



## The Effect of Different Grape Varieties and Adding Different Ratios of Mustard Seeds on the Phenolic Compounds, Antioxidant Capacity, and Bioaccessibility Values of Hardaliye under *In Vitro* Digestion

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### ABSTRACT

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Hardaliye, grape-based fermented beverage, rich in antioxidant phenolic compounds. Bioaccessibility and antioxidant capacity of bioactive compounds in hardaliye, produced using varying amounts of mustard seeds (1%, 1.5%, and 2%) with Merlot and Papazkarası grape varieties, were evaluated under *in vitro* gastrointestinal digestion conditions. After digestion, Merlot and Papazkarası samples with 2% addition of mustard seed showed significantly higher total phenolic compounds (TPC) (358.48±14.73 and 89.01±2.42 mg GAE/L, respectively) compared to other samples (P<0.05). 2% mustard seed added Merlot samples resulted in the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) and cupric reducing antioxidant capacity (CUPRAC) values (19.06±3.91 and 9.96±1.83 mmol TEAC/L, respectively) which differed significantly from other samples (P<0.05). The Merlot sample with 2% addition of mustard seed showed significantly higher TPC, total flavonoid compounds (TFC), DPPH, and CUPRAC bioaccessibility values compared to other Merlot samples (P<0.05). For Papazkarası samples with 2% mustard seed addition, significant differences were observed only in terms of TPC and TFC bioaccessibility values (15.87±2.30% and 15.27±1.29%, respectively) compared to samples with 1% and 1.5% mustard seed addition (P<0.05). The study demonstrated that the bioaccessibility of bioactive compounds in hardaliye can vary depending on the grape variety and to some extent, the use of mustard seed. This suggests that the food matrix and interaction with other food matrices in the environment can affect the stability and bioavailability of bioactive compounds during simulated digestion.

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### Introduction

Grape (*Vitis vinifera* L.) is one of the most consumed and economically important fruits worldwide either processed or unprocessed. Grapes are rich in phenolic compounds and flavonoids (anthocyanins and procyanidins), which are mainly found in red grapes and their products (Dani et al., 2007). Grapes are a naturally rich source of phenolic compounds, so they have been associated with important health benefits such as antioxidant, anti-carcinogenic, heart protective and anti-inflammatory properties (Toaldo et al., 2014). One of the most widely consumed products of grapes is grape juice. Hardaliye, produced using lactic acid fermentation, is a non-alcoholic grape juice. It is a traditional beverage that has been produced and consumed throughout history, especially in the Thrace region of Turkey. During its production, especially dark colored (red, black) grapes,

which form the characteristic of hardaliye, are preferred. In addition, mustard seeds added as 1-2% whole/ground in order to prevent alcoholic fermentation by preventing yeast activity during the production of hardaliye are effective in the formation of the unique taste and smell of hardaliye (Arici and Coskun, 2001).

In addition to mustard seeds, chemical additives (benzoates and sorbates) are used to limit alcohol formation during the fermentation of hardaliye (Askin and Atik, 2016; Coskun et al., 2012). Due to its production technique and potentially high grape polyphenol content, hardaliye is thought to provide antioxidant effects. However, the functionality of these ingredients with potential health benefits does not only depend on the initial amount present in the food, but the effects of the digestive process must also be considered. The assessment of

beneficial effects of phenolic compounds involves the analysis of bioaccessibility and bioavailability. Bioavailability encompasses the processes of release, absorption, metabolism, distribution, and elimination of compounds (Carbonell-Capella et al., 2014). To evaluate the bioaccessibility of bioactive compounds, various models can be employed, including *in vitro*, *in situ*, *ex vivo*, and *in vivo* models. Among these models, *in vitro* digestion procedures are advantageous because they are fast, cost-effective, safe, more reproducible, and do not experience the ethical restrictions imposed on *in vivo* models (Minekus et al., 2014). Throughout the process of digestion, phenolic compounds have the potential to undergo transformations, leading to the formation of different compounds with varied bioaccessibility and biological activity. Additionally, in some cases, these compounds may remain bound within the food matrix and not be released. This diminishes their positive impact on human health since bioactive compounds yield beneficial effects solely when they are prepared for absorption following the completion of the entire digestive process (Rein et al., 2013). Therefore, the post-digestion amount and bioavailability of phenolic compounds may differ according to their release from the food matrix, their stability under digestive conditions between biochemical and physicochemical factors (Sęczyk et al., 2021).

The aim of this study was to investigate the effect of the difference in grape variety preference and mustard seed addition on the bioaccessibility and antioxidant capacity of bioactive components in hardaliye, taking into account the changes caused by *in vitro* gastrointestinal digestion conditions. The Merlot and Papazkarası grape varieties were chosen for the study due to their suitability for making hardaliye, a grape-based beverage, in addition to their frequent use in grape juice and wine production.

## Material and Method

### Material

Grapes (Merlot and Papazkarası) used in making hardaliye were obtained from Tekirdağ Viticulture Research Institute of the Ministry of Agriculture and Forestry. The chemicals used in the analyzes are ethyl alcohol, methanol, Folin-Ciocalteu reagent, sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), sodium nitrite ( $\text{NaNO}_2$ ), aluminium chlorid ( $\text{AlCl}_3$ ), sodium hydroxide ( $\text{NaOH}$ ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), copper (II) chloride ( $\text{CuCl}_2$ ), neocuproine (Nc), ammonium acetate ( $\text{NH}_4\text{Ac}$ ), sodium chloride ( $\text{NaCl}$ ), hydrochloric acid ( $\text{HCl}$ ), Potassium Chloride ( $\text{KCl}$ ), sodium bicarbonate ( $\text{NaHCO}_3$ ), Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) were obtained from Sigma-Aldrich. Urea, bovine serum albumin, pepsin, pancreatin, mucin used in *in vitro* digestion were obtained from Merck, uric acid,  $\alpha$ -amylase, lipase were obtained from Sigma-Aldrich.

### Preparation of Hardaliye Samples

Hardaliye samples were made with Merlot and Papazkarası grapes provided from Tekirdağ Viticulture Research Institute of the Ministry of Agriculture and Forestry. The sample pattern consists of hardaliyes produced in two replicates using Papazkarası grape variety with 1%, 1.5% and 2% mustard seeds, and hardaliyes

produced in two replicates using Merlot grapes with 1, 1.5% and 2% mustard seeds. Production was carried out as stated by Arici and Coskun (2001). The finished samples were bottled and stored at  $+4^\circ\text{C}$ .

### Determination of Physicochemical Properties of Hardaliye Samples

#### pH, Acidity and Dry matter determination

The pH value of the samples was measured with the WTW-Inolab Weilheim (Germany) pH meter. Total acidity was determined according to the titration method reported by Cemeroglu (2007). In order to determine the total acidity values of the samples, 5 mL of each sample was taken into a 100 mL flask and the samples were made up to volume with distilled water. After shaking well, 10 mL sample was taken and titrated up to 8.1 with a pH meter, and the titratable acidity of each sample was determined. The amount of water-soluble dry matter ( $^\circ\text{Brix}$ ) of the samples was determined using a handheld refractometer (Anekella and Orsat, 2013).

#### Alcohol test

Gas chromatography mass spectroscopy technique was used for quantitative determination of ethyl alcohol. GCMS-QP2010 (Shimadzu, Milan, Italy) gas chromatography-mass spectroscopy device was used. Commercial libraries of the device (NIST27 and WILEY7) were used for component accuracy testing. CTC-Combi-PAL-autosampler was used in conjunction with GCMS-QP2010. Restec (Bellefonte, USA) Rtx-5MS silica capillary column (30 m  $\times$  0.25 mm (inner diameter)  $\times$  0.25  $\mu\text{m}$ ) was used to achieve chromatographic separation. The carrier gas in the system was Helium with a flow rate of 1 mL/min. Injector and detector temperatures are set to  $2500^\circ\text{C}$ . The temperature of the column program was set at  $4^\circ\text{C}$  for 10 minutes, the ionization voltage value was preferred as 70 eV in the range of 35-375 amu mass scanning. A pure (GC-grade) ethyl alcohol standard (Sigma-Aldrich) suitable for gas chromatography was used. Ethyl alcohol standards were prepared at concentrations of 100 ppm, 50 ppm, 25 ppm, 5 ppm and 1 ppm for the calibration curve. A five-level calibration curve was drawn. Pure water was used as the solvent. Calibration standards were injected into the device using 1.5 mL vials.

The calibration curve for ethyl alcohol was obtained by using the total ion chromatograms obtained. The calibration  $R^2$  coefficient was determined as 0.99. The calibration equation was calculated as  $Y=1758.858X+235234.7$ .

#### Determination of invert sugar and total sugar in hardaliye samples

Determination of invert sugar and total sugar in hardaliye samples was made according to the Lane-Eynon method stated by Cemeroglu (2007).

#### Extraction

The method reported by Mulero et al. (2010) was modified and applied. 5 mL of hardaliye or digestive sample was mixed with 15 mL of solvent (0.1 HCl + 79.9 mL of methanol + 20 mL of  $\text{H}_2\text{O}$ ) and incubated in a shaking water bath at  $25^\circ\text{C}$  in darkness for 15 hours. After the incubation period, the samples were kept in an ultrasonic water bath for 15 minutes and then centrifuged

at 1075 x g for 10 minutes. The clear parts obtained from the first centrifugation were subjected to a second centrifugation at 5645 x g for 20 minutes. The extracts obtained after the extraction process were stored separately in sample tubes at -20°C.

#### Determination of Total Phenolic Compounds

The total phenolic compounds (TPC) of the samples was measured using the Folin-Ciocalteu method (Singleton and Rossi, 1965). 0.5 mL of sample extract was mixed with 2.5 mL of Folin-Ciocalteu reagent that had been diluted with distilled water at a ratio of 1:10. The resulting mixture was allowed to incubate for a period of 5 minutes. Subsequently, the mixture was further supplemented with 2 mL of 7.5% (w/v) sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) solution and homogenized by means of a vortex mini-shaker. For the analysis, a solution consisting of 80% methanol was also prepared, along with the extract. After a 90-minute incubation period in the dark at room temperature, the mixture was analyzed at a wavelength of 760 nm using a UV-visible spectrophotometer (Shimadzu UV-1700 UV-Vis, Japan). The obtained absorbance value was compared against the blank sample and correlated with a previously constructed calibration curve. The results were ultimately presented in terms of milligrams per liter (mg L<sup>-1</sup>) as gallic acid equivalent (GAE).

#### Determination of Total Flavonoids

The total flavonoid compounds (TFC) of the samples was determined according to the method of Zhishen et al. (1999). 1 mL of the sample was mixed with 5 mL of distilled water, followed by the addition of 0.3 mL of 5% (w/v) NaNO<sub>2</sub>, and mixed again. After waiting for 5 minutes, 0.3 mL of 10% (w/v) AlCl<sub>3</sub> was added and mixed again. After waiting for an additional 6 minutes, 2 mL of 1 M NaOH solution and 1.4 mL of distilled water were added. The absorbance values of the samples were measured at 510 nm wavelength using a UV spectrophotometer (Shimadzu UV-1800, Japan). The obtained absorbance value was compared against the blank sample and correlated with a previously constructed calibration curve for catechin. The final results were expressed in units of mg L<sup>-1</sup> catechin equivalent (CE).

#### Total Antioxidant Capacity

The total antioxidant capacity was assessed by conducting DPPH and CUPRAC tests. Both tests used Trolox as a reference standard, and Trolox equivalent antioxidant capacity (mmol TEAC/L) was used to describe the results. The procedure for the DPPH (1,1-diphenyl-2-

picrylhydrazyl) assay was carried out following the method outlined by Kumaran and Karunakaran (2006). A volume of 100 µL of the extract/standard was added to 3.9 mL of a methanol solution containing 0.1 mM DPPH. The blank sample was created by mixing 100 µL of an 80% methanol solution with 3.9 mL of the DPPH solution. A UV-Visible spectrophotometer (Shimadzu UV-1700 UV-Vis, Japan) was used to measure the mixture's absorbance at 517 nm after it had been incubated at room temperature in the dark for 30 minutes. The absorbance was then compared to a blank. The CUPRAC (Cupric Reducing Antioxidant Capacity) analysis was carried out using the methodology established by Apak et al. (2004). Initially, 1 mL of 0.01 M copper (II) chloride (CuCl<sub>2</sub>), 1 mL of 0.0075 M neocuproine (Nc), and 1 mL of ammonium acetate (NH<sub>4</sub>Ac) buffer (pH 7.0) were combined. Then, 100 µL of the extract or Trolox standard was added to this mixture. To reach a final volume of 4.1 mL, 1 mL of distilled water was added to the mixture. To provide a control for analysis, a blank solution consisting of 80% methanol was prepared, just like the extract/Trolox standard. Afterwards, the mixture was incubated in the dark at 25 °C for 1 hour, following which the absorbance was measured at 450 nm using a UV-visible spectrophotometer (Shimadzu UV-1700 UV-Vis, Japan) against the blank sample.

#### In vitro Digestion Procedure

The procedure originally published by Lee et al. (2016) was modified slightly for *in vitro* simulated digestion. 5 mL of the sample was added to 50-mL Falcon tubes. *In vitro* digestion was performed by sequentially adding solutions simulating the conditions of the mouth, stomach, and small intestine. Enzymes, organic compounds, and inorganic compounds were used to prepare saliva solution, gastric juice, duodenal juice, and bile juice, as presented in Table 1.

The samples in Falcon tubes were incubated with 5 mL of saliva solution in a shaking water bath at 37°C for 5 minutes. The samples were then mixed with 10 mL of gastric juice and subjected to incubation for 30 minutes at 37°C in a shaking water bath. After the incubation with gastric juice, the samples were further treated by adding 10 mL of duodenal juice and 5 mL of bile juice. The resulting mixture was then subjected to incubation in a shaking water bath at 37°C for 2 hours. Once the digestion process was finished, the volume of the mixture was adjusted to 50 mL by adding deionized water. Subsequently, the samples were centrifuged at 1073 x g for 10 minutes, followed by filtration through a 0.45-µm cellulose acetate (CA) filter. The materials were filtered and then stored for subsequent analysis at -20°C in a freezer.

Table 1. The components and amounts of saliva, gastric, duodenal, and bile secretions utilized in a human *in vitro* digestion model.

Saliva	Gastric juice	Duodenal juice	Bile juice
1.7 mL NaCl (175,3 g/L)	16.5 mL HCl (37 g/L)	6.5 mL KCl (89.6 g/L)	68 mL NaHCO <sub>3</sub> (84.7 g/L)
8 mL urea (25 g/L)	18 mL CaCl <sub>2</sub> ·2H <sub>2</sub> O (22.2 g/L)	9 mL CaCl <sub>2</sub> ·2H <sub>2</sub> O (22.2 g/L)	10 mL CaCl <sub>2</sub> ·2H <sub>2</sub> O (22.2 g/L)
15 mg uric acid	1 g bovine serum albümin	1 g bovine serum albümin	1.8 g bovine serum albümin
280 mg α-amylase	2.5 g pepsin	9 g pancreatin	
25 mg mucin	3 g mucin	1.5 g lipase	
pH 6,8 ± 0,2	pH 1.50 ± 0.1	pH 8.0 ± 0.2	pH 7.0 ± 0.2

Calculation of bioaccessibility: The bioaccessibility index (BI) was employed to determine the percentage of compounds that were bioaccessible during gastrointestinal digestion. In accordance with the definition provided by Ortega et al. (2011), the abbreviation B represents the concentration of compounds in the intestinal phase, while C represents the initial concentration of the undigested sample.

$$\% \text{ BI} = (\text{B/C}) \times 100$$

### Statistical Analysis

All measurements and tests were carried out three times, and the average value was taken as the final result. The statistical analysis was performed using SPSS version 21 (SPSS, Chicago, IL, USA). A single component analysis of variance was used in the statistical study to assess the differences between the groups at a significance level of 5% (ANOVA;  $P < 0.05$ ). After conducting the analysis of variance, if deemed necessary, the means of different sources of variation were compared using a multiple comparison test (Tukey's test,  $P < 0.05$ ).

## Results and Discussion

### Physicochemical Analysis Results of Hardaliye Samples

The physicochemical analysis results of hardaliye samples are shown in Table 2. The pH values of hardaliye samples prepared with Merlot and Papazkarası grape varieties were found to be similar. The difference in the ratio of added mustard seeds did not create a statistically significant difference in both grape varieties. The pH values of the Hardaliye samples are consistent with the values reported in the literature (Coskun et al., 2018; Gündüz et al., 2019). Although the total acidity values were similar in Merlot and Papazkarası hardaliye samples, the difference in the addition of mustard seed indicated a statistically significant difference in the acidity values of the samples ( $P < 0.05$ ). The acidity values of the samples are in consistent with the literature values reported in previous studies (Askin and Atik 2016; Coskun et al., 2018). The dry matter contents were higher in Merlot hardaliye compared to Papazkarası hardaliye. The °Brix value was the highest in hardaliyes with 2% mustard seed addition for both grape varieties. Alcohol contents of Merlot and Papazkarası hardaliyes were similarly affected by the addition of mustard seeds. The highest alcohol value in hardaliyes was determined in the samples with 1.5% mustard seed (Merlot and Papazkarası respectively;  $0.53 \pm 0.08$  and  $0.50 \pm 0.07$ ) and differed statistically from the samples with 1% and 2% mustard seeds ( $P < 0.05$ ). The

highest total sugar value was found in Merlot hardaliye with 1% mustard seed and Papazkarası hardaliye with 1.5% mustard seed (respectively;  $19.75 \pm 0.77$  and  $16.06 \pm 1.19$ ). Similar to the total sugar results, the highest invert sugar content was determined in Merlot hardaliye with 1% mustard seed and Papazkarası hardaliye with 1.5% mustard seed (respectively;  $18.75 \pm 6.25$  and  $14.55 \pm 2.05$ ). Total sugar and invert sugar values of hardaliyes differed statistically significantly with the inclusion of mustard seeds ( $P < 0.05$ ). The acidity values of the samples were found to be similar to the literature values (Coskun and Arici, 2011; Coskun et al., 2018).

### Bioactive Compounds Before and After In Vitro Digestion

Total phenolic compounds (TPC) and total flavonoid compounds (TFC) contents in hardaliye extracts were determined before *in vitro* digestion and at each step of *in vitro* digestion application (mouth, stomach and intestinal digestive extracts). The results were shown in Table 3. Initially, TPC contents of Merlot hardaliye extracts were found to be significantly higher than Papazkarası hardaliye extracts. For Merlot hardaliyes the highest value was at 1.5% mustard seed added sample with  $1356.37 \pm 12.95$  mg GAE/L which is more than twice that of Papazkarası samples. While TPC values differed significantly depending on grape type ( $P < 0.05$ ), the change in mustard seed ratio was not cause a statistically significant difference in TPC values. TFC content before digestion changed significantly with the change of mustard seed ratio in Merlot hardaliyes ( $P < 0.05$ ). The highest TFC content in Merlot and Papazkarası samples was determined in samples with 1% mustard seed (respectively  $231.11 \pm 34.95$  and  $84.29 \pm 11.27$  mg CE/L). This value for Papazkarası did not differ statistically from the sample with 2% mustard seed added ( $P > 0.05$ ), while the sample with 1.5% mustard seed added statistically differed from the other Papazkarası samples ( $P < 0.05$ ). The TFC content in Hardaliye samples showed a significant difference depending on the grape type, similar to the pattern observed in TPC values ( $P < 0.05$ ).

Aksoy et al. (2022) determined the TPC of the hardaliye extracts of Cabernet Sauvignon, Merlot and Shiraz with 1.5% mustard seed were in the range of  $1639.74 \pm 82.85$  -  $2471.92 \pm 37.85$  mg GAE/L, and the TFC contents in the range of  $199.35 \pm 12.9$  -  $358.85 \pm 13.28$  mg CE/L. Coskun et al. (2018) determined TPC values between  $368.8 \pm 0.10$  and  $2727.5 \pm 1.50$  mg GAE/L in hardaliyes collected from different houses, and between  $961.8 \pm 0.40$  and  $2286.8 \pm 2.15$  mg GAE/L in hardaliyes produced in the laboratory.

Table 2. The physicochemical analysis results of hardaliye samples.

Analyses	Grape Variety and Addition of Mustard Seeds					
	Merlot			Papazkarası		
	% 1	% 1.5	% 2	% 1	% 1.5	% 2
pH	$3.49 \pm 0.08^a$	$3.54 \pm 0.02^a$	$3.53 \pm 0.08^a$	$3.56 \pm 0.04^a$	$3.64 \pm 0.07^a$	$3.62 \pm 0.07^a$
Acidity (%)	$1.14 \pm 0.01^b$	$1.26 \pm 0.02^{ab}$	$1.39 \pm 0.03^a$	$1.09 \pm 0.05^a$	$1.02 \pm 0.07^a$	$0.81 \pm 0.16^b$
Dry Matter Conten (°Briks)	$21.55 \pm 0.63^a$	$21.90 \pm 0.56^a$	$22.25 \pm 0.77^a$	$18.15 \pm 0.35^b$	$18.50 \pm 0.70^b$	$19.45 \pm 0.49^a$
Alcohol (%)	$0.36 \pm 0.01^b$	$0.53 \pm 0.08^a$	$0.33 \pm 0.03^b$	$0.37 \pm 0.04^b$	$0.50 \pm 0.07^a$	$0.32 \pm 0.01^b$
Total Sugar (g/100mL)	$19.75 \pm 0.77^a$	$15.25 \pm 0.77^b$	$14.7 \pm 1.55^c$	$9.43 \pm 1.26^c$	$16.06 \pm 1.19^a$	$12.45 \pm 0.49^b$
Invert Sugar (g/100mL)	$18.75 \pm 6.25^a$	$14.2 \pm 0.00^b$	$9.45 \pm 1.64^c$	$7.37 \pm 0.43^c$	$14.55 \pm 2.05^a$	$11.29 \pm 1.86^b$

\*The difference between the means indicated with different letters for the same grape variety in the same row was found to be statistically significant ( $P < 0.05$ ).

Table 3. The content of bioactive compounds and values of antioxidant capacity of hardaliye samples before and after *in vitro* digestion.

	Grape Variety and Addition of Mustard Seeds					
	Merlot		Papazkarası			
	% 1	% 1.5	% 2	% 1	% 1.5	% 2
Analyses: TPC (mg GAE/L)						
Initial	1354.29±74.07 <sup>Aa</sup>	1356.37±128.95 <sup>Aa</sup>	1322.56±233.54 <sup>Aa</sup>	549.97±181.16 <sup>Ba</sup>	587.84±233.00 <sup>Ba</sup>	560.88±94.30 <sup>Ba</sup>
Mouth	615.79±16.42 <sup>Ab</sup>	633.01±53.26 <sup>Aab</sup>	656.65±48.54 <sup>Aa</sup>	210.85±25.96 <sup>Bb</sup>	246.65±38.75 <sup>Ba</sup>	257.05±46.75 <sup>Ba</sup>
Stomach	470.22±32.42 <sup>Ab</sup>	488.75±45.24 <sup>Ab</sup>	521.57±35.13 <sup>Aa</sup>	142.05±18.45 <sup>Bb</sup>	167.57±28.21 <sup>Ba</sup>	177.75±24.26 <sup>Ba</sup>
Intestine	301.08±17.35 <sup>Ab</sup>	318.47±25.56 <sup>Ab</sup>	358.48±14.73 <sup>Aa</sup>	69.84±4.52 <sup>Bc</sup>	82.51±2.48 <sup>Bb</sup>	89.01±2.42 <sup>Ba</sup>
Analyses: TFC (mg CE/L)						
Initial	231.11±34.95 <sup>Aa</sup>	181.22±60.78 <sup>Ab</sup>	184.57±8.31 <sup>Ab</sup>	84.29±11.27 <sup>Ba</sup>	80.95±24.28 <sup>Bb</sup>	83.99±19.92 <sup>Ba</sup>
Mouth	117.49±35.43 <sup>Aa</sup>	94.74±26.46 <sup>Aa</sup>	98.30±13.54 <sup>Ab</sup>	37.83±24.83 <sup>Bab</sup>	33.94±8.33 <sup>Bb</sup>	40.22±3.93 <sup>Ba</sup>
Stomach	85.97±13.64 <sup>Aa</sup>	73.30±15.88 <sup>Ab</sup>	74.87±24.98 <sup>Ab</sup>	25.74±6.89 <sup>Bb</sup>	23.42±3.46 <sup>Bc</sup>	28.22±2.79 <sup>Ba</sup>
Intestine	44.58±1.37 <sup>Aa</sup>	39.71±7.69 <sup>Aa</sup>	44.07±4.94 <sup>Aa</sup>	11.32±0.86 <sup>Bb</sup>	10.52±1.06 <sup>Bc</sup>	12.82±0.84 <sup>Ba</sup>
Analyses: DPPH (mmol TEAC/L)						
Initial	13.06±1.99 <sup>Aa</sup>	13.53±1.24 <sup>Aa</sup>	13.16±1.07 <sup>Aa</sup>	6.97±0.55 <sup>Ba</sup>	7.04±0.24 <sup>Ba</sup>	6.87±0.75 <sup>Ba</sup>
Mouth	12.32±1.48 <sup>Aa</sup>	13.08±0.64 <sup>Aa</sup>	13.07±2.10 <sup>Aa</sup>	6.01±1.82 <sup>Ba</sup>	5.71±0.94 <sup>Ba</sup>	5.86±1.71 <sup>Ba</sup>
Stomach	12.61±1.01 <sup>Aa</sup>	13.59±2.04 <sup>Aa</sup>	13.43±1.15 <sup>Aa</sup>	5.86±0.73 <sup>Ba</sup>	5.50±1.18 <sup>Ba</sup>	5.73±1.83 <sup>Ba</sup>
Intestine	16.89±0.79 <sup>Ab</sup>	17.70±2.76 <sup>Ab</sup>	19.06±3.91 <sup>Aa</sup>	7.99±0.34 <sup>Ba</sup>	8.04±1.86 <sup>Ba</sup>	8.15±0.43 <sup>Ba</sup>
Analyses: CUPRAC (mmol TEAC/L)						
Initial	15.15±0.68 <sup>Aa</sup>	14.64±1.93 <sup>Aa</sup>	14.38±0.58 <sup>Aa</sup>	6.11±0.41 <sup>Ba</sup>	6.18±0.57 <sup>Ba</sup>	6.34±0.64 <sup>Ba</sup>
Mouth	6.34±0.48 <sup>Aa</sup>	6.43±0.76 <sup>Aa</sup>	7.24±0.91 <sup>Aa</sup>	2.35±0.16 <sup>Ba</sup>	2.12±0.83 <sup>Ba</sup>	2.43±0.72 <sup>Ba</sup>
Stomach	5.91±1.04 <sup>Aa</sup>	6.07±0.47 <sup>Aa</sup>	6.84±1.71 <sup>Aa</sup>	2.12±0.82 <sup>Ba</sup>	1.95±0.46 <sup>Ba</sup>	2.21±0.16 <sup>Ba</sup>
Intestine	8.85±0.36 <sup>Ab</sup>	8.37±1.90 <sup>Ab</sup>	9.96±1.83 <sup>Aa</sup>	2.99±0.09 <sup>Ba</sup>	2.83±0.18 <sup>Ba</sup>	3.19±0.23 <sup>Ba</sup>

\*Samples prepared with the same grape variety and varying amounts of mustard seed addition on the same line showed statistically significant differences between the means denoted by different lowercase letters (P<0.05); \*\*Samples prepared with the same amount of mustard seed addition and different grape varieties on the same line showed statistically significant differences between the means denoted by different capital letters (P<0.05).

Milinčić et al. (2021) determined the TFC values of grape pomace extracts obtained from Prokupac red grapes as ranging from 5.6±0.16 to 14.5±0.24 mg CE/g dw. Meanwhile, Moreno-Montoro et al. (2015) found that the TFC values in the juices of white and red grapes were on average 62.7±12.4 and 98.0±9.8 mg CE/L, respectively. Additionally, they found mean TFC values of 28.7±7.2 and 228±11.8 mg CE/L in white and red wines, respectively.

TPC and TFC values; may depend on the characteristics of the grape such as the type, size, skin thickness, seed amount and fruit juice rate, the climatic conditions of the region where it is grown, the soil structure and factors such as the fermentation process. Therefore, the differences in TPC and TFC values of Merlot and Papazkarası hardaliye are mainly due to grape type. Although mustard seeds stand out with their antimicrobial properties, they also contain phenolic compounds, which are plant secondary metabolites (Diosady et al., 2007).

However, small increases in mustard seed addition were not effective in increasing the TPC and TFC values of hardaliyes. The TPC values of all samples were significantly reduced due to the effect of *in vitro* digestion. Hardaliyes produced with different grape varieties were affected by the *in vitro* digestion stages at different rates, and the results showed statistical differences (p<0.05). The greatest decrease in TPC values after oral digestion was seen in samples with 1% mustard seed: 54.53% in Merlot hardaliye and 61.66% in Papazkarası hardaliye. The TPC of the hardaliye samples was less affected by the stomach phase than the mouth phase. The smallest decrease in TPC values during the transition from the mouth phase to the stomach phase was observed in samples containing 2% mustard seed, with a decrease of 20.57% and 30.85% in the Merlot and Papazkarası samples, respectively. Intestinal

digestion results were also similar to results determined at other stages of digestion. The TPC values of Merlot hardaliyes exhibited a lower level of decrease in the intestinal stage, as well as in other digestion stages, compared to Papazkarası hardaliyes and showed a statistically significant difference (P<0.05). In both Merlot and Papazkarası hardaliyes, the highest values after intestinal digestion were found in the samples with 2% mustard seed (respectively; 358.48±17.35 and 89.07±4.52 mg GAE/L), and the lowest values were found in the samples with 1% mustard seed (respectively; 301.08±14.73 and 69.84±2.42 mg GAE/L).

Costa et al. (2019) determined the TPC content in Shiraz grape pomace extract as 239.01 ± 4.44 mg GAE/g before digestion and 13.59 ± 0.23 mg GAE/g after digestion. Aksoy et al. (2022) determined that the TPC values of hardaliyes produced with 1.5% mustard seed Shiraz, Merlot and Cabernet Sauvignon grapes decreased at each stage of *in vitro* digestion and at the end of digestion this value respectively was 751.02±9.92, 808.14±33.19 and 760.79±3.15 mg GAE/L.

The conditions of *in vitro* digestion affected the TFC values of Merlot and Papazkarası hardaliyes at different rates, and the results differed statistically between grape varieties (P<0.05). After the oral phase, the highest reductions in TFC were observed in Papazkarası hardaliyes, while the lowest TFC value was 33.94±8.33 mg CE/L in the Papazkarası sample with 1.5% mustard seeds. Gastric digestion was the stage where the smallest reductions in TFC occurred compared to the oral and intestinal phases. The greatest reductions in the stomach digestion were also found in samples with 1% mustard seeds. The lowest value was determined in Papazkarası hardaliye with 1.5% mustard seed as in oral digestion. The

lowering effect of intestinal digestion was greater than the effect of gastric digestion and was similar to the effect of oral digestion. TFC values of hardaliye made with varying grape varieties after intestinal digestion were statistically significantly different ( $P < 0.05$ ). The highest post-digestion value was in Merlot hardaliye with 1% mustard seeds with  $44.58 \pm 1.37$  mg CE/L, consistent with initial. For Papazkarası, the highest value was found with  $12.82 \pm 0.84$  mg CE/L in Papazkarası sample with 2% mustard seeds.

Corona-Leo et al. (2021) investigated the effect of *in vitro* digestion on the phenolic contents of different apple varieties and reported that the amount of total flavonoids was found to be lower after digestion. Xie et al. (2020) determined the total flavonoid content of passion fruit juices after *in vitro* digestion in the range of 49.57-190.98 mg CE/L.

Decreases in the amount of phenolic compounds during digestion may be due to interactions with the pH of the digestive environment, digestive enzymes and other components originating from the food matrix, deterioration in the chemical structure or solubility changes. Bermudez-Soto et al. (2007) stated that some phenolic compounds are very sensitive to intestinal conditions. The instability of flavonoids under alkaline conditions in the gut may be associated with their reduction (Ketnawa et al., 2021) also, as in hardaliye, phenolics in liquid food matrix are more prone to degradation and more unstable (Podsędek et al., 2014). Merlot and Papazkarası grape varieties may have differences in terms of phenolic compound profile and the rate of hydrolysis in digestion also depends on the phenolic profile. Therefore, depending on the grape type, the rate of being affected by the digestion stages may vary. The reductions in TP and TF contents of Merlot hardaliyes due to the effect of digestion stages were smaller with the increase of mustard seed addition. In Papazkarası hardaliyes, this situation was not as evident as in Merlot hardaliyes. This may be due to the changes in the phenolic compound profile depending on the differentiation of grape varieties. On the other hand, lipids can capture the polyphenols with which they interact during the transit through the gastrointestinal tract and protect these polyphenols from interaction with other molecules (Pinarli et al., 2020). The increase in the amount of mustard seed, which is a kind of oilseed, may have relatively reduced the decrease in the polyphenol content through the lipids it contains, albeit in small amounts.

#### **Antioxidant Capacity Before and After In Vitro Digestion**

Antioxidant capacities of hardaliye samples were determined before and after *in vitro* digestion and the results were shown in Table 3. The initial DPPH and CUPRAC antioxidant capacity values of Merlot hardaliye extracts were found to be approximately twice as high as those of Papazkarası hardaliye extracts, and the results showed statistically significant differences depending on the grape diversity ( $P < 0.05$ ). The lowest antioxidant capacity value was determined to be  $6.87 \pm 0.75$  mmol TEAC/L in Papazkarası hardaliye extract with 2% mustard seed, while the highest antioxidant capacity value was  $13.53 \pm 1.24$  mmol TEAC/L in Merlot hardaliye extract with 1.5% mustard seed, according to the DPPH method. According to the CUPRAC method, the lowest value was determined with  $6.11 \pm 0.41$  mmol TEAC/L in Papazkarası

with 1% mustard seed and the highest values were determined to be  $15.15 \pm 0.68$  mmol TEAC/L in the Merlot sample with 1% mustard seed.

Aksoy et al. (2022) reported that antioxidant capacity values determined by DPPH and CUPRAC methods in hardaliye extracts produced from Shiraz, Merlot and Cabernet Sauvignon grapes with 1.5% mustard seed differ significantly depending on grape variety. Panceri et al. (2015) determined the antioxidant capacity of Merlot wine as  $2.67 \pm 0.08$  mmol TEAC/L by DPPH test. Askin et al. (2016) determined the antioxidant activities of hardaliye samples as  $8.53 \pm 0.05$  mM TE/mL with the ABTS<sup>•+</sup> test, and  $7.45 \pm 0.08$  mM TE/mL after 60 days of storage at 4°C.

Differences in antioxidant capacity values of hardaliye samples may be partly due to different assay methodologies used as well as due to the genetic variations within grape varieties (Romero-Díez et al., 2018). Different antioxidant capacity methods measure a different aspect of antioxidant activity so that values can change regardless of the content of phenolic compounds (Frankel and Meyer, 2000).

In the post-digestion samples, there was a similar situation to the pre-digestion in terms of grape type. The results showed that the antioxidant capacity values of post-digestion extracts from Merlot hardaliye were significantly higher than those of Papazkarası samples in both methods. Moreover, the antioxidant capacity values differed significantly between the two grape varieties ( $p < 0.05$ ). After digestion, all samples showed an increase in their DPPH antioxidant capacity values. Small decreases observed in the DPPH values of the samples after oral digestion, and the values of the gastric digest samples were found to be quite close to the values of the oral stage. The transition from the oral phase to the stomach phase resulted in small increases in the antioxidant capacity values of Merlot hardaliyes, while small decreases were observed in the values of Papazkarası hardaliyes. After intestinal digestion, all hardaliyes exhibited the highest DPPH antioxidant capacity values. The highest increase was determined with a value of 46.09% in Papazkarası hardaliye with 1.5% mustard seed, however, the highest overall value of  $19.06 \pm 3.91$  mmol TEAC/L was obtained for Merlot hardaliye with 2% mustard seed addition. At the end of digestion, Papazkarası hardaliyes did not differ statistically significantly with the addition of mustard seeds ( $P > 0.05$ ), while the sample Merlot hardaliye with 2% mustard seed differed statistically from other Merlot samples ( $P < 0.05$ ).

In contrast to the DPPH values, a decrease in CUPRAC antioxidant capacity values was observed after digestion. There was no statistically significant change in the CUPRAC values after oral and gastric digestion with the change of mustard seed addition in hardaliyes made with both grape. The highest values in the mouth and stomach digestion stages belonged to Merlot hardaliye with 2% mustard seed ( $7.24 \pm 0.91$  and  $6.84 \pm 1.71$  mmol TEAC/L, respectively). Although CUPRAC values remained below the initial values after intestinal digestion, these values increased in hardaliye made with both grape varieties, unlike the other digestion stages. Although the highest increase was observed in the Merlot sample with 1% mustard seed, the highest overall value of  $9.96 \pm 1.83$  mmol TEAC/L was found in the Merlot sample with 2% mustard seed ( $P < 0.05$ ).

Table 4. The content of bioactive compounds and values of antioxidant capacity of hardaliye samples before and after *in vitro* digestion.

%Bioaccessibility	Grape Variety and Addition of Mustard Seeds					
	Merlot			Papazkarası		
	% 1	% 1.5	% 2	% 1	% 1.5	% 2
TPC	22.23±2.04 <sup>Ab</sup>	23.47±1.35 <sup>Ab</sup>	27.10±2.43 <sup>Aa</sup>	12.70±1.86 <sup>Bb</sup>	14.03±2.00 <sup>Bab</sup>	15.87±2.30 <sup>Ba</sup>
TFC	19.29±1.05 <sup>Ac</sup>	21.91±2.89 <sup>Ab</sup>	23.87±2.17 <sup>Aa</sup>	13.43±1.79 <sup>Bb</sup>	13.00±2.48 <sup>Bb</sup>	15.27±1.29 <sup>Ba</sup>
DPPH	129.36±21.90 <sup>Ab</sup>	130.88±30.04 <sup>Ab</sup>	144.84±24.07 <sup>Aa</sup>	114.75±10.57 <sup>Ba</sup>	114.34±29.14 <sup>Ba</sup>	118.75±17.54 <sup>Ba</sup>
CUPRAC	58.42±11.82 <sup>Ab</sup>	57.21±10.37 <sup>Ab</sup>	69.32±10.80 <sup>Aa</sup>	49.04±09.14 <sup>Ba</sup>	45.83±08.76 <sup>Ba</sup>	50.36±09.44 <sup>Ba</sup>

\*Samples prepared with the same grape variety and varying amounts of mustard seed addition on the same line showed statistically significant differences between the means denoted by different lowercase letters (P<0.05); \*\*Samples prepared with the same amount of mustard seed addition and different grape varieties on the same line showed statistically significant differences between the means denoted by different capital letters (P<0.05).

Aksoy et al. (2022) reported that the amounts of hardaliye produced with Cabernet Sauvignon, Merlot and Shiraz grapes with 1.5% mustard seed increased after gastric and intestinal digestion and were significantly higher than the initial value after digestion. They reported that the CUPRAC value was lower than the initial, although it increased with the transition from the gastric phase to the intestinal phase. Mihaylova et al. (2021) noticed that the antioxidant capacity of commercial fruit juice containing 10% black grape and 12% aloe vera, as determined by CUPRAC and DPPH tests, decreased after simulated digestion. Zoubiri et al. (2019) determined the CUPRAC value in red fresh grapes as 3359.4±101.8 mg TAC/100 g DW before digestion and 433.7±35.1 mg TAC/100 g DW after digestion.

While TPC decreased after digestion, antioxidant capacity increased especially after intestinal digestion. During the digestion of foodstuffs, especially with alkaline pH in the intestinal phase, the release of polyphenols embedded in the matrix or bound to components such as dietary fiber and carbohydrates occurs. This may cause an increase in antioxidant activity after digestion. The increase in antioxidant activity may be due to other components in the digestive samples such as proteins, carbohydrates and vitamins, as well as due to the molecular changes of phenolic substances, and additionally due to the hydrolyzing activity of digestive enzymes (Liu et al., 2020, Su et al., 2019).

#### Bioaccessibility Values

The % bioaccessibility values of the samples after *in vitro* digestion were calculated and the results were shown in Table 4. When evaluated in terms of grape type, it was determined that Merlot hardaliyes showed higher bioaccessibility compared to Papazkarası hardaliyes and the results differed statistically (p<0.05). TPC and TFC % bioaccessibility were found to be highest in both Merlot and Papazkarası hardaliye samples with 2% mustard seed; 27.10±2.43% and 15.87±2.30% for TPC, and 23.87±2.17% and 15.27±1.29% for TFC, respectively. In all Hardaliye samples, DPPH bioaccessibility was higher than CUPRAC bioaccessibility. For DPPH, 2% mustard seed added samples differed significantly from other samples (P<0.05); it was found 144.84±24.07% and 118.75±17.54% for Merlot and Papazkarası, respectively. While Papazkarası samples did not differ significantly for CUPRAC values, hardaliye with 2% mustard seed differed significantly in Merlot samples (P<0.05). Similar to other bioaccessibility results, the highest CUPRAC values belong to samples with 2% mustard seed addition:

69.32±10.80% and 50.36±09.44% for Merlot and Papazkarası, respectively.

Aksoy et al. (2022) determined the TP and TF % bioaccessibility of 1.5% mustard seed added hardaliyes the highest in Shiraz (45.87±2.92% and 39.51±3.98%) and the lowest in Cabernet Sauvignon (32.41±1.09 and 27.3±0.61%) hardaliye. They also reported that DPPH antioxidant capacity bioaccessibility was higher than CUPRAC antioxidant capacity bioaccessibility. Corona-Leo et al. (2021) determined the TF bioaccessibility of Starking and Granny Smith apple cultivars as 50.08% and 43.08%, respectively, determined the bioaccessibility of antioxidant capacity as 28.45% and 24.05%, respectively, with the DPPH test, and 22.44% and 21.98%, respectively, with the ABTS test. Zoubiri et al. (2019) reported the antioxidant capacity % recovery values of red and white grapes as 15±1.6% and 15.5±1.9%, respectively, by the DPPH method, and as 12.9±1.4% and 13.7±0.6%, respectively, by the CUPRAC method.

Phenolic compounds, typically considered as secondary metabolites, are commonly either bonded with certain macromolecules like proteins and carbohydrates, forming copolymers, or obstructed by these macromolecules. During digestion, the impacts of acid, alkali, as well as certain digestive enzymes can lead to the breakdown of the plant cell wall and the hydrolysis of bonds (Liu et al., 2020). As with the stability of food polyphenols, their bioaccessibility is highly dependent on their interaction with the matrix (Da Silva Haas et al., 2018). This explains the significant variation of bioaccessibility depending on the grape variety from which the hardaliye is produced, and the variation depending on the addition of mustard seed in hardaliyes produced with the same grape variety for some parameters. Thus, the results indicate that the bioaccessibility of bioactive compounds is highly influenced by the food matrix and its interaction with other added food matrices, which is consistent with the findings of previous studies.

#### Conclusions

TPC, TFC, DPPH and CUPRAC values of hardaliye samples before and after digestion varied significantly depending on the grape variety (p<0.05). Merlot hardaliye stands out from Papazkarası hardaliye in terms of bioactive components and antioxidant capacity. Bioactive components of Merlot hardaliye were more stable under *in vitro* digestion conditions compared to Papazkarası hardaliye. Likewise, bioactive compounds and antioxidant capacity of Merlot hardaliyes exhibited higher

bioaccessibility. Thus, its bioactive compounds may be more bioavailable and have the potential to exert beneficial health effects however further analysis and studies are needed to confirm this. On the other hand, the effect of change in mustard seed addition rate was similar in Merlot and Papazkarası hardaliye. Overall, the bioactive components in the samples with 2% mustard seed addition were relatively more stable under *in vitro* digestion conditions and exhibited higher bioaccessibility. The results confirmed that the content of bioactive ingredient, the effect of the *in vitro* gastrointestinal simulated environment on these bioactive components, and the bioaccessibility are strongly dependent on the food matrix and the its interaction with other added food matrices. In order to gain a better understanding of the interaction between bioactive components and macro and micro elements in the food matrix, further *in vivo* studies should be conducted to obtain more definitive results. In addition, using more detailed research techniques (for example, High-Performance Liquid Chromatography), the phenolic compounds of the research samples and which components are stable under *in vitro* conditions should be defined.

### Competing Interests

The author declare that have no competing interests.

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