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A response surface methodology study on the effects of some phenolics and storage period length on vegetable oil quality: change in oxidation stability parameters

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Abstract: In the present study, response surface methodology was conducted for the determination of effects of some phenolics (gallic acid, ellagic acid, and quercetin) on some stability parameters of mixed oil prepared with sunflower and hazelnut oils (50:50, v/v). In this respect, peroxide value (PV), free fatty acids (FFAs), *p*-anisidine value, induction period, and refractive index of mixed oil were investigated. Predictive regression equations were constructed for the estimation of each studied parameter ($R^2 > 0.861$). The storage period caused a significant increase in the peroxide value, FFAs, *p*-anisidine, and refractive index values of the mixed oil, while it decreased the induction period value of oil ($P < 0.01$). The addition of gallic acid significantly retarded oxidation ($P < 0.05$), and in general gallic acid and quercetin were found to be effective for preserving oil against oxidation.

Key words: Vegetable oil, phenolics, storage, oxidation, response surface methodology

1. Introduction

Edible oils are crucial for the human diet due to their high energy value, essential fatty acid content, ability to provide good solubility for some vitamins (Papadopoulou and Roussis, 2008), and cholesterol-lowering effect (Yalcin, 2011). The preservation of vegetable oils is very difficult because of their high unsaturated fatty acid content. The reaction between oxygen and unsaturated fatty acids causes the deterioration of lipids or lipid-containing products (Erkan et al., 2009). The possibility of inhibiting this reaction has been a source of interest because lipid deterioration affects consumer acceptance and adversely influences other food components such as proteins, carbohydrates, pigments, and fat-soluble vitamins, and results in development of an off-flavor, loss of nutritional value, discoloration, and the formation of toxic compounds (Chung et al., 2004). These compounds formed during oxidation can cause health problems, such as heart failure, cataract, and brain dysfunction, by cytotoxic action (Lambropoulos and Roussis, 2007). Moreover, it has been claimed that free radicals formed as a result of lipid oxidation may lead to cancer development (Kawanishi et al., 2002). The harmful effects of the oxidation depend on the lipid composition (fatty acid profile; number; position, geometry, and conjugation of double bonds; antioxidants) and environmental conditions during processing and storage such as oxygen, light, heat, and moisture (Çolakoğlu, 2007).

There are many hazardous effects of lipid oxidation. The addition of antioxidants into food formulations is the most general, easy, and accepted method to prevent or retard lipid oxidation (Halliwell et al., 1995). Butylated hydroxytoluene, butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ), and propyl gallate are synthetic antioxidants that are very commonly used in the food industry. However, recent studies have reported that these synthetic antioxidants may have toxic effects for health (Jeong et al., 2004). For that reason, the use of TBHQ in food formulations is prohibited in Japan, Canada, and Europe (Mohdaly et al., 2010). BHA has been removed from the list of "generally recognized as safe" (GRAS) compounds (Goli et al., 2005).

In light of safety concerns about synthetic antioxidants, researchers recently have focused on exploring natural antioxidant compounds, which are safer and more effective than synthetic antioxidants. Of these natural

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antioxidant compounds, phenolic compounds have mostly been used. Phenolic compounds are known as secondary metabolites of the plants. One of the most important biological activities of these compounds is their antioxidant activity (Cook and Samman, 1996). There are many studies in the literature dealing with the antioxidant capacity of different phenolics (Armando et al., 1998). In this study, we examined the effect of gallic acid, quercetin, and ellagic acid, and their interactions on the oxidation of lipids using response surface methodology (RSM). RSM is an effective statistical tool for observation of different independent variables that affect the quality characteristics of the product. This technique gives information about the relationship between the dependent and independent variables (Krazhiyan et al., 2011).

We observed the effect of storage period and different phenolic compounds (gallic acid, ellagic acid, and quercetin) and their interactions on the primary (peroxide value, PV) and secondary (*p*-anisidine value) oxidation reactions, induction period, free fatty acid (FFA) content, and refractive index values of mixed oil (sunflower: hazelnut oil) using response surface methodology.

2. Materials and methods

2.1. Materials

Refined sunflower (*Helianthus annuus*) and hazelnut (*Corylus avellana*) oils were purchased from local markets and these oils were mixed at the ratio of 50:50 (v/v). Gallic acid, ellagic acid, and quercetin were obtained from Sigma-Aldrich (Sweden). Standard fatty acid methyl esters were from ACCU Standard Inc. (USA).

2.2. Addition of phenolic compounds to mixed oil

Each phenolic compound, solved in ethanol, was added to 100 mL of the oil mixture depending on the central composite rotatable design (Table 1). Oil samples were stored at 50 °C in a hot-air oven (Nuve EN 120, Turkey) and exposed to constant sunlight and air during the storage period. Sample analysis was carried out according to a central composite rotatable design with 5-center repetitive points (runs 10–14, Table 1). In addition, mixed oil with no additive was also stored as a control sample in the same conditions, and analysis of this sample was also performed.

2.3. Peroxide value determination

PVs of oil samples were determined according to the method proposed by the AOAC (2000). Approximately 5 g of mixed oil sample was dissolved in 10 mL of chloroform. Fifteen milliliters of acetic acid and 1 mL of saturated solution of potassium iodide (KI) were added and the resulting mixture was shaken manually and kept in the dark at room temperature for 5 min. Seventy-five milliliters of distilled water was added and the mixture was again shaken vigorously. After addition of 1 mL of starch solution (1%), the mixture was titrated with 0.01 N sodium thiosulfate until the blue color disappeared. The PV expressed as milliequivalent of available oxygen per kg of oil ($meqO_2$/kg oil) was calculated according to the following formula:

$$PV = \frac{[(V_1 - V_0) \times N]}{M}, \quad (1)$$

where V_1 and V_0 are the volume of sodium thiosulfate used for the titration of the sample and the blank, respectively. N is the normality of the sodium thiosulfate solution and M is the amount of the mixed oil (g). All analyses were carried out in triplicate.

2.4. Free fatty acids (FFA)

FFA values of samples were determined according to the method of AOAC (2000). First, 5–10 g of mixed oil sample was dissolved in 100 mL of ethanol-diethyl ether mixture (1:1, v/v). Phenolphthalein (1%) was then dropped to the solution, which was shaken by hand. The mixture was titrated with 0.1 N standardized sodium hydroxide (in ethanol) until a permanent pink color appeared. The FFA values, expressed as oleic acid, of the oil samples were calculated as follows:

$$FFA\ \% = \frac{[(V \times 2.82]}{M}, \quad (2)$$

where V is the volume of the 0.1 N sodium hydroxide used during titration, 2.82 represents the gram molecule of oleic acid divided by 100, and M is the weight of the oil sample. All analyses were carried out in triplicate.

2.5. *p*-Anisidine value

The *p*-anisidine value was determined according to the IUPAC standard method (IUPAC, 1992) with some modification. Approximately 0.2 g of the mixed oil sample was dissolved in 25 mL of isooctane. The absorbance of this solution (Ab) was then measured at 350 nm using a UV-Vis spectrophotometer (Cary 100 Conc, Varian Australia PTY Ltd, Australia). After this, 5 mL of oil solution was mixed with 1 mL of *p*-anisidine reagent prepared by dissolving 0.25 g of *p*-anisidine in 100 mL of glacial acetic acid and shaking the mixture vigorously. After 10 min, the absorbance of the mixture (As) was determined at 350 nm. The *p*-anisidine values were calculated using the following formula:

$$p\text{-anisidine value} = 25 \times (1.2As - Ab)/m, \quad (3)$$

where m is the mass of the oil sample (g). Analyses were conducted in triplicate for all samples.

2.6. Refractive index values of the oil samples

The refractive index value of the oil samples was determined using an automatic refractometer (Reichert AR, USA) and the results were reported at 20 °C. All analyses were carried out in triplicate.

Table 1. Central composite rotatable design for the independent variables (actual and coded levels).

Run	Coded level				Actual level			
	X_1	X_2	X_3	X_4	Storage period (day)	Ellagic acid (g)	Gallic acid (g)	Quercetin (g)
1	-1.483	0	0	0	0.00	0.05	0.05	0.05
2	-1	-1	-1	-1	19.54	0.02	0.02	0.02
3	-1	-1	-1	1	19.54	0.02	0.02	0.08
4	-1	1	1	-1	19.54	0.08	0.08	0.02
5	-1	-1	1	-1	19.54	0.02	0.08	0.02
6	-1	1	-1	1	19.54	0.08	0.02	0.08
7	-1	1	1	1	19.54	0.08	0.08	0.08
8	-1	1	-1	-1	19.54	0.08	0.02	0.02
9	-1	-1	1	1	19.54	0.02	0.08	0.08
10	0	0	0	0	60.00	0.05	0.05	0.05
11	0	0	0	-1.483	60.00	0.05	0.05	0.00
12	0	0	0	0	60.00	0.05	0.05	0.05
13	0	1.483	0	0	60.00	0.10	0.05	0.05
14	0	0	0	0	60.00	0.05	0.05	0.05
15	0	0	0	1.483	60.00	0.05	0.05	0.10
16	0	0	0	0	60.00	0.05	0.05	0.05
17	0	0	-1.483	0	60.00	0.05	0.00	0.05
18	0	0	0	0	60.00	0.05	0.05	0.05
19	0	-1.483	0	0	60.00	0.00	0.05	0.05
20	0	0	1.483	0	60.00	0.05	0.10	0.05
21	1	1	-1	1	100.46	0.08	0.02	0.08
22	1	-1	-1	-1	100.46	0.02	0.02	0.02
23	1	1	1	1	100.46	0.08	0.08	0.08
24	1	-1	1	-1	100.46	0.02	0.08	0.02
25	1	-1	1	1	100.46	0.02	0.08	0.08
26	1	1	1	-1	100.46	0.08	0.08	0.02
27	1	-1	-1	1	100.46	0.02	0.02	0.08
28	1	1	-1	-1	100.46	0.08	0.02	0.02
29	1.483	0	0	0	120.00	0.05	0.05	0.05

X_1: Storage period (day); X_2: ellagic acid (g); X_3: gallic acid (g); X_4: quercetin (g).
Oil volume was 100 mL.

2.7. Rancimat oxidation

A Rancimat device (743, Metrohm, Switzerland) was used for determination of induction period, which is a measure of the stability index (in hours) of the oil samples. Three grams of mixed oil sample was placed into the glass tube. The airflow was set at 20 L/h, and the temperature at 130 °C. All samples prepared according to Table 1 and the control sample were subjected to oxidation using a Rancimat device. The oil stability index values of the oils were determined as the hour at the end of the induction period. Experiments were carried out in duplicate.

2.8. Experimental design and statistical analyses

The effect of storage period on the parameters of the control sample and the significant differences between the samples were determined by performing ANOVA using SPSS 17.0.1. The Tukey test was used for determination of the differences between the dependent variables of the oil samples.

RSM was used to observe the effect of independent variables (storage period, amount of gallic acid, ellagic acid, and quercetin added to the 100-mL oil sample) on the dependent variables (PV, FFA, *p*-anisidine, refractive index value, and induction time). A rotational central composite design was used and the range of the storage time and phenolic amounts were determined to be 0–120 days and 0–0.1 g per 100 mL oil, respectively.

All experiments were replicated 3 times and there were 5-center points in the experimental design. The relationship between the independent and dependent variables was explained by the following second-degree polynomial equation:

$$y = \sum_{i=1}^{4} \beta_{ki} X_i + \sum_{i=1}^{4} \beta_{kii} X_i^{\,2} + \sum_{i,j \le 2}^{4} \beta_{kij} X_i X_j \,, \qquad (4)$$

where y is the dependent variable and β_{ki}, β_{kii}, and β_{kij} are the coefficients of linear, quadratic, and interaction terms, respectively. X_i and X_j represent the independent variables. Analysis of variance was performed to determine the effect and regression coefficients of linear, quadratic, and interaction terms. P-values of less than 0.05 were accepted as statistically significant. The model adequacies were examined by R^2 values. RSM was applied using the JMP 5.0.1 statistical package program.

3. Results and discussion

3.1. Physicochemical properties of the control mixed oil

Some physicochemical properties of the control oil sample at different storage periods are listed in Table 2. The results showed that the storage period caused a significant change in the physicochemical characteristics of the oil ($P < 0.05$). The peroxide value of the control oil sample was 19.50 $meqO_2$/kg oil at the beginning of the storage and a significant increment was observed, up to the maximum value of 448.10 $meqO_2$/kg oil after 120 days ($P < 0.05$). Yalcin (2011) reported that the peroxide values of hazelnut oil that did not contain any antioxidant were 34.5, 74.8, and 531.4 at 40 °C after 30, 60, and 120 days of storage, respectively. Yalcin et al. (2011) also investigated the efficiency of some natural waste extracts on sunflower oil and reported that the PV of control sunflower oil was found to be 140.82 $meqO_2$/kg oil after 45 days of storage at 40 °C. As is known, exposure of oil to the air (Paz and Molero, 2001), heat (Matalgyto and Al-Khalifa, 1998), and light (Xiaoying and Ahn, 1998) causes a significant increase in the oxidation of oil. The main reason for the deterioration of vegetable oils is the oxidative rancidity that occurs at the double bond of the fatty acids in the triglyceride structure (Akhtar et al., 1985). Similar results were observed in the FFA values of our control oil sample.

Table 2. Effect of storage period on the peroxide value, free fatty acids, *p*-anisidine values, induction time, and refractive index of the control sample.*

Storage period (days)	Physicochemical properties				
	Peroxide value ($meqO_2$/kg oil)	Free fatty acid (%)	*p*-Anisidine value	Induction time (h)	Refractive index
0	19.50 ± 0.81^{e}	0.25 ± 0.02^{d}	7.04 ± 0.25^{c}	1.74 ± 0.02^{a}	1.4737 ± 0.0000^{d}
24	152.57 ± 4.03^{d}	0.23 ± 0.00^{d}	8.29 ± 0.16^{c}	0.21 ± 0.02^{b}	1.4740 ± 0.0004^{d}
60	368.38 ± 3.76^{c}	0.78 ± 0.01^{c}	141.12 ± 2.33^{b}	0.07 ± 0.01^{c}	1.4759 ± 0.0001^{c}
96	412.04 ± 9.06^{b}	2.11 ± 0.03^{b}	162.13 ± 1.22^{a}	0.06 ± 0.01^{c}	1.4776 ± 0.0001^{b}
120	448.10 ± 15.43^{a}	4.84 ± 0.21^{a}	162.91 ± 4.30^{a}	0.05 ± 0.00^{c}	1.4793 ± 0.0000^{a}

*Means ± standard deviation.
Column values with different lowercase letters in superscript are significantly different at $P < 0.05$.

The FFA value of the control sample was determined to be 0.25% at the beginning of the storage. It increased to 4.84% after 120 days of storage at 50 °C, a significant increase ($P < 0.05$). Rehman (2006) reported that the FFA values of the control corn oil stored at 25 °C and 45 °C for 120 days were 7.0% and 11%, respectively. A similar trend was reported by Yalcin (2011). The first initiating step in deterioration of oils is the formation of FFA, which is sensitive to oxygen attacking in the presence of light, air, or heat. This reaction results in the formation of many organic compounds and FFA, which are responsible for the development of rancidity and off-flavors (Rehman, 2006). Rehman (2006) also reported that the formation of FFA and increases in peroxide value are the best indicators of fat deterioration and can be used to monitor the extent of fat deterioration.

The *p*-anisidine values of the control oil sample were 7.04 and 162.91 at the beginning and end of the storage period, respectively. Crapiste et al. (1999) reported that the *p*-anisidine value of fresh extracted sunflower oil was 1.91. In the same study, the *p*-anisidine value of pressed and stored sunflower oil at 67 °C for 54 days was found to be 130, while this value was 0.96 at the beginning of storage. Hydroperoxides are the primary products that occur during oil oxidation but they are labile chemical substances that decompose into many different secondary oxidation products. *p*-Anisidine is an indicator of aldehyde content that is constant at the beginning of the oxidation and sharply increases following the peroxide decomposition. For that reason, *p*-anisidine is commonly used to determine oil oxidation levels (Crapiste et al., 1999).

Induction times of the control oil samples decreased with the increment of storage period ($P < 0.05$). Induction time was 1.74 h at 130 °C at the beginning of the storage and 0.05 h at the end of storage. Läubli and Bruttel (1986) reported that the induction time, which is determined by Rancimat, is based on the conductometric determination of volatile degradation products and features automatic plotting of conductivity versus time. This technique has many advantages compared to other procedures, such as the active oxygen method. Läubli and Bruttel (1986) reported that the induction time of oils decreased with the increase of set temperature in Rancimat.

A significant increase was observed in refractive index values of control oil during storage, and it increased to 1.4793 at the 120th day of storage. Farag et al. (2007) reported that the refractive index of the control oil samples was 1.4724 at the beginning of the heating period and a significant increase was observed in the refractive index of oil with the increase in heating period because of oxidation; it increased to approximately 1.4760 after 5 days of heating. Yoon et al. (1985) reported that the refractive index of rice bran oil and double fractioned palm olein increased almost linearly with the increase in heating time. In another study, Johnson and Kummerow (1957) investigated the chemical changes that take place in corn oil during thermal oxidation, and they reported that the corresponding refractive index of samples increased with thermal treatment duration at a constant temperature and with the increase of temperature at a constant treatment time.

According to the correlation analysis results, a few significant correlations were determined among the physicochemical properties of mixed oil stored at 50 °C for 120 days. The peroxide value showed a significant positive correlation with the *p*-anisidine ($r = 0.966$) and refractive index ($r = 0.912$) values of mixed oil ($P < 0.05$). A significant positive correlation was also determined between the FFA values and refractive indexes. Since the *p*-anisidine results were parallel to peroxide values, it also showed significant correlation with the refractive index of mixed oil ($P < 0.05$).

3.2. Oxidation kinetics of control mixed oil

In general, vegetable oil oxidation was fitted to half-order kinetics (Colakoglu, 2007). However, the addition of antioxidants caused a change in the half order to the first order (Labuza and Bergquist, 1983). The following equation is used to describe the half-order reaction kinetics (Erkan et al., 2009):

$$Y^{1/2} - Y_0^{1/2} = (1/2)k_M[A]t, \quad (5)$$

where Y and Y_0 are the concentration of the oxidation products at time t and the initial concentration of the oxidation products, [A] is the concentration of the lipid substrate, and t is the storage time (days). According to our results, peroxide formation during the 120 days storage was found to be:

$$Y^{1/2} - Y_0^{1/2} = 0.1660t \ (R^2 = 0.8704) . \quad (6)$$

The half-order reaction kinetics were also performed for determination of *p*-anisidine formation of the mixed oil sample during storage. The equation for *p*-anisidine formation is as follows:

$$Y^{1/2} - Y_0^{1/2} = 0.0990t \ (R^2 = 0.9134). \quad (7)$$

The high R^2 values indicate that the formation of peroxide and *p*-anisidine was well fitted to the half-order reaction kinetics, which is in agreement with the finding of Erkan et al. (2009). The formation of peroxide and *p*-anisidine was compared and it was seen that peroxide formation was faster than that of *p*-anisidine. This result was expected because *p*-anisidine gives information about the secondary oxidation products (Erkan et al., 2009).

3.3. Physicochemical properties of mixed oil treated with phenolics

Table 3 shows the physicochemical properties of an oil sample treated with different phenolics according to the central composite rotatable design given in Table 1. Significant changes were observed in the peroxide, FFA,

p-anisidine, induction time, and refractive index values of the oil sample depending on the storage period and added phenolic type ($P < 0.05$). Three different phenolics (gallic acid, ellagic acid, and quercetin) were incorporated at different ratios into the mixed oil based on the given design (Table 1) and oils were stored at 50 °C for 120 days in an incubator continually exposed to oxygen and light. Figure 1a illustrates the effect of storage period, gallic acid, and quercetin on the peroxide values of oil samples. It is clear from the figure that storage period caused a significant increase in the peroxide values of oil containing phenolics ($P < 0.05$). The peroxide value of the oil containing 0.05 g/100 mL gallic acid, 0.05 g/100 mL ellagic acid, and 0.05 g/100 mL quercetin was 17.54 meqO_2/kg oil at the beginning of the storage and increased to 393.95 meqO_2/kg oil at the end of the 120 days of storage (Table 3). Gallic acid showed a significant reduction effect ($P < 0.05$) on the formation of peroxides and decreased the peroxide values during storage (Figure 1a). The linear effect of quercetin provided no reduction in peroxide formation ($P > 0.05$) but the quadratic effect of quercetin and gallic acid provided a significant decrease in peroxide value (Table 4, $P < 0.05$). The linear, interaction, and quadratic effects of ellagic acid on the reduction of

Table 3. Experimental physicochemical quality values of the mixed oil samples stored at 50 °C for different periods.*

Runs	Physicochemical properties				
	Peroxide value (meqO_2/kg oil)	Free fatty acid (%)	*p*-Anisidine value	Induction time (h)	Refractive index
1	17.54 ± 0.46	0.63 ± 0.03	4.1 ± 0.35	5.85 ± 0.60	1.4737 ± 0.0000
2	56.07 ± 2.36	0.37 ± 0.02	7.86 ± 0.03	0.85 ± 0.05	1.4744 ± 0.0000
3	65.66 ± 3.25	0.43 ± 0.01	8.55 ± 0.14	1.28 ± 0.05	1.4744 ± 0.0000
4	88.34 ± 2.89	0.56 ± 0.00	8.93 ± 0.14	2.04 ± 0.04	1.4744 ± 0.0000
5	81.53 ± 4.00	0.49 ± 0.02	9.71 ± 0.35	2.38 ± 0.04	1.4744 ± 0.0000
6	84.32 ± 1.84	0.41 ± 0.01	7.51 ± 0.20	0.78 ± 0.01	1.4743 ± 0.0000
7	86.06 ± 3.17	0.50 ± 0.03	10.09 ± 0.65	1.35 ± 0.05	1.4745 ± 0.0000
8	68.03 ±4.15	0.45 ± 0.01	8.07 ± 0.07	1.59 ± 0.09	1.4741 ± 0.0001
9	80.48 ± 4.80	0.53 ± 0.02	9.00 ± 0.26	1.53 ± 0.01	1.4745 ± 0.0000
10	119.25 ± 3.14	0.55 ± 0.05	29.47 ± 1.32	0.12 ± 0.00	1.4739 ± 0.0000
11	120.03 ± 0.35	0.55 ± 0.01	23.85 ± 0.30	0.11 ± 0.00	1.4742 ± 0.0000
12	121.94 ± 1.22	0.56 ± 0.00	22.00 ± 0.11	0.13 ± 0.00	1.4743 ± 0.0000
13	122.00 ± 2.40	0.57 ± 0.02	21.48 ± 1.49	0.12 ± 0.00	1.4741 ± 0.0000
14	120.03 ± 5.41	0.54 ± 0.03	23.19 ± 0.42	0.13 ± 0.00	1.4744 ± 0.0000
15	94.52 ± 3.52	0.43 ± 0.02	13.90 ± 0.24	0.09 ± 0.00	1.4738 ± 0.0000
16	307.33 ± 1.66	0.59 ± 0.03	85.26 ± 1.69	0.06 ± 0.00	1.4751 ± 0.0000
17	319.70 ± 5.93	0.59 ± 0.02	112.71 ± 1.97	0.05 ± 0.00	1.4754 ± 0.0000
18	120.05 ± 4.75	0.46 ± 0.02	18.23 ± 0.49	0.13 ± 0.00	1.4739 ± 0.0000
19	226.53 ± 3.36	0.44 ± 0.01	36.75 ± 1.18	0.08 ± 0.00	1.4743 ± 0.0000
20	117.60 ± 2.40	0.49 ± 0.03	13.95 ± 0.88	0.14 ± 0.00	1.4739 ± 0.0000
21	385.87 ± 9.15	1.03 ± 0.02	170.13 ± 0.30	0.05 ± 0.00	1.4761 ± 0.0000
22	399.37 ± 3.58	0.99 ± 0.05	120.24 ± 2.17	0.06 ± 0.00	1.4756 ± 0.0000
23	234.25 ± 6.22	0.56 ± 0.04	27.52 ± 0.58	0.09 ± 0.00	1.4741 ± 0.0000
24	351.65 ± 3.20	0.81 ± 0.05	72.85 ± 0.18	0.07 ± 0.00	1.4750 ± 0.0000
25	366.51 ± 4.85	1.33 ± 0.01	183.98 ± 1.07	0.04 ± 0.00	1.4765 ± 0.0000
26	355.57 ± 9.14	0.74 ± 0.03	102.95 ± 0.07	0.07 ± 0.00	1.4753 ± 0.0000
27	305.44 ± 2.89	0.56 ± 0.04	59.73 ± 0.31	0.08 ± 0.00	1.4746 ± 0.0000
28	281.95 ± 7.42	0.78 ± 0.02	52.46 ± 0.60	0.07 ± 0.00	1.4746 ± 0.0000
29	393.95 ± 15.71	1.07 ± 0.01	140.58 ± 5.99	0.07 ± 0.00	1.4760 ± 0.0001

*Means ± standard deviation, For each run, all analyses were carried out in triplicate.

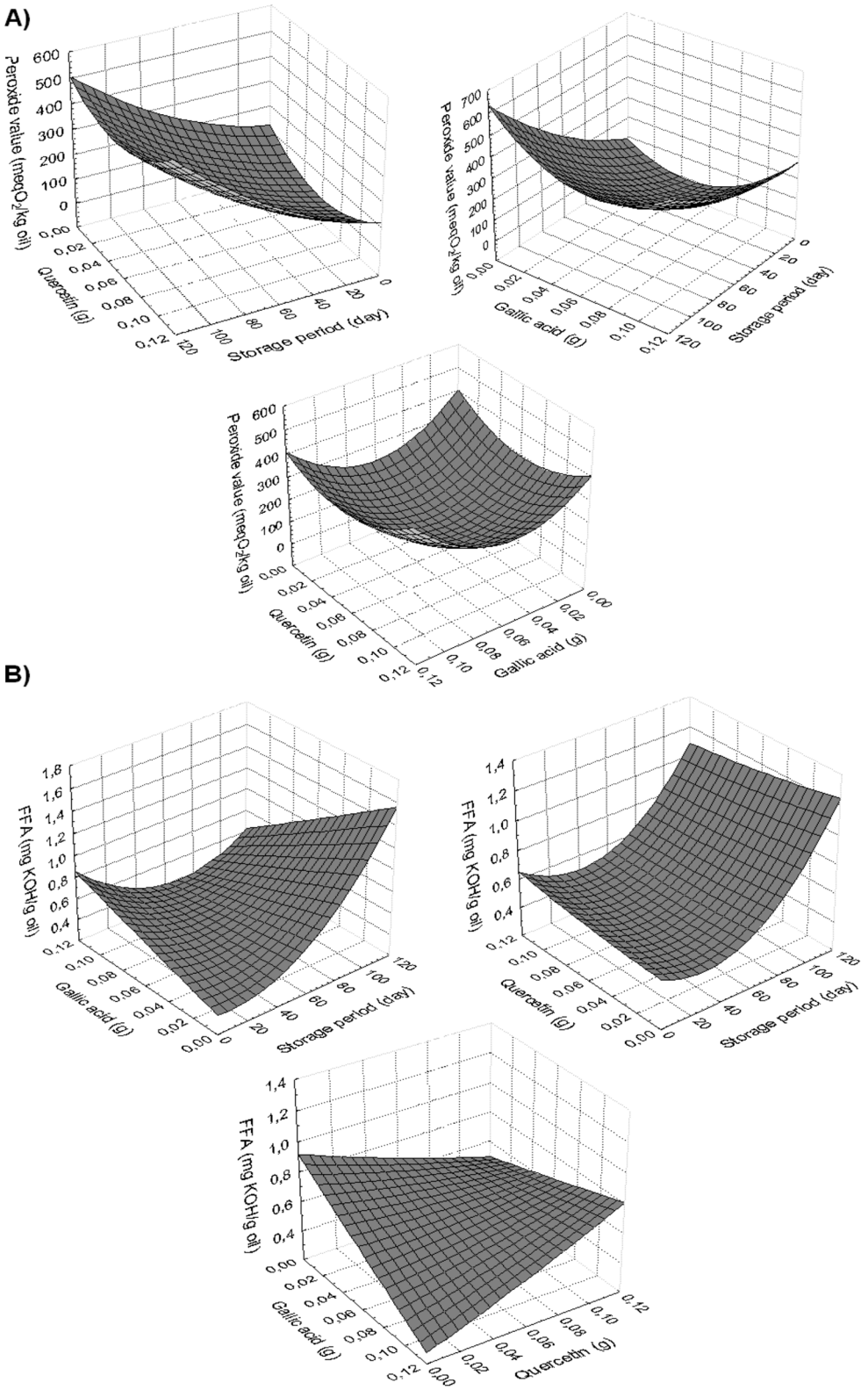

Figure 1. The response surface plots showing the effect of storage period, gallic acid, and quercetin on the peroxide values (A) and free fatty acids (B) of mixed oil stored at 50 °C for 120 days.

Table 4. Significance of the regression models (F-values) and the effects of processing variables on physicochemical quality parameters of mixed oil stored at 50 °C for 120 days.

Source[a]	Physicochemical properties				
	Free fatty acid (%)	Peroxide value ($meqO_2$/kg oil)	*p*-Anisidine value	Induction time (h)	Refractive index
X_1	46.437***	169.156***	100.840***	55.756***	36.795***
X_2	0.083	0.002	0.008	0.192	0.000
X_3	5.538**	4.510**	22.927***	1.478	14.045***
X_4	0.152	1.096	7.004**	0.259	2.245
$X_1 \times X_2$	0.002	0.274	0.012	0.286	0.046
$X_1 \times X_3$	12.565***	4.126*	21.049***	1.749	11.513***
$X_1 \times X_4$	0.294	0.000	0.025	0.295	0.017
$X_2 \times X_3$	0.445	0.187	1.501	0.277	0.802
$X_2 \times X_4$	0.696	0.701	1.046	0.009	0.525
$X_3 \times X_4$	4.007*	0.243	4.394**	0.107	3.129*
$X_1 \times X_1$	15.874***	4.109*	9.199***	36.705***	8.105**
$X_2 \times X_2$	0.375	1.275	2.117	0.371	0.241
$X_3 \times X_3$	0.001	15.715***	3.523*	0.511	6.498**
$X_4 \times X_4$	0.024	6.122**	4.809**	0.446	6.217**
Model	6.18***	14.820***	12.745***	7.034***	6.438***
Lack of fit	154.41***	2144.935***	54.124***	7270.962***	2.586
R^2	0.861	0.937	0.924	0.876	0.866

[a] X_1: Storage period (days); X_2: ellagic acid (g); X_3: gallic acid (g); X_4: quercetin (g).
***: $P < 0.01$, **: $P < 0.05$, *: $P < 0.1$.

peroxide value were found to be insignificant (Table 4, $P > 0.05$). Yalcin (2011) reported that the addition of gallic acid and quercetin showed a significant reduction in the peroxide value of hazelnut oil during storage compared to the control sample. In addition, ellagic acid did not provide a significant reduction in peroxide value after 45 days storage, and the peroxide values of the control sample and the sample containing ellagic acid were found to be similar (Table 4, $P > 0.05$). In general, synergistic or antagonistic effects can be observed among the phenolics when they are used in combination. Ellagic acid was found to be more effective compared to retinol and beta-carotene in hazelnut oil for the retardation of peroxide formation (Yalcin, 2011). It can be speculated that the ellagic acid was not found to be effective in the present study because of the antagonistic effect. Freeman et al. (2010) investigated the synergistic and antagonistic interactions of phenolic compounds present in navel oranges and reported that many antagonistic effects were observed on the oxygen radical absorbance capacity when combinations of 2, 3, or 4 phenolic compounds were used. Roussis et al. (2008) investigated the effect of some phenolics on the oxidative stability of corn oil and concluded that the gallic acid and caffeic acid showed strong inhibitory effects on the formation of oxidation. As can be seen from Table 3, the peroxide values of samples increased to 393.95 $meqO_2$/kg oil. The main reason for this high peroxide value is a hydrogen atom from the active methylene group forming a free radical, which is a reaction accelerated by the addition of radical source such as light, heat, or oxygen. In the present study, peroxide values increased significantly because the oil samples were exposed to intensive light and oxygen at a rather high temperature (50 °C) for 120 days. Figure 1b shows the effect of storage period and addition of

gallic acid and quercetin on the FFA values of oil samples. In parallel with peroxide values, the storage period caused a significant increment in FFA values of samples (Table 3, $P < 0.01$). As stated before, the first initiating step in deterioration of oils is the formation of FFAs, which are sensitive to oxygen. The effect of ellagic acid and quercetin was found to be insignificant ($P > 0.05$) on the formation of FFA in oil. Yalcin (2011) reported that the addition of gallic acid increased the acidity of the hazelnut oil sample at day 60 of the 120-day storage period, but at the end of the storage, the differences between the FFA values of the control oil and the oil containing gallic acid were found to be insignificant ($P > 0.05$). Quercetin was found to be most effective phenolic compound for reducing FFA formation. It can be speculated that the incorporation of phenolics together in a model system can cause an antagonistic effect (Freeman et al., 2010). The effect of storage period, gallic acid, and quercetin on the *p*-anisidine values of the oil samples is illustrated in Figure 2. As shown in the figure, the storage period caused a significant increase in the *p*-anisidine value of the sample. The *p*-anisidine value of the oil incorporated with phenolics was determined to be 4.1 at the beginning of the storage and reached 140.58 at the end of the storage (Table 3). Adding gallic

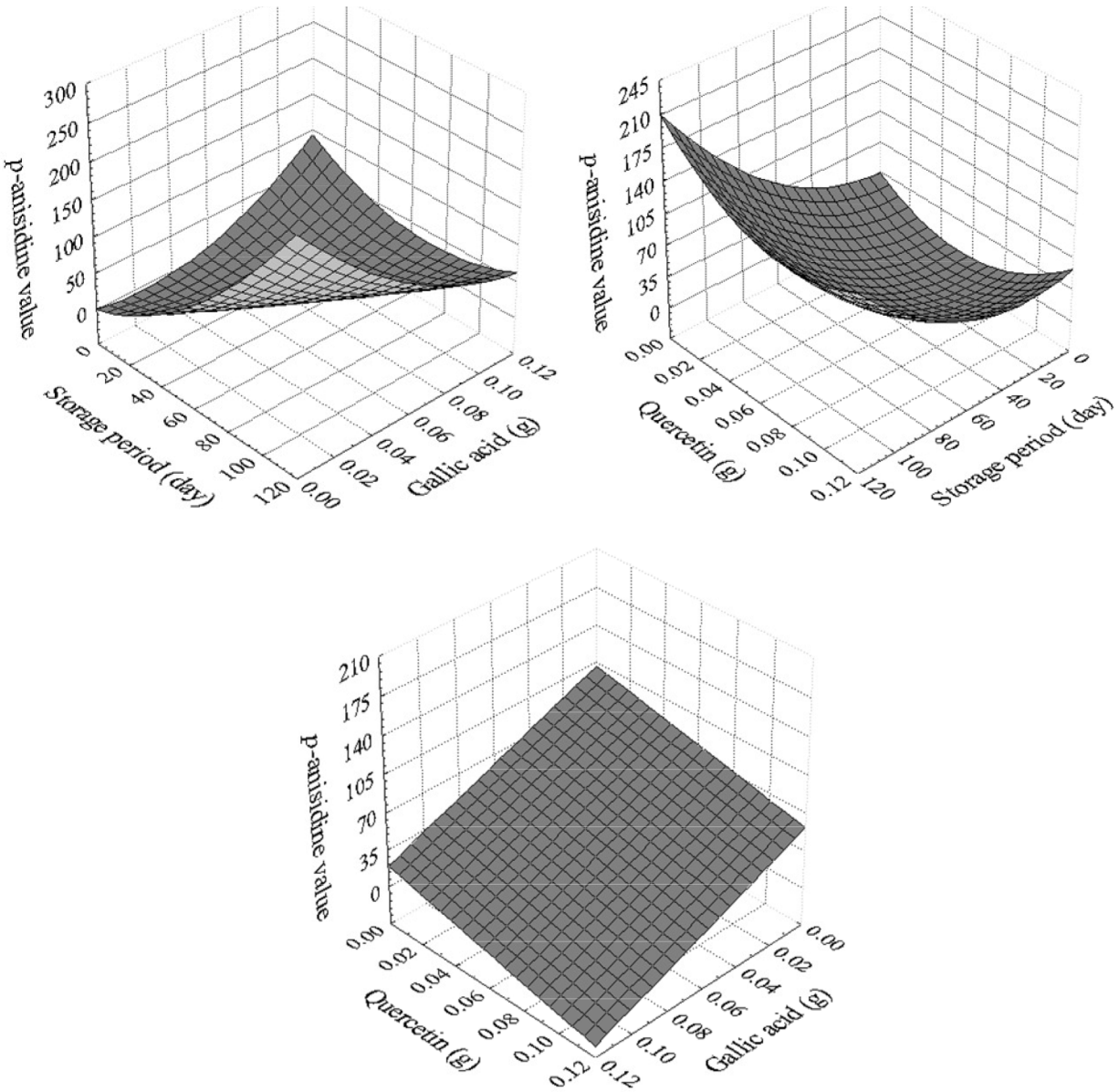

Figure 2. The response surface plots showing the effect of storage period, gallic acid, and quercetin on the *p*-anisidine values of mixed oil stored at 50 °C for 120 days.

acid and quercetin provided a significant decrease in *p*-anisidine values. As stated before, the *p*-anisidine value is the measure of secondary oxidation products and is significantly correlated with peroxide values (r = 0.966). Ahn et al. (2008) reported that the formation of secondary oxidation products, which was measured through the *p*-anisidine test, was effectively inhibited in sunflower oil using natural plant extracts such as rosemary, broccoli sprout, and citrus extracts. Figure 3 illustrates the effect of storage period, gallic acid, and quercetin on the induction period values of the oil samples. In general, storage period decreased the induction period values of samples (Table 3, $P < 0.05$). The effects of gallic acid and quercetin were found to be insignificant on the induction period. It can be speculated that the high temperature (130 °C) may cause degradation of phenolics. The induction period of the oil sample containing phenolics was determined to be 5.85 h at the beginning of the storage period and 0.07 h at the end of the storage. Because of the long storage period and exposure of the samples to the rather intensive oxygen, light, and heat, the induction period was found to be very low. Ahn et al. (2008) reported that the induction period of control sunflower oil was 7.03 h and the addition of different plant extract mixtures into the oil provided a significant ($P < 0.05$) increase in induction period (16.26 h). The effect of storage period, gallic acid, and quercetin on the refractive

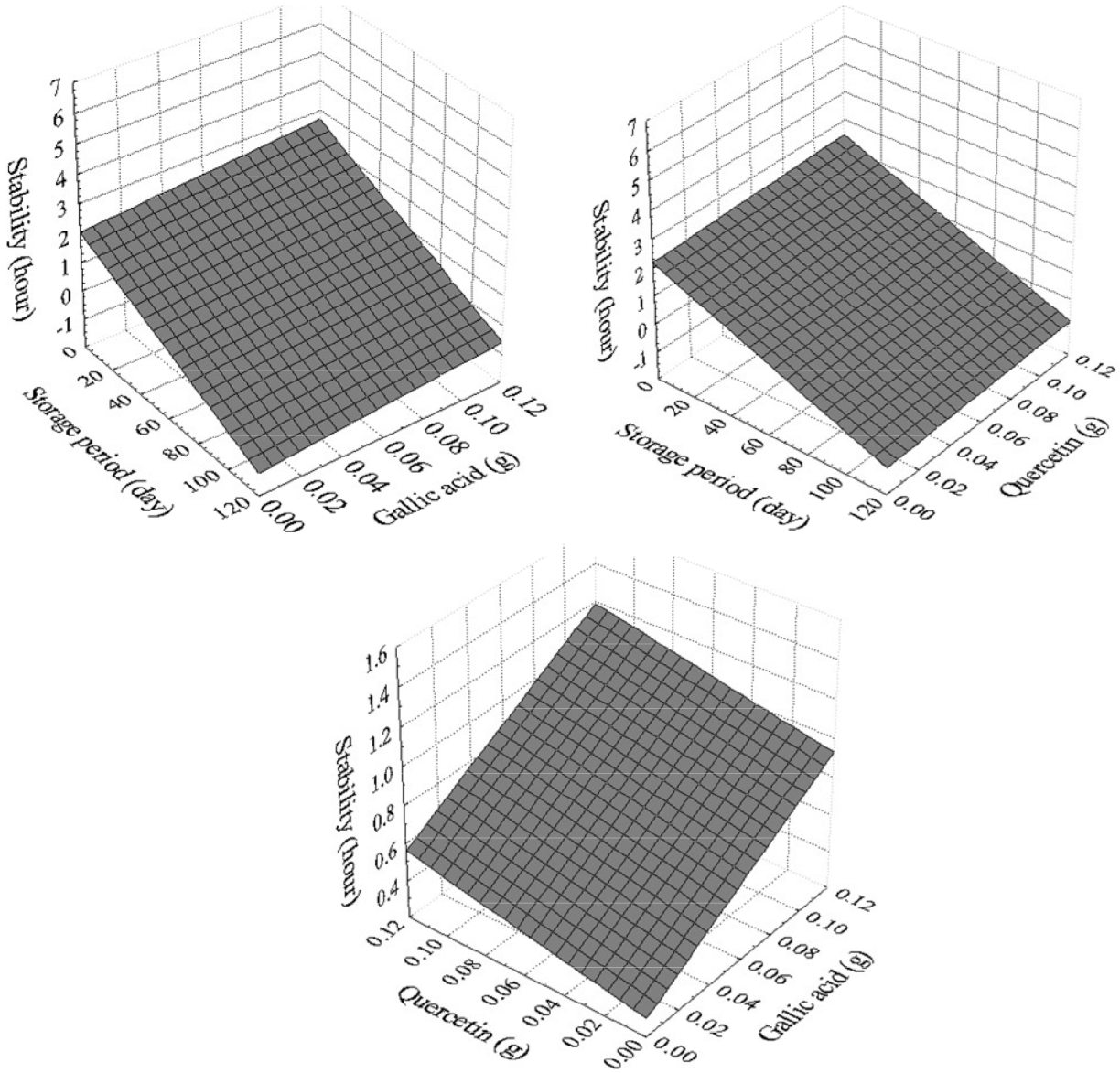

Figure 3. The response surface plots showing the effect of storage period, gallic acid, and quercetin on the induction time values of mixed oil stored at 50 °C for 120 days.

index values of mixed oil samples is illustrated in Figure 4. The refractive index values of the oil samples significantly ($P < 0.05$) increased with storage period. Refractive index values were measured as 1.4737 at the beginning of the storage and 1.4760 at the end of the storage. As expected, gallic acid inhibited the increase in refractive index values of the oil, which occurs due to the formation of oxidation products. It was reported that the refractive index of rice bran oil and palm olein increased linearly with heating time (Yoon et al., 1985). Farag et al. (2007) reported that the addition of olive leaf juice to the sunflower oil as a natural antioxidant retarded the refractive index increase in terms of formation of oxidation products, and it was determined that the higher level of polyphenolics induced a lowering effect on sunflower oil refractive index.

3.4. Predictive regression models

Figure 5 illustrates the regression coefficients and statistical significance levels for the linear, interaction, and quadratic effects of each physicochemical parameter. As can be seen from the figure and as stated before, storage period caused a significant increment in peroxide value, FFA, *p*-anisidine, and refractive index and a decrease in induction period

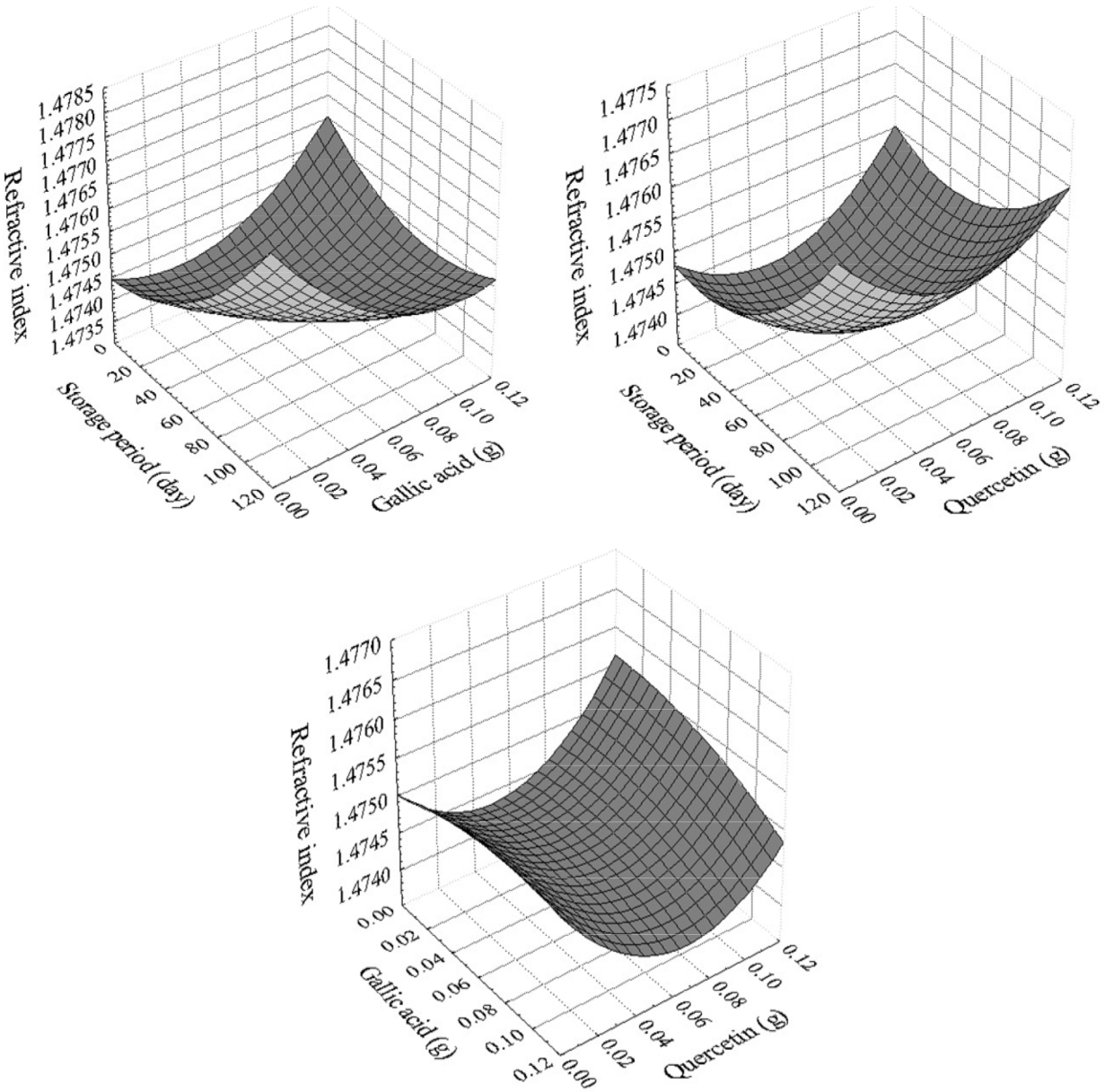

Figure 4. The response surface plots showing the effect of storage period, gallic acid, and quercetin on the refractive index values of mixed oil stored at 50 °C for 120 days.

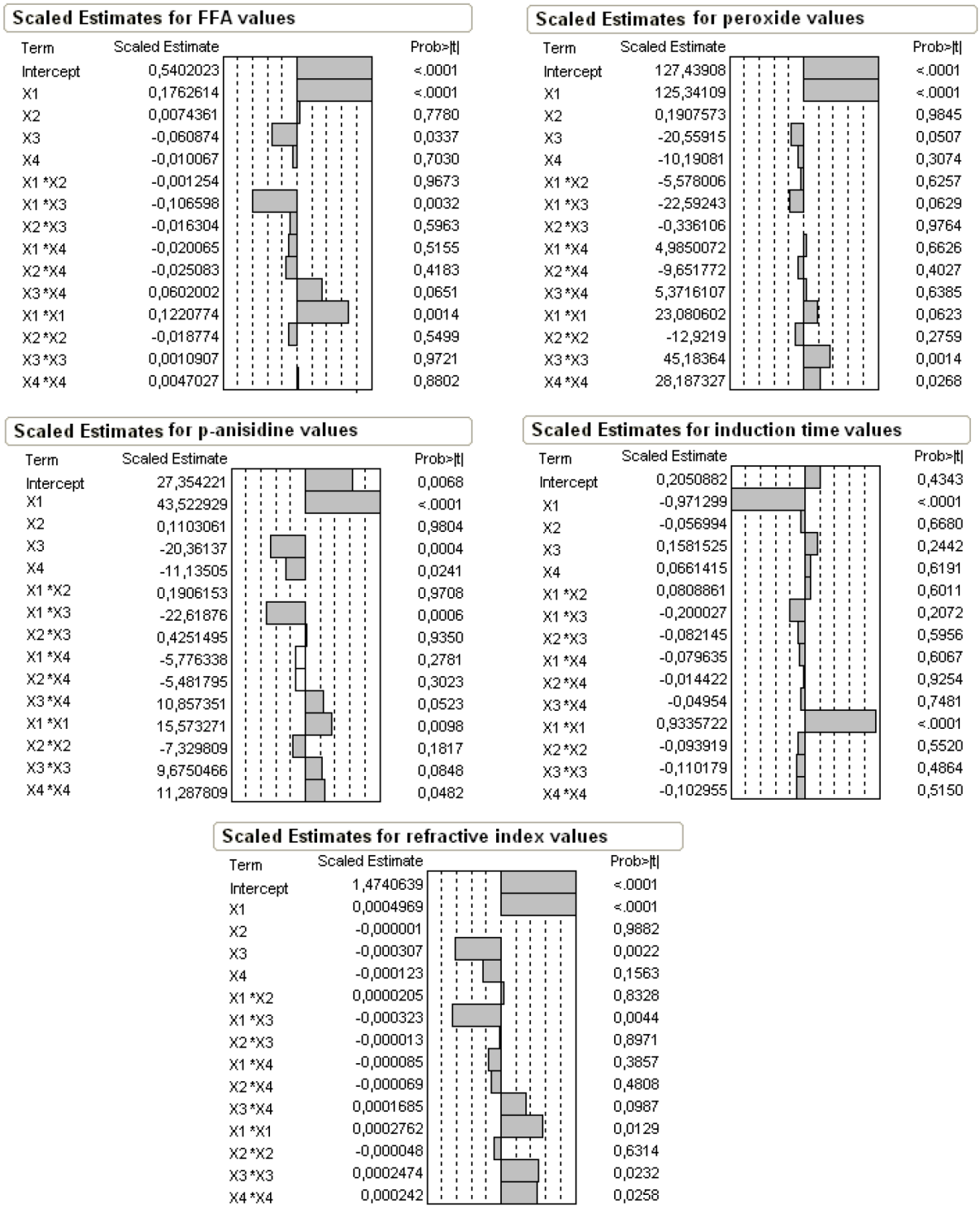

Figure 5. Scaled estimates for physicochemical quality parameters showing the direction of linear, interaction, and quadratic effects of the processing variables: X_1, storage period (days); X_2, ellagic acid (g); X_3, gallic acid (g); X_4, quercetin (g). Positive and negative scaled estimates values indicate the direction of the increase and decrease, respectively.

($P < 0.05$). Among the phenolics, the most effective one was gallic acid, which provided a significant retardation in oxidation. The constructed regression models can be effectively used for the estimation of oxidation parameters

in different storage periods with the addition of phenolics at different concentrations, because quite high coefficients of determination were obtained for the parameters. As can be seen in Table 4, the coefficients of determination were calculated as 0.861, 0.937, 0.924, 0.876, and 0.866 for FFA, peroxide values, *p*-anisidine value, induction period, and refractive index, respectively. The lack-of-fit values for studied parameters was found to be significant, which means that the order of the regression was not secondary (Table 4). However, Box and Draper (1987) reported that a model with significant lack of fit could still be used when a large amount of data was included in the analysis. It was also reported that the high coefficient of determination values (R^2) is evidence of the applicability of the regression model between the ranges of variables included (Martínez and Pilosof, 2012). It is apparent from the study that storage period caused a deterioration of mixed oil because of high levels of oxidation. Peroxide value, FFA value, *p*-anisidine, refractive index, and induction period changed significantly due to the oxidation reaction. The addition of gallic acid significantly retarded oxidation ($P<0.05$) and in general gallic acid and quercetin were found to be effective on the preservation of oil against oxidation. Response surface methodology was successfully used to determine the effects of storage period and phenolic compounds on the oxidation parameters of mixed oil. Predictive regression equations were constructed to estimate each of the studied parameters with different storage periods and phenolic concentrations with rather high determination coefficients ($R^2 \geq 0.861$).

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