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The effect of bread-making process on the antioxidant activity and phenolic profile of enriched breads

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RESEARCH ARTICLE

Abstract

In this study, the effect of fermentation time and baking temperature on the antioxidant activity and phenolic profile of bread enriched with grape and pomegranate seed were investigated. It was determined that total phenolic content (TPC) and antioxidant activity improved through prolonged fermentation time. TPC in the control dough ranged from 280 to 376 mg gallic acid equivalent (GAE)/kg; the TPC in the pomegranate seed-containing dough ranged from 402 to 466 mg GAE/kg; the TPC content in the grape seed-containing dough ranged from 551 to 591 mg GAE/kg. The Trolox equivalent antioxidant capacity (TEAC) value of the control samples increased from 525 to 1,017 µmol Trolox, the TEAC value of the pomegranate seed-containing samples increased from 1,059 to 2,575 µmol Trolox, and the TEAC value of the grape seed-containing samples increased from 1,992 to 2,950 µmol Trolox. The ferulic acid contents in the control, pomegranate seed, and grape seed-containing doughs did not change with increasing fermentation time. However, gallic acid content of the dough increased significantly as the fermentation period increased. It was also determined that baking increased antioxidant activity and gallic acid content showed a 10-fold increase. The TPC and TEAC values in the pomegranate seed and grape seed-containing breads also significantly increased. Fermentation and baking process significantly influence bioactive components and antioxidant activity.

Keywords: antioxidant activity, bread, baking, fermentation, phenolics

1. Introduction

Enriched foods are mainly used to address malnutrition in developing countries and promote a healthier and more balanced diet in the rest of the world. They cannot substitute medicines in case of a disease. In recent years, there has been a global trend towards the use of the natural substances in the food as a source of antioxidant and functional ingredients. Especially natural antioxidants have received considerable interest because of their nutritional and functional properties. Bread plays key role in the human nutrition. Generally, wheat bread is considered to be a good source of energy and irreplaceable food for many people (Skrbic and Filipcev, 2008). However bread made with white flour is a food with a low antioxidant capacity. Enrichment of bread with bioactive ingredients has been commonly used in order to enhance their pro-health properties. Due to its widespread consumption, bread which in 37 developed communities provides more than 50% of the total energy intake, is considered to be the best vehicle for functional supplements (Akhtar *et al.*, 2011). Currently, there are several successful studies concerning the improvement of the functional properties of bread via enrichment. However, the majority of these studies observed the antioxidant activity and phenolic profile of breads enriched with bioactive ingredients. Very few studies have been conducted to determine the effects of fermentation and baking on antioxidant activity and phenolic compounds.

Fermentation has historically been one of the processes that are used to improve the properties of various foods and produce new products (Katina *et al.*, 2007). The fermented products such as beer and bread that are obtained from

cereals date back to ancient Egypt. Although fermentation was first used to achieve brewing, aroma development, and stability improvement, with the development of industrial baking, the use of baker's yeast has gained an international popularity (Poutanen *et al.,* 2009). During fermentation, various biochemical events changing the digestibility and bioavailability of the products occur and large numbers of metabolites are formed (Katina *et al.,* 2007). Previous studies have shown that fermentation increased the antioxidant activity. For example, Virtanen *et al.* (2007) determined that radical scavenging effect increased in milk fermented with different lactic acid bacteria strains. Moktan *et al.* (2008) determined that the radical scavenging effect and ferric reducing power of fermented soy beans increased. Fermentation also contributes to the release of phenolic compounds. The phenolic compounds are released from food matrix by the action of microbial enzymes (Moore *et al.,* 2007). It is well known the phenolic compounds in the cereals such as barley, wheat, and corn are in the free form or in the form of conjugates with sugars, sugar alcohols, or amines (Chandrasekara and Shahidi, 2012). Phenolic compounds in cereals are believed to have low bioavailability because of their insoluble form (Kern *et al.,* 2003). To release the phenolics using enzymes, chemicals, or fermentation prior to consumption is one of the ways to increase the bioavailability of the phenolics (Moore *et al.,* 2007).

Thermal process is one of the most important steps of bread making process. Certain complex events that affect quality properties such as storage stability, sensory properties, and nutritional properties occur during heat treatment (Ceylan, 2018; Ceylan *et al.,* 2018; Meral, 2017). Some food components are destroyed during the thermal process while some components can be released and new components can be formed during treatment (Meral, 2017). It has been reported that antioxidant properties of bread improved with thermal treatment (Meral and Doğan, 2013; Peng *et al.,* 2010). However, there was no detailed study about the effects of thermal treatment on antioxidant activity and phenolic compounds.

The present study aimed to evaluate the effect of different fermentation times and baking temperatures (for wheat bread, grape seed (GS) and pomegranate seed (PS) enriched breads) on antioxidant properties and phenolic compounds. GS and PS were added the bread formulation to determine the effect of bread-making process on antioxidant activity and phenolics. Extracts from GS are popular plant extracts that have been widely used in various food and beverage applications. GS has attracted more attention in the recent years due to its health benefits like antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory properties (Perumella and Navam, 2011). Meral and Doğan (2013) stated that the antioxidant activities of the bread increased significantly with the increased GS substitution. The incorporation of GS into bread formulation provides a novel method to solve a waste disposal problem while adding extra health benefits (Hoye and Ross, 2011).

PS is one of the most important industrial waste. In the recent years several works have indicated PS has potent antioxidant activity and it prevents the formation of cancer and tumors. Foodstuffs prepared with PS have increased as PS has strong antioxidant activity. In their study, Meral *et al.* (2016) revealed that the antioxidant activity and phenolic compounds (gallic acid and catechin) of the PS-containing breads increased. For the above mentioned reasons we used GS and PS. The incorporation of GS and PS in bread formula would have the advantage as GS and PS were a rich source of a number of phenolic compounds (Jayaprakasha *et al.*, 2003). While choosing the fermentation times and baking temperatures, the volumes of the dough and bread and the colour of the bread crust were taken into account and fermentation times that will not decrease the volumes of the dough and bread and baking temperatures that will not cause the bread crust to become darker were selected.

2. Materials and methods

Materials

All chemicals used were of analytical grade. Gallic acid, vanillic acid, ferulic acid, *p*-coumaric acid, syringic acid, caffeic acid, catechin, epicatechin, quercetin, rutin, chlorogenic acid, pyrocatechuic acid, phloridzin, 2,2-diphenylpcrylhydrazyl (DPPH), Folin-Ciocalteu reagent, 2,2-azinobis (3-ethylenebenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and potassium persulfate ($K_2S_2O_8$) were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). Wheat bread flour was purchased from Taşdemirler Flour Plant (Van, Turkey). The bread additive including emulsifier, alpha amylase, ascorbic acid and citric acid was obtained from Puratos, (Istanbul, Turkey). Instant yeast was obtained from Pakmaya A.Ş. (Kocaeli, Turkey). GS and PS were obtained from Velioğlu Baharat (Van, Turkey). These seeds were ground with a laboratory mill (Perten LM 120, Hägersten, Sweden) just before using. After GS and PS were ground (particle size <0.5 mm) wheat flour was replaced with ground seeds at 5.0% level.

Bread making

The formulation proposed by Meral and Dogan (2013) was used in the making of the control bread samples. Basic dough formula depends on 100 g flour basis. Flour (100 g), water (57 g) salt (1.5 g), yeast (1 g) and bread additive (0.8 g) were mixed. All the solid components used in bread making were placed in a kneader (Yücebaş Makine, İzmir, Turkey). The doughs that were kneaded for an optimal

time were fermented 45 min. in a fermentation cabinet at 30 °C and 90% relative humidity and after this period, the dough cut in pieces weighing 200 g were rested on a counter for 10 min. and following the AACC (10-10B), moulded into loaves in a moulding machine (Şimşek Laborteknik, Ankara, Turkey). The moulded doughs were placed in dough pans of 14.29×7.94 cm (Teksan Makine A.Ş. Bursa, Turkey) and went through fermentation in a fermentation cabinet at 30 °C and 90% relative humidity. The fermented doughs were baked in a convection oven (Özkesoğlu, İstanbul, Turkey). To better observe the effects of fermentation time and baking temperature, some of the doughs were added 5% PS and some doughs were added 5% GS. These components were added to flour by means of replacement: flour in an amount corresponding to the amount of the added natural components was removed to make the doughs. The samples that did not contain any additional component were represented by C, the samples that contained pomegranate seeds were represented by PS, and the samples that contained grape seeds were represented by GS.

Preparation of the samples for analysis

To determine the effect of fermentation, samples were collected 0, 60, 90, 120, and 150 min. after kneading and to stop the fermentation, the samples were placed in a deep freezer at -18 °C (Arçelik, İstanbul, Turkey). The doughs fermented for different durations were coded 0, 60, 90, 120 and 150. Fermented dough samples were separated into two portions. A portion of the fermented doughs was baked for 25 min. in a convection oven at 210 °C (Özkesoğlu), while another portion of the doughs was baked for 17 min. at 218 °C. To determine the effect of baking, the samples put in the deep freezer immediately after kneading and the samples baked at 210 °C and 218 °C were compared with each other. The samples were coded 1, and 2, respectively. To avoid discrepancy in the analysis results, dough samples and baked samples were kept 48 hours in a freeze dryer (Labconco, Kansas City, MO, USA) to ensure their moisture levels reached 4-5%. The dried samples were milled in a laboratory-type mill (Perten LM 120) to reduce their particle size.

Extraction of the phenolic compounds

The phenolic compounds were extracted to determine the antioxidant activity and total phenolic compound content of the doughs and breads and reveal the phenolic compound profile using HPLC. The method proposed by Meral and Doğan (2013) was modified and used to extract the phenolic compounds. The 5-g sample collected from the milled samples was placed in centrifuge tubes and, then, 15 ml of methanol was added to the tubes. Using a shaking incubator at 35 °C, the tubes were kept 22 hours in a shaking incubator at 35 °C and a rotational speed of 3,500 rpm. Then, the tubes were centrifuged at 12,000×*g* for 15 min. After centrifugation, the supernatant was transferred to amber bottles and the extraction was repeated up to four times. The supernatants were pooled and made up to a final volume of 50 ml with methanol. Each sample extracted for HPLC analysis was filtered with 0.45 µ pore-size cellulose filters (Millipore), transferred to amber vials, and kept at -20 °C until analysis.

Determination of total phenolic compounds

TPC were determined using the Folin-Ciocalteu method. For this purpose, 300 µl sample and 3 ml Na_2CO_3 (2%) were added to test tubes and about 2 min. later, 150 µl of the Folin-Ciocalteu reagent that was 1:1 diluted with ultra-pure water was added to the tubes. The mixture was mixed and kept 45 min. at room temperature in darkness and, then, the absorbance of the mixture was read in a spectrophotometer at 765 nm (T80 UV/VIS, PG Instrument, UK). TPC was calculated using the calibration graph plotted using gallic acid and the results were expressed as gallic acid equivalent (GAE) (Meral and Doğan, 2013).

Determination of DPPH radical scavenging effects

The extracts at five different concentrations (100, 250, 500, 750, and 1000 µl) were transferred to a test tube and the final volume was brought to 1 ml with methanol. Then, 3 ml of the freshly prepared methanolic DPPH solution (0.004%, w/v) was added to the samples. Upon mixing, the test tubes were incubated in darkness and their absorbance was read against a control sample (the sample containing 1 ml methanol and 3 ml DPPH solution) on a spectrophotometer at 517-nm wavelength using a quartz cuvette. The measurements were performed in two repetitions and the radical scavenging property of each sample was calculated using the following equation (Meral and Doğan, 2013).

$$\% \text{ Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}}$$

where $A_{control}$ is the absorbance of control at 517 nm and A_{sample} is the absorbance of sample at 517 nm.

Determination of the Trolox equivalent antioxidant capacity

The method proposed by Re *et al.* (1999) was employed. To determine the Trolox equivalent antioxidant capacity (TEAC) value, first, the 2.45 mM potassium persulfate-containing 7 mM $ABTS^+$ radical solution was prepared. Ethanol was used for dilution so that the solution can give an absorbance of 0.70±0.2 at 734 nm and 1,980 µl of the diluted radical solution was transferred to a test tube and 20 µl of the sample solution were added; the solution was

rapidly vortex-mixed and after 6 min., the absorbance of the solution was read at 734 nm on a spectrophotometer. The same procedure was repeated for Trolox and the antioxidant activity value was expressed as μmol Trolox equivalent.

Phenolic compounds determination

The method proposed by Coloric *et al.* (2005) was employed to separate the phenolic compounds using HPLC. The chromatographic separation was conducted in the Shimadzu (Kyoto, Japan) HPLC system, using a C18 (GLC, Tokyo, Japan) ODS-3 Inertsil column (150×4.6 mm, particle size 5 μm). As the mobile phase, 2% acetic acid-containing water (A) and acetonitrile:water (0.5% acetic acid-containing) (50:50) (B) were used and gradient elution was applied (0. min. 15% B, 25. min. 25% B, 33. min. 35% B, 40. min. 50% B, and 42. min. 70% B). The flow-rate of the mobile phase was adjusted to 1 ml/min and column temperature was set to 25 °C and for the analyses of gallic acid, syringic acid, protocatechuic acid, and *p*-hydroxybenzoic acid, a wavelength of 280 nm was used, while a wavelength of 320 nm was used for caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, catechin, and epicatechin.

Statistical analyses

Data were analysed using the StatGraphics Centrium 15.1 1 (StatGraphics, 2006) for one-way ANOVA. All trials were carried out in triplicate and all data were reported as means±SD (standard deviation). Differences among means were evaluated using Duncan's multiple range tests at a significance level of $P<0.05$. The changes in the samples in terms of fermentation times and baking temperatures were compared.

3. Results and discussion

The effect of fermentation time on the total phenolic compounds, antioxidant activity and the phenolic profile of the doughs

Table 1 shows the effects of fermentation time on the TPC and TEAC contents of the doughs. Each sample was compared based on the fermentation times, regardless of the other variables. In all samples, the TPC and TEAC values increased with increasing fermentation time and the increases were statistically significant ($P<0.05$). The TPC in the control sample ranged from 280 to 376 mg GAE/kg; the TPC in the pomegranate seed-containing samples ranged from 402 to 466 mg GAE/kg; the TPC in the grape seed-containing samples ranged from 551 to 591 mg GAE/kg. The TEAC value of the control samples increased from 525 to 1,017 μmol Trolox, the TEAC value of the pomegranate seed-containing samples increased from 1,059 to 2,575 μmol Trolox, and the TEAC value of the grape seed-containing samples increased from 1,992 to 2,950 μmol Trolox. Previous studies showed that the antioxidant activity and TPC changed during fermentation. In their study, Liukkonen *et al.* (2003) revealed that the antioxidant activity and TPC of the rye flour-containing doughs changed during fermentation. Other previous studies have revealed that *Aspergillus candida* and certain lactic acid bacteria released antioxidants (Yen *et. al.,* 2003). Dordevic *et al.* (2010) reported that the conjugated and free radical contents of the rye samples fermented with *Saccharomyces cerevisiae* increased. Starzynska-Janiszewska *et al.* (2016) stated that antioxidant activity increased with fermentation. We determined remarkable increases in TEAC and TPC values, as newly compounds were formed and phenolic extraction was improved due to alcohol production by yeast (Poutanen *et al.,* 2009; Wang *et al.,* 2014). In this study, we determined TEAC and TPC values of doughs enriched with GS were higher than control doughs and doughs enriched with PS. However the percentage increase of these values in dough containing GS was lower than others. For TEAC, while percentage increase in dough containing GS was 32.4%, percentage increase in control and dough containing PS was 48.3 and 58.8%, respectively. Similar trend was observed for TPC. The increase in TPC values, for control was 25.5%; for doughs containing PS was 13.73%, for doughs

Table 1. The effect of fermentation time on total phenolic content (TPC) and Trolox equivalent antioxidant capacity (TEAC).[1]

Sample[2]	Fermentation time (min)[3]	TPC (mg GAE/kg sample)	TEAC (μmol/kg sample)
C	0	280±7.07^{b}	525±11.31^{c}
	60	280±2.82^{b}	667±23.33^{b}
	90	360±10.60^{a}	1,008±12.02^{a}
	120	359±14.84^{a}	1000±24.04^{a}
	150	376±16.26^{a}	1,017±00.03^{a}
PS	0	402±12.72^{b}	1,059±12.02^{e}
	60	426±17.67^{b}	1,109±12.02^{d}
	90	475±2.82^{a}	1,367±23.33^{c}
	120	475±3.53^{a}	1,859±12.02^{b}
	150	466±2.12^{a}	2,575±11.31^{a}
GS	0	551±15.55^{b}	1,992±12.02^{d}
	60	558±5.65^{b}	2,200±0.00^{c}
	90	594±7.07^{a}	2,750±24.04^{b}
	120	592±7.07^{a}	2,717±23.33^{b}
	150	591±5.65^{a}	2,950±24.04^{a}

[1] The values are given as mean±SD (n=3). The samples with different letters in the same column are significantly different from each other. ($P<0.05$).
[2] C = Control; GS = bread including grape seed; PS = bread including pomegranate seed.
[3] Each sample was compared based on fermentation time, independent of other variables.

containing GS was 6.76%. To date, there was no publication determining the effects of bread-making process on the functional properties. Therefore, it was difficult to compare our results. However, it can be claimed that the antioxidant activity and TPC were mainly affected by fermentation time and the type of antioxidant compounds. While there were many studies revealing the antioxidant activity of GS, a few studies focused on antioxidant activity of PS. GS is rich source of phenolic compounds and it has been used in functional food formulation. The high phenolic content of GS provides several advantages such as strengthening effect on dough (Meral and Doğan, 2013). Similarly, it was stated that PS could be used in bread formulation as it showed powerful antioxidant activity against free-radicals (Meral *et al.,* 2016). The initial higher antioxidant activity of dough containing GS indicated as soon as GS was added in dough formula showed the antioxidant activity against the free-radicals. The antioxidant activity was high at the initial of fermentation period, as the majority of phenolics found in GS were in soluble forms. Nevertheless, further studies are needed to understand the reason of differences between GS and PS.

Figure 1 shows the DPPH radical-scavenging effect in doughs fermented for different durations. The DPPH-scavenging effect of the control samples did not change with increasing fermentation time. Taking the highest sample concentration of 10 mg/ml into account, the inhibition was determined to be between 28 and 29%. The antioxidant activity of the pomegranate seed-containing doughs showed a statistically significant increase with increasing fermentation time (P<0.05). The initial DPPH-scavenging effect of 37% in the non-fermented 10 mg/ml sample-pomegranate seed-containing dough reached 45% in the doughs fermented for 90 min. Fermentation increased the DPPH radical scavenging effect by 20%. In the grape seed-containing doughs, the DPPH radical scavenging effect significantly increased with increasing fermentation time. The scavenging effect of doughs ranged from 72 to 84%. This increase during fermentation agrees with the results obtained by Moore *et al.* (2007) who reported that the DPPH radical scavenging effect of wheat increased with yeast fermentation and by Dordevic *et al.* (2010) who reported that antioxidant activity increased with fermentation with *S. cerevisiae*. In this study, the DPPH scavenging effect of control dough did not change; however, the TPC and TEAC values increased with increasing fermentation time. The antioxidant activity in the control dough was due to the antioxidant compounds in flour and the doughs did not contain any additional antioxidant compound. Cereals contain bioactive compounds such as phenolic compounds, lignans, and hemicelluloses. The majority of these compounds are concentrated in the external layers of cereals and the endosperm contains only a small fraction of bioactive compounds (Poutanen *et al.,* 2009). According to the results, the non-changing DPPH radical

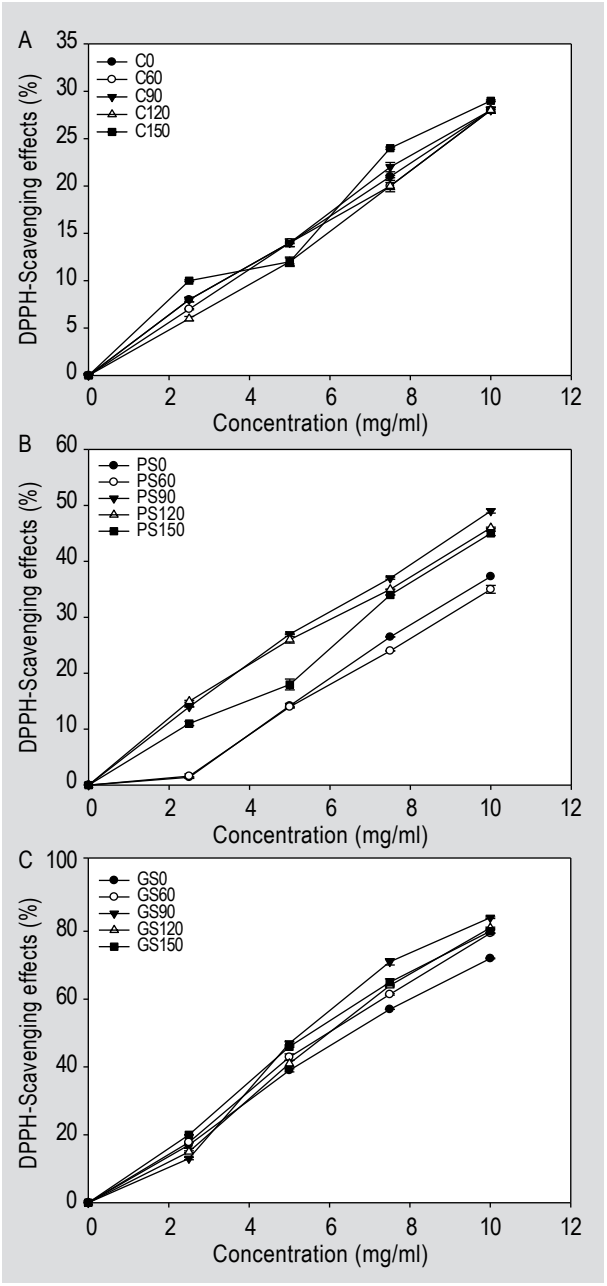

Figure 1. The 2,2-diphenylpcrylhydrazyl (DPPH)-scavenging effect of fermentation time in doughs (A) control; (B): pomegranate seed; and (C) grape seed.

scavenging effect is attributable to the low levels of the currently available antioxidant compounds in the control dough. The DPPH method, albeit a common method, has certain disadvantages in practice, which include the reaction time, the presence of compounds such as carotenoids that have strong absorbance at the same wavelength as DPPH (Holtz, 2008). Moreover, it also has disadvantages such as the higher antioxidant activity of smaller molecules and the limited measurement of the polymeric antioxidants in extracts (Amorati and Valgimigli, 2015).

Moore *et al.* (2007) showed that antioxidant activity increased considering the results obtained with the oxygen radical absorbance capacity assay, however did not significantly change according to the ABTS assay and it was determined that the antioxidant activity measurement method was effective in the determination of the effect of fermentation. Using a single antioxidant activity method is not sufficient when developing a procedure. These results indicate that multiple methods are required when measuring antioxidant activity. In this study, we determined that antioxidant activity and TPC increased with fermentation time. Also, we demonstrated that the highest antioxidant activity and TPC values were found in dough containing GS. Following of doughs containing GS, antioxidant activity and TPC were highest in doughs containing PS. Our data were consistent with previous observations. Meral and Doğan (2013) revealed that antioxidant activity and TPC values of bread with enriched GS were higher than control bread. In another study, Meral *et al.* (2016) stated that incorporation of PS into bread formulae improved the antioxidant activity of bread. The extended fermentation time and the antioxidant compounds incorporated into formulation improved functional properties of breads. The results of the present work indicated it was important that the fermentation time as well as presence of compounds possessing antioxidant activity. The difference in the antioxidant activity of the doughs containing GS and PS might be ascribed to their different phenolic compositions.

Table 2 shows the effect of fermentation time on the phenolic profile of the doughs. Ferulic acid and gallic acid were determined in the control doughs and pomegranate seed-containing doughs, while the grape seed-containing doughs contained catechin and epicatechin in addition to these phenolics. The ferulic acid contents in the control, pomegranate seed, and grape seed-containing doughs did not change with increasing fermentation time. However, the gallic acid content significantly increased with increasing fermentation time ($P<0.05$). The gallic acid content in the control ranged from 1.27 to 2.03 mg/kg, gallic acid content in the pomegranate seed-containing samples ranged from 1.68 to 2.16 mg/kg, gallic acid content in the grape seed-containing samples ranged from 5.36 to 7.56 mg/kg. The catechin and epicatechin amounts in the grape seed-containing doughs were not affected by the fermentation time. Meral and Doğan (2013) demonstrated that bread including GS contained gallic acid and epicatechin.

The previous studies carried out by different researchers revealed that the fermentation stage modified the phenolic profile of cereals (Katina *et al.,* 2007; Wang *et al.,* 2014). Our findings were different from the results of Katina *et al.* (2007) who determined that ferulic acid content of rye bread increased with yeast fermentation. The observed differences were because of the higher ferulic acid amount in rye than in wheat. Wang *et al.* (2014) determined that fermented cereals had a better antioxidant activity and higher phenolic content than non-fermented cereals.

Table 2. The effect of fermentation time on the phenolic profile of the doughs (mg/kg).[1]

Sample[2]	Fermentation time (min)[3]	Ferulic acid	Gallic acid	Catechin	Epicatechin
C	0	1.57±0.15^{a}	1.27±0.01^{d}		
	60	1.46±0.08^{a}	1.53±0.04^{c}		
	90	1.51±0.01^{a}	1.96±0.00^{b}		
	120	1.51±0.05^{a}	1.97±0.00^{ab}		
	150	1.43±0.02^{a}	2.03±0.04^{a}		
PS	0	1.61±0.09^{a}	1.68±0.03^{c}		
	60	1.55±0.00^{a}	1.69±0.06^{c}		
	90	1.68±0.06^{a}	1.90±0.07^{b}		
	120	1.65±0.04^{a}	2.00±0.70^{b}		
	150	1.69±0.01^{a}	2.16±0.02^{a}		
GS	0	1.60±0.00^{a}	5.36±0.02^{c}	24.22±0.00^{a}	25.95±0.02^{a}
	60	1.63±0.11^{a}	6.80±0.45^{b}	23.32±2.88^{a}	23.26±2.68^{a}
	90	1.54±0.04^{a}	7.36±0.21^{ab}	25.45±1.23^{a}	25.51±2.44^{a}
	120	1.64±0.08^{a}	7.61±0.03^{a}	24.04±1.93^{a}	25.01±0.58^{a}
	150	1.64±0.06^{a}	7.56±0.24^{a}	26.06±0.02^{a}	25.03±0.32^{a}

[1] The values are given as mean±SD (n=3). The samples with different letters in the same column are significantly different from each other. ($P<0.05$).

[2] C = Control; GS = bread including grape seed; PS = bread including pomegranate seed.

[3] Each sample was compared based on fermentation time, independent of other variables.

The results of the study showed that antioxidant activity and phenolic compound amount of the dough increased with fermentation and fermentation changed the phenolic profile. Studies carried out by different researchers revealed that fermentation increased antioxidant activity and phenolic compounds. These results agree with the results obtained in the present study. The increasing effect of fermentation on antioxidant activity and phenolic compounds can be explained in different ways:

- Glutathione is an antioxidant molecule that has a vital role in the protection of living cells against oxidation. A study showed that glutathione was an important molecule that protects the *S. cerevisiae* cells against oxidation (Duncan and Jamieson, 1996). Glutathione, which is present in yeast cells and has antioxidant properties that protects the cells against oxidation, is released during fermentation and thus can increase antioxidant activity (Wei *et al.,* 2003).
- The metabolites formed due to the metabolic activities of microorganisms during fermentation interact with the components in cereals. pH decreases and ethanol is produced during fermentation. The changing conditions during fermentation enable the activation of the already present enzymes and the enzymes activated with microbial metabolites improve the technological and nutritional quality of the foods (Poutanen *et al.,* 2009).
- Protein hydrolysates that are known to have antioxidant activity are formed as a result of the hydrolysis of proteins due to the extracellular proteinase enzymes released by microorganisms during fermentation. Antioxidant activity can increase because of the formation of free amino groups during fermentation as well as the release of metabolites (Virtanen *et al.,* 2007).
- Some studies have shown that new metabolites are formed during fermentation due to the starter cultures (Wang *et al.,* 2014). These metabolites modify bioactive compounds and result in the formation of new bioactive compounds. Furthermore, antioxidant activity increases because of the various bioactive compounds that are released due to the disruption of the cell walls of cereals, the changing of the composition of the foods by the enzymes such as amylase, xylanase, and proteinase that are present in foods and microorganisms, and the release of the conjugated bioactive compounds with the decrease in pH (Katina *et al.,* 2007).
- Fermentation can change the structure, composition, and polarity of the antioxidant compounds in fermented products. Therefore, the antioxidant properties of fermented and non-fermented products vary (Wang *et al.,* 2014).
- The alcohol formation due to fermentation can increase the antioxidant activity (Perez-Gregorio *et al.*, 2011). The ethanol formation during fermentation can facilitate the extraction of the phenolic compounds (Jayaram *et al.,* 2014);
- Antioxidant compounds of microbial origin can form in various fermented products. Antioxidant activity is increased by the intracellular antioxidants, peptides, and hydrogen-donating ability of microorganisms (Moktan *et al.,* 2008).

These results indicate that when the fermentation time increases, the antioxidant compounds in the dough are released and the bioavailability of the dough increases.

The effect of baking temperature on the total phenolic compounds, antioxidant activity and the phenolic profile of the breads

Table 3 shows the effects of baking temperatures on the TPC and TEAC values. The doughs obtained after kneading were compared with the breads baked at different temperatures. In the unbaked control group, the initial TPC value of 280 mg GAE /kg sample reached 538 mg GAE /kg sample after baking. In these breads, the initial TEAC value of 525 µmol Trolox reached 1,585 µmol Trolox. The TPC and TEAC values in the pomegranate seed and grape seed-containing breads also significantly increased, while the baking temperatures did not have a statistically significant effect on the TPC and TEAC values.

Figure 2 shows the effect of baking temperature on the DPPH radical scavenging effect of the breads. Baking temperature significantly increased the DPPH scavenging effect. The DPPH scavenging effect ranging from 8 to

Table 3. The effect of baking temperature on the TPC and TEAC contents of the breads.[1]

Sample[2]	Baking temperature[3]	TPC (mg GAE /kg)	TEAC (µmol Trolox/kg)
C	Unbaked	280±7.07^{b}	525±11.31^{b}
	1	536±14.84^{a}	1,598±5.65^{a}
	2	538±5.65^{a}	1,585±8.48^{a}
PS	Unbaked	402±12.72^{b}	1,059±12.02^{b}
	1	451±1.51^{a}	2,215±25.45^{a}
	2	445±2.12^{a}	2,195±9.19^{a}
GS	Unbaked	551±15.55^{b}	1,992±12.02^{b}
	1	771±8.48^{a}	5,062±18.38^{a}
	2	758±3.53^{a}	5,066±26.87^{a}

[1] The values are given as mean±SD (n=3). The samples with different letters in the same column are significantly different from each other. (P<0.05).

[2] C = Control; GS = bread including grape seed; PS = bread including pomegranate seed.

[3] Each sample was compared based on fermentation time, independent of other variables.

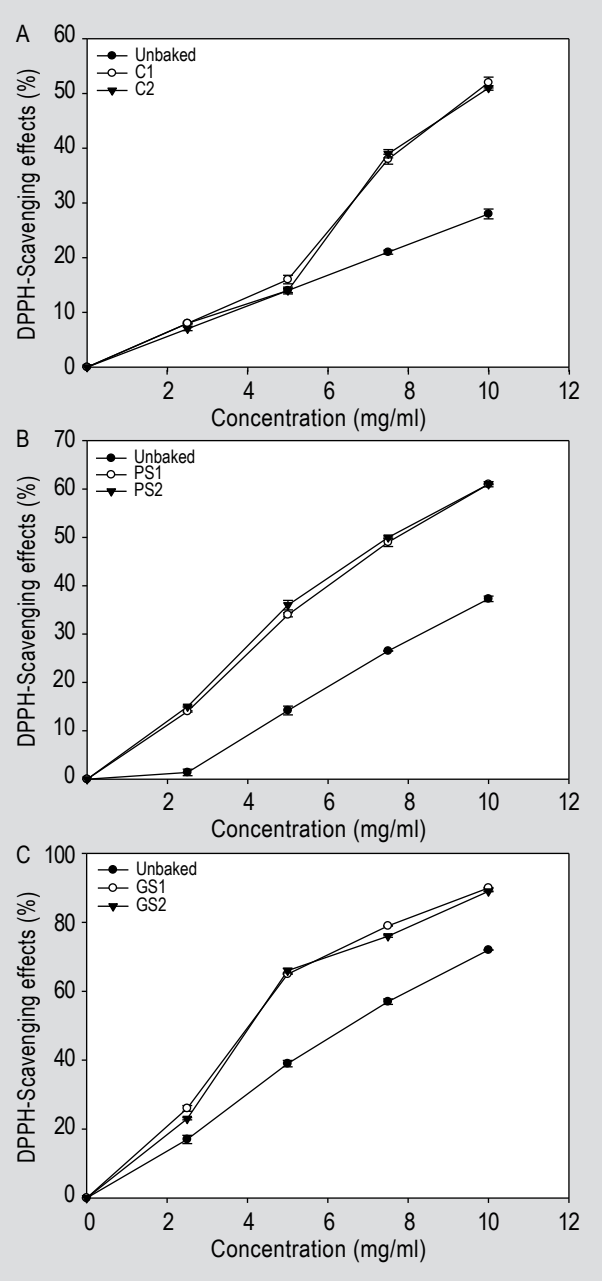

Figure 2. The 2,2-diphenylpcrylhydrazyl (DPPH)-scavenging effect of baking temperature (A) control; (B) pomegranate seed; and (C) grape seed.

25% of the unbaked doughs reached 8-51 and 7-52% in the baked breads, respectively. In the case of 10-mg/ml sample concentration, the initial scavenging effect of 28% reached 52% and showed an increase of about 2-fold. The DPPH scavenging effects of the pomegranate seed-containing breads ranged from 1 to 37%, 14 to 61%, and 15 to 61%, respectively. The antioxidant activity significantly increased with baking but baking temperature did not have a significant effect on the antioxidant activity.

The DPPH scavenging effects of the grape seed-containing breads significantly increased. After baking, the DPPH scavenging effect ranging from 17 to 72% reached 26-90 and 23-89%, respectively. Taking the highest sample concentration into account, the initial DPPH scavenging effect of 72% was determined to reach 90%.

Gahler *et al.* (2003) stated that the antioxidant activity of heat-treated tomato increased because of the release of phytochemicals such as lycopene from the food matrix. Tukmen *et al.* (2005) revealed that boiling, microwave cooking, and steaming caused significant increases in the antioxidant activities of broccoli, pepper, green beans, and spinach. Tsai *et al.* (2005) demonstrated that heating increased the ferric reducing ability of the mulberry extracts but the TEAC value did not change significantly. Gelinas and McKinnon (2006) determined that the TPC of the breads including whole wheat flour was higher than that of the flour used during the bread-making process (Dietrych-Szostak and Oleszek, 1999). It has been stated that Maillard reaction products have antioxidant activity. Michalska *et al.* (2008) determined that the intermediate products formed during the early stages of the Maillard reaction did not have an antioxidant activity and the products forming in the later stages of the Maillard reaction were free radical scavengers. The researchers reported that the antioxidant properties improved with the browning reaction.

Table 4 shows the effect of baking temperature on the phenolic profile of the breads. In the control breads, baking did not change the ferulic acid content; however, gallic acid was not determined in the breads, which is possibly attributable to its destruction due to baking. In the pomegranate seed-containing breads, the ferulic acid content did not change but the gallic acid content significantly increased with baking. The initial gallic acid content of the unbaked doughs was 1.68 mg/kg sample and with a 10-fold increase, it reached 17.23 mg/kg sample after baking. A statistically significant decrease occurred in the ferulic acid content of the grape seed-containing breads after baking. In these samples, gallic acid, catechin, and epicatechin contents showed a significant increase. Baking temperature did not cause a change in the ferulic acid, gallic acid, catechin, and epicatechin contents. In these samples, gallic acid content ranged from 5.36 to 59.30 mg/kg sample; catechin content ranged from 24. to 37.55 mg/kg sample; epicatechin content ranged from 25.95 sample to 30.03 mg/kg sample. The gallic acid content increased by about 11-fold with baking. The increase in gallic acid content may be associated with some gallate derivatives which, in natural components, convert to gallic acid as a result of heat and are thought to contribute to the increase in the gallic acid content. Heat causes gallate derivatives to disintegrate and convert to gallic acid (Velioğlu, 2006). Grape seed contains high amounts of polyphenol proanthocyanidins, which are

Table 4. The effect of baking temperature on the phenolic profile of the breads (mg/kg).[1]

Sample[2]	Baking temperature[3]	Ferulic acid	Gallic acid	Catechin	Epicatechin
	Unbaked	1.57±0.15^{a}	1.27±0.01		
C	1	1.26±0.28^{a}	0		
	2	1.12±0.03^{a}	0		
	Unbaked	1.61±0.09^{a}	1.68±0.03^{b}		
PS	2	1.03±0.05^{a}	17.33±0.28^{a}		
	2	0.99±0.03^{a}	17.23±0.42^{a}		
	Unbaked	1.6±0.00^{a}	5.36±0.02^{b}	24.22±0.00^{b}	25.95±0.02^{b}
GS	1	1.42±0.00^{b}	60.72±1.66^{a}	40.75±2.07^{a}	31.35±1.12^{a}
	2	1.47±0.02^{b}	59.30±0.74^{a}	37.55±0.63^{a}	30.03±0.05^{a}

[1] The values are given as mean±SD (n=3). The samples with different letters in the same column are significantly different from each other. (P<0.05).
[2] C = Control; GS = bread including grape seed; PS = bread including pomegranate seed.
[3] Each sample was compared based on fermentation time, independent of other variables.

the oligomers of flavan-3-ol units, especially catechin and epicatechin (Yılmaz and Toledo, 2004)

In a study conducted by Meral (2011), rhubarb plant was added to the composition of the bread and the phenolic profiles of the control breads and rhubarb-added breads were examined. According to their results, the gallic acid content of the bread significantly increased with the addition of rhubarb. Compared to the control group, the gallic acid content increased by 112-fold with the addition of rhubarb. The results of HPLC showed that gallic acid was released due to heat treatment.

In a study carried out by Zielinski *et al.* (2001), the effects of extrusion cooking on the amounts of vanillic, syringic, *p*-coumaric, ferulic, and caffeic acids either in the free or conjugated form in wheat, barley, rye, and oat were investigated. A significant change occurred in the phenolic composition of the cereals after heat treatment. The amounts of the free and conjugated forms of vanillic, syringic, and ferulic acid in wheat increased with heat treatment, while a decrease in the free vanillic and ferulic acid content of barley was determined. The free and conjugated vanillic acid in rye decreased with increasing temperature, while the free and conjugated ferulic acid amounts showed an increase. Although the heat treatment caused an increase in the free forms of the vanillic, syringic, and ferulic acid in oat, the amount of the conjugated forms of these phenolics decreased with increasing temperature. In the light of their findings, the researchers concluded that phenolic acids and the conjugated forms of the phenolic acids can take different forms under different physiological and technological conditions.

In a study carried out by Meral (2017), the TPC, TEAC, and DPPH scavenging capacities and the phenolic profiles of the rhubarb samples that were dried at different temperatures were investigated. Their results showed that the TPC and antioxidant activity of the rhubarb samples decreased with increasing temperature. The *p*-coumaric acid, ferulic acid, rutin, and quercetin contents of the samples decreased with increasing temperatures used in the heat treatment, while the gallic acid, *o*-coumaric acid, phloridzin, pyrocatechuic acid, and caffeic acid amount increased with increasing temperature. Since high temperatures cause the degradation of certain phenolic compounds, antioxidant activity in foods can decrease. However, the products of the Maillard reaction, especially melanoidins, can compensate for the decrease in activity stemming from the loss of the phenolic compounds and can even increase the activity (Delgado-Andrade and Morales, 2005; Ramirez-Jimenez *et al.*, 2000). Therefore, the Maillard reaction products play an important role in antioxidant activity. It is reported that some phenolics are released due to heat treatment, whereas some are destroyed. Furthermore, various compounds can enable the release of phenolic compounds as well as inhibiting them (Gelinas and McKinnon, 2006). In addition, the phenolic compounds with lower molecular weights in the functional groups of the phenolic components can be released after heat treatment (Meral, 2017).

4. Conclusions

In this study, the effects of fermentation time and baking temperature on the antioxidant activity and phenolic profile of the breads enriched with pomegranate and grape seeds were investigated. The analyses showed that TPC, TEAC, and radical scavenging effect increased with increasing fermentation time. With the increasing fermentation time, the ferulic acid content of the samples did not change but the gallic acid content of the samples significantly increased. With baking, TPC, TEAC, and DPPH radical

scavenging effect significantly increased, while, among the phenolic compounds, a 10-fold increase in gallic acid was determined. The study revealed that the baking process increased bioactive compounds. However, different baking conditions did not produce significantly different results. The study revealed that fermentation and baking had a role in the increase in bioactive compounds and antioxidant activity. Choosing a longer fermentation time during the bread-making process will probably increase antioxidant activity but it should be noted that one of the main goals in bread making is maintain organoleptic characteristics including the pore structure, volume, and texture. It should not be forgotten that prolonged fermentation will cause gas escape from dough and thus will negatively affect the volume and colour of the bread and therefore, the doughs should be fermented for as long as the formulation and process allow. This study revealed that fermentation and baking are processes that improve antioxidant properties.

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