils (olive, sunflower, and palm), their antioxidant capacity and oxidative stability improved remarkably, conforming to the results of the present study.

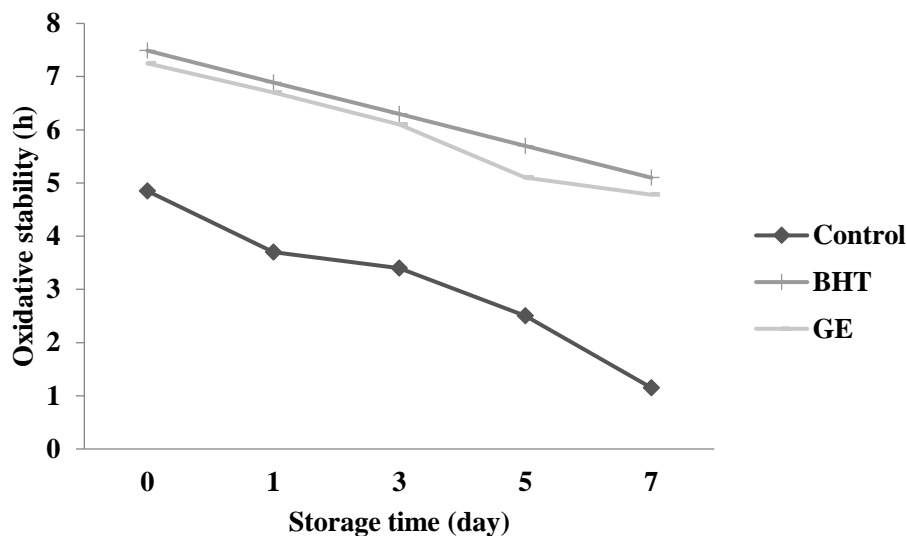


Figure 5- Effects of antioxidant type and storage time on oxidative stability of soybean oil

## Conclusions

The main purpose of this research was to enhance the oxidative stability of soybean oil using the antioxidants of grape pomace extracted with subcritical water. It was concluded that the use of subcritical water gave rise to the major phenolic compounds and thus to the antioxidant activity of the grape pomace extract. Accordingly, addition of the grape pomace extract to soybean oil improved its oxidative stability. In conclusion, it can be declared that the grape pomace antioxidant extract prepared through SWE is a suitable alternative to commercial synthetic antioxidants.

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