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RESEARCH ARTICLE

Effects of Aging Time on the Antioxidant Activity of Pomelo Wine

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Abstract Variations in amino acid and volatile component contents, and in total flavonoid, polyphenol contents and their vitro antioxidant activities at different aging times of pomelo wine were investigated; scavenging rates against DPPH, ABTS, and ferric reducing antioxidant powers were also explored. Most amino acids reached maximum values in the third aging year; and volatile components of pomelo wine also indicated good wine quality in the third year. Total flavonoid and polyphenol contents in pomelo wine were increased and antioxidant activities enhanced during the first 3 years. Samples aged for 3 years had the highest total flavonoid and total polyphenol contents, and the strongest antioxidant activities (38.23±2.16% for DPPH· scavenging effect, 91.84±4.14% for ABTS· scavenging effect, and 0.90±0.07 mmol/L for FRAP value). These parameters gradually decreased after the 3 year period. A significant (p<0.05) correlation between antioxidant activity, and total flavonoid and polyphenol contents was identified.

Keywords: aging time, pomelo wine, antioxidant activity

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Introduction

Pomelo (*Citrus maxima* or *Citrus grandis*) is a citrus fruit of the Rutaceae family. Citrus fruits are known for high vitamin C contents that reduce the risk of many diseases originating from oxidative stress when consumed in the diet. Moreover, citrus fruits contain abundant phenolic compounds that are beneficial to human health due to antioxidant activities of scavenging free radicals (1). Pomelo is eutrophic, juicy, and delicious and can be consumed fresh or in processed forms. Pomelo can contribute to a healthy human diet due to important functions for prevention of cancer and chronic diseases (2,3), and the fruit reportedly has antimicrobial activities (4). Consumption of processed fruit juice instead of citrus fruit has recently increased in developed countries (5). China is the leading world producer of pomelo and production is continually growing. However, with production growth, market saturation has become a problem for further development of the pomelo industry. Therefore, more diverse uses of pomelo and advanced processing techniques should be developed for generation of higher value-added products. Brewing of pomelo-based wine will contribute to advancement of pomelo use in the food industry.

Pomelo wine is an alcoholic beverage produced via fermentation of fresh pomelo. Newly brewed pomelo wine is turbid and dull, has a spicy and bitter taste and is, therefore, not favorable for drinking. After storage and appropriate processing, the liquor can be clear, bright in color, and aromatic. Pomelo wine has a unique taste and a high nutritional value, containing 17 amino acids, vitamins and minerals, including VC, VB_1, Fe, and Zn. Variations in amino acid and volatile component contents are described in this study. Flavonoids and phenolic compounds in cherries (6), blueberries (7), jujube (8), bayberry (9), apple (10), and lychee (11,12) have antioxidant activities. Therefore, total flavonoid, total polyphenol contents, and the *in vitro*

antioxidant activity of pomelo wine at different aging times were determined in this study. In addition, relationships among these factors were also determined to provide a scientific basis for development and comprehensive processing of pomelo wine.

Materials and Methods

Materials Pomelo wines used in this study were brewed from 2008 to 2012 at the College of Food Science, Southwest University, Chongqing, China. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,4,6-tripyridyl-S-triazine (TPTZ) were supplied by Sigma Chemical Company. Rutin and gallic acid were obtained from the National Institute for Food and Drug Control. Folin-Ciocalteu reagent was purchased from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China. H-type amino acid mixed with a standard was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other reagents used were of analytical grade and were used as received. Deionized, ultrapure water was used throughout.

Amino acid analysis An automated amino acid analyzer (L-8900; Hitachi High-Technologies Corp., Tokyo, Japan) was used (13). The analytical column was an HPLC Packed column (Hitachi High-Technologies Corp.) (Ion-exchange resin, 4.6 mm i.d., 60 mm length, 3 μm particle size) with sulfone (SO^3) groups as the active exchange site. A visible analytical detector (Hitachi High-Technologies Corp.) was used.

For sample preparation, 20 mL of pomelo wine was centrifuged (LG10-2.4A; Beijing Jingli Centrifuge Co., Ltd., Beijing, China) at 4,400×*g* for 10 min at 25°C. The supernatant was filtered using a 0.22 μm microporous membrane to obtain a test solution. Then, 20 μL of test solution and an amino acid-mixed standard solution were injected into the amino acid analyzer for measurement.

Gradient elution mode was used for chromatograms. The temperature of the separation column was 57°C and the reaction column was 135°C. The flow rates for the buffer were 0.35 and 0.35 mL/min for the ninhydrin solution. For Channel 1, the detection wavelength was 570 nm and for Channel 2 the detection wavelength was 440 nm. The injection volume was 20 μL.

Volatile compound analysis Volatile compounds in pomelo wine was analyzed by solid phase microextraction-combine with gas chromatograpy/mass spectrum (SPME-GC-MS) according to previous report with some modification (14). Briefly, 2 g of NaCl was added to 8 mL of wine sample in a solid phase microextraction bottle. An octanol solution was used as an internal standard (0.1 mL of octanol mixed with alcohol for a total volume of 100 mL). A total of 1.0 μL of this mixture in wine samples was used as an internal standard. An SPME probe was placed in the solid phase microextraction bottle at 20 mm above the wine sample surface. The bottle was heated at 40°C for 10 min. Wine samples were subjected to extraction for 40 min at 40°C. Then the volatile compounds was performed on a Shidmadzu GC-2010 gas chromatograpy system equipped with a mass spectrometry detector (Shidmadzu Co., Ltd., Kyoto, Japan) and a DB-5 MS capillary column (30 m ×0.25 mm ID with a 0.25 μm film thickness).

GC-MS parameters were set as follows: The probe inserted into the GC-MS injection port was removed for 5 min of desorption to start the GC-MS analysis. The injector temperature for chromatograms was 250°C and the column was heated at 40°C for 2 min, then increased by 5°C/min to 130°C, increased by 4°C/min to 220°C, and maintained at 220°C for 10 min, then increased by 20°C/min to 280°C, and maintained at 280°C for 2 min. The helium carrier gas flow rate was 1.0 mL/min. An MS electron ionization mode at an ionization energy of 70 eV was used with a mass range of 40 to 450 amu/s. The GC-MS interface temperature was 280°C with a split ratio of 5:1. Extraction and injection of each sample were conducted thrice. Compounds were identified based on comparison with commercial reference compounds provided by Sigma-Aldrich using previously reported RI values and comparison of wine sample mass spectra with reference spectra obtained from the NIST 08.

Determination of total flavonoid contents The method used for determination of total flavonoid contents (TFC) in pomelo wine was described previously (15) and used with modification. Briefly, 0.2 mL of a wine sample was added to 300 μL of a sodium nitrite solution (5%), then mixed with 300 μL of an aluminium nitrate solution (5%), followed by incubation (water bath; Shanghai Jing Hong Laboratory Instrument Co., Ltd., Shanghai, China) for 6 min at room temperature. Subsequently, the wine sample solution was mixed with 2 mL of a sodium hydroxide solution (4%) and 6.4 mL of ethanol (30%). The absorbance of the reaction mixture was measured using a spectrophotometer at 510 nm after incubation (water bath; Shanghai Jing Hong Laboratory Instrument Co., Ltd.) at room temperature for 15 min. Rutin was used for establishment of a calibration curve.

Determination of total phenolic contents The total phenolic contents (TPC) of wine samples were colorimetrically measured using the Folin-Ciocalteu method (16). First, 0.05 mL of a wine sample solution was transferred to a 10

mL colorimetric tube and mixed with 4.8 mL of distilled water. Then 0.5 mL of Folin-Ciocalteu reagent (Beijing Solarbio Science & Technology Co., Ltd.) (50%, v/v) was added, and the mixture was blended. The absorbance of the reaction mixture was measured using a spectrophotometer (Beijing Purkinje General Instrument Co., Ltd. Beijing, China) at 765 nm after incubation (water bath; Shanghai Jing Hong Laboratory Instrument Co., Ltd.) at 45°C for 15 min. Gallic acid was used for construction of a calibration curve. The TPC value was expressed as gallic acid equivalents (GAE).

DPPH free radical scavenging assay The DPPH scavenging activity was determined based on an assay modified from Ranilla *et al.* (17). First, 100 μL of a wine sample was added to 2.0 mL of a 200 μM DPPH in an ethanol solution. The mixture was then blended and incubated (water bath; Shanghai Jing Hong Laboratory Instrument Co., Ltd.) for 30 min in the dark at room temperature. The absorbance was monitored (Beijing Purkinje General Instrument Co., Ltd.) at 517 nm (A_{samp}) against a blank of ethanol. The absorbance of the control (an ethanol solution instead of a wine sample) was also recorded at the same wavelength (A_{cont}). Therefore, the percentage of inhibition was calculated as:

$$\text{DPPH}\cdot \text{ Scavenging effect (\%)}=\left(1-\frac{A_{sample}}{A_{control}}\right)\times 100$$

An ABTS free radical scavenging assay A modified version of the assay described by Arnao *et al.* (18) was followed. The radical cation was prepared by mixing a 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v), and the mixture was left to stand for 12 to 16 h in the dark at room temperature. The $ABTS^+$ solution was diluted using ethanol to an absorbance value of 0.700±0.02 at 734 nm. The photometric assay was applied to a mixture of the $ABTS^+$ solution (2.85 mL) and wine samples (150 μL) that were incubated for 10 min in the dark at room temperature. Wine (A_{samp}) and control samples (ethanol in place of sample, A_{cont}) absorbance values were measured (Beijing Purkinje General Instrument Co., Ltd.) at 734 nm. The scavenging effect was calculated as:

$$\text{ABTS}\cdot \text{ Scavenging effect (\%)}=\left(1-\frac{A_{sample}}{A_{control}}\right)\times 100$$

Ferric reducing antioxidant power assay The ferric reducing antioxidant power (FRAP) was measured following a modified method of Deighton *et al.* (19). Briefly, FRAP reagent was mixed with a 0.3 M acetate buffer (pH 3.6), a 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3$ at a ratio of 1:1:10. The mixture was freshly prepared. An wine sample aliquot of 100 μL was mixed with 2.9 mL of FRAP reagent. The absorbance of the reaction mixture was measured using a spectrophotometer at 593 nm after incubation at 37°C for 10 min. Ferrous sulfate was used for construction of a calibration curve and results were expressed as mg/mL of ferrous sulfate equivalents per mg of fresh weight.

Statistical analysis Simple regression analysis was performed for determination of the dose-response relationship of standard solutions and wine samples. All analysis was performed in triplicate. Data were recorded as means± standard deviation (SD) and analyzed using SPSS. Significance of differences were determined using Scheffe's test, and values at $p<0.05$ were considered significant.

Results and Discussion

Amino acids of pomelo wine The composition and amount of free amino acids varied between brewing years. Free amino acids, which exist naturally in food, determine the taste, flavor, and quality of foods (20,21). The amino acids present in pomelo wine for different aging times are shown in Table 1. Pomelo wine contained 17 amino acids, including 7 essential and 10 non-essential amino acids. Threonine, Glycine, Alanine, Isoleucine, Leucine, Tyrisine, Lysine, Histidine, and Arginine contents reached a maximum in the year of 2009. Glutamic acid, Proline, Methionine, and Phenylalanine contents were highest in the year of 2010. The proline content was higher than for other amino acids for all brewing years and reached a significantly higher maximum value of 664.74±4.24 mg/L ($p<0.05$) in the year of 2010. The Cysteine content began to decline in the year of 2010, then showed an ascending trend thereafter.

Volatile compositions in different pomelo wines The key aromatic compounds of pomelo wine were identified and grouped into esters, alcohols, and acids (Table 2). The composition of aromatic compounds differed both qualitatively and quantitatively among wine samples. There were 18 different aromatic components in pomelo wine samples in third brewing year, more than for other years. The total volatile ester content of pomelo wine samples in third brewing year was also higher than that of other years, whereas the acid content was lowest.

Volatile esters were the main compounds affecting fruity flavors and had effects on the organoleptic characteristics of pomelo wine samples. The alcohol contents of pomelo wine samples in the fourth brewing year were higher than for other years. Liquor with a high alcohol content is spicy and possesses a pungent odor. A high alcohol content

Table 1. Amino acids present in pomelo wine at different aging times (mg/L)[1)]

Pk #	RT	Name	2008	2009	2010	2011	2012
1	4.700	Asp	63.37±2.45^{b}	57.11±3.55^{c}	68.85±3.32^{a}	19.46±1.73^{d}	10.61±0.73^{e}
2	5.347	Threonine	35.54±1.65^{c}	51.54±2.73^{a}	40.06±1.96^{b}	13.71±1.45^{d}	14.97±2.64^{d}
3	5.933	Serine	46.57±1.49^{a}	43.66±2.32^{ab}	42.60±1.85^{b}	12.15±1.41^{c}	7.48±0.43^{d}
4	6.653	Glutamic acid	53.77±1.35^{c}	59.19±2.86^{b}	79.87±1.45^{a}	44.66±2.73^{d}	36.37±1.81^{e}
5	7.267	Proline	216.08±5.57^{e}	387.32±6.24^{d}	664.74±4.24^{a}	612.17±9.58^{b}	449.12±13.43^{c}
7	9.553	Glycine	49.55±2.28^{b}	76.16±4.86^{a}	42.02±3.38^{c}	23.39±2.01^{d}	15.37±1.76^{e}
8	10.240	Alanine	105.01±5.40^{b}	159.42±3.97^{a}	75.47±4.96^{c}	29.35±1.37^{e}	50.68±2.88^{d}
10	12.180	Cystine	ND	ND	ND	2.34±0.51^{b}	23.32±2.39^{a}
11	12.633	Valine	53.77±3.50^{b}	86.52±5.76^{a}	49.58±4.25^{b}	16.11±2.91^{c}	15.74±1.70^{c}
13	13.780	Methionine	25.17±2.70^{b}	30.50±5.35^{b}	39.18±3.58^{a}	27.29±2.63^{b}	13.95±1.49^{c}
15	15.907	Isoleucine	42.47±2.23^{b}	71.62±1.33^{a}	39.11±1.33^{c}	9.26±0.44^{d}	9.08±0.34^{d}
16	17.000	Leucine	97.69±3.09^{b}	139.14±4.22^{a}	91.68±3.96^{c}	35.23±1.81^{d}	33.79±3.32^{d}
17	18.447	Tyrosine	38.60±4.31^{b}	45.69±4.99^{a}	33.13±2.17^{b}	16.58±3.02^{c}	10.61±0.88^{c}
18	19.533	Phenylalanine	52.92±2.43^{b}	50.94±2.60^{a}	62.77±2.89^{b}	49.07±4.01^{a}	60.20±4.86^{b}
22	21.980	Lysine	106.40±5.34^{b}	137.21±5.88^{a}	110.94±3.57^{b}	72.68±3.94^{c}	44.74±1.93^{d}
24	24.127	Histidine	17.02±3.39^{cd}	32.57±2.68^{a}	25.21±3.38^{b}	19.47±2.17^{c}	11.97±2.22^{d}
28	28.073	Argininev	75.47±4.88^{b}	97.38±4.47^{a}	71.21±5.83^{b}	56.52±1.30^{c}	32.31±1.60^{d}

[1)]Means±SD. Different letters represent significant differences among groups (p<0.05); ns = not significantly different.

Table 2. Volatile component contents of pomelo wine at different aging times[1)]

Compounds		Concentration (%)				
		2008	2009	2010	2011	2012
Ester	Isopentyl acetate	4.50±1.39^{b}	6.50±1.49^{a}	3.48±0.61^{c}	3.25±1.31^{c}	2.97±0.20^{c}
	2-Methylbutyl acetate	1.38±0.23^{a}	1.26±0.17^{a}	0.85±0.12^{b}		
	Ethyl hexanoate	2.45±0.34^{c}	1.84±0.08^{a}	1.42±0.30^{ab}	1.80±0.15^{a}	1.58±0.22^{ab}
	Ethyl benzoate	1.97±0.19^{b}	2.07±0.33^{b}	0.94±0.05^{c}	2.22±0.20^{b}	3.72±0.56^{a}
	Diethyl succinate	13.54±2.07^{b}	19.13±2.10^{a}	4.82±0.75^{c}	7.06±1.61^{c}	
	Ethyl octanonate	1.71±0.15^{c}	1.28±0.14^{cd}	0.95±0.12^{d}	2.61±0.50^{b}	3.40±0.70^{a}
	Terpinyl acetate			0.76±0.23		
	Benzenepropanoic acid, ethyl ester	1.11±0.24				
	Hydrocinnamic acid ethyl ester		0.81±0.12			
	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1.15±0.23				
	Phthalic acid diisobutyl ester		1.29±0.22^{b}	2.31±0.33^{a}		
	L-ascorbyl dipalmitate			19.67±1.70		
	Methyl 14-methylhexadecanoate			1.84±0.35^{c}	5.89±1.12^{b}	13.07±0.59^{a}
	Stearic acid glycidyl dster			1.21±0.28		
	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	1.42±0.34				
	Adipic acid bis(2-ethylhexyl) ester					2.39±0.50
Alcohol	a-Methyl-a-[4-methyl-3-pentenyl] oxiranemethanol	3.95±0.55^{c}	6.56±0.46^{b}	1.03±0.20^{d}	10.58±1.71^{a}	
	Octanol			2.18±0.19^{c}	2.99±0.33^{b}	3.68±0.19^{a}
	Phenylethyl Alcohol	1.65±0.15^{c}	2.65±0.57^{c}	8.66±1.54^{a}	5.68±0.61^{b}	
Acid	Octanoic Acid	1.49±0.34^{c}	2.34±0.49^{b}	2.50±0.38^{b}	3.46±0.46^{a}	2.43±0.43^{b}
	Decanoic acid	1.90±0.65^{ab}	1.06±0.24^{b}	1.48±0.34^{b}	1.90±0.33^{a}	2.24±0.40^{a}
	Hexadecanoic acid	23.13±2.56^{b}	18.79±2.07^{c}	17.96±2.41^{c}	26.05±1.10^{b}	29.67±2.23^{a}
	Oleic Acid	29.63±2.06^{a}	23.93±2.00^{b}	21.79±2.29^{b}	30.76±2.89^{a}	29.95±2.38^{a}
	Octadecanoic acid	6.12±0.75^{a}	5.07±0.79^{ab}	4.40±0.70^{b}		

[1)]Means±SD. Different letters represent significant differences among groups (p<0.05); ns = not significantly different.

causes negative effects on wine flavor. However, phenylethyl alcohol (a phenol derivative of fatty alcohol) has a rose aroma and has a positive effect on wine aroma. The phenylethyl alcohol contents of pomelo wine samples in the third brewing year were highest among all years. The quality of pomelo wine samples in the third brewing year was best based on volatile component contents.

TFC and TPC of pomelo wine Flavonoids and phenolic compounds are secondary metabolites that are present in many plants as potent antioxidants, free radical scavengers, and inhibitors of lipid peroxidation (22). The antioxidant activities of plant materials are correlated with flavonoid and phenolic contents (23). Therefore, the effect of TFC and TPC on the antioxidant activities of pomelo wine should be considered.

The Forint-phenol reagent can react with phenolic compounds to produce a blue color. Phenolic contents are proportional to the color depth as the deeper the blue color, the higher the phenolic content (24). TFC and TPC contents in pomelo wine, expressed as μg of lutins and μg of GAE per mL of wine sample, respectively, are shown in Table 1. TPC and TFC values increased with aging time and reached respective maximum values of 220.0±6.24 and 220.0±6.24 μg/mL in the year of 2010. TPC and TFC values decreased with longer aging times after these maxima were reached.

Antioxidant activities of pomelo wine Different assays have been used to measure the antioxidant activities of foods and biological samples. Spectrophotometric assays have recently been used to measure antioxidant activities of foods. Popular assays are ABTS, DPPH, FRAP, and oxygen radical absorbance capacity assays (25,26). The antioxidant activity describes the capability of redox molecules in foods and biological systems to scavenge free radicals. The antioxidant activity of pomelo wine was assessed using DPPH, ABTS, and FRAP assays (Fig. 1-3). The free radical scavenging and ferric reducing activities of pomelo wine, based on the scavenging value (%) and the FRAP value (mmol/L), respectively, differed for different aging times. Over the first three years the antioxidant activities improved gradually and reached maximum values in the year of 2010 (38.23±2.16% for the DPPH· Scavenging effect, 91.84±4.14% for the ABTS· Scavenging effect, and 0.90±0.07 mmol/L for the FRAP value). However, the antioxidant activities declined thereafter.

Relationship among TPC, TFC, and antioxidant activity values The antioxidant activities of wine samples were measured based on mass, TPC, and TFC

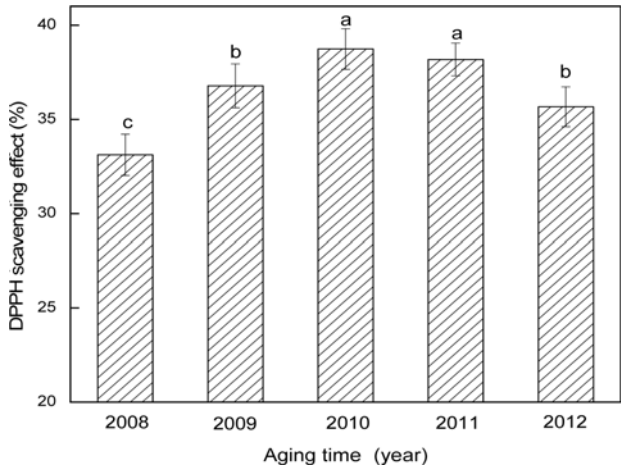

Fig. 1. Scavenging activities against DPPH free radicals of pomelo wine at different aging times.

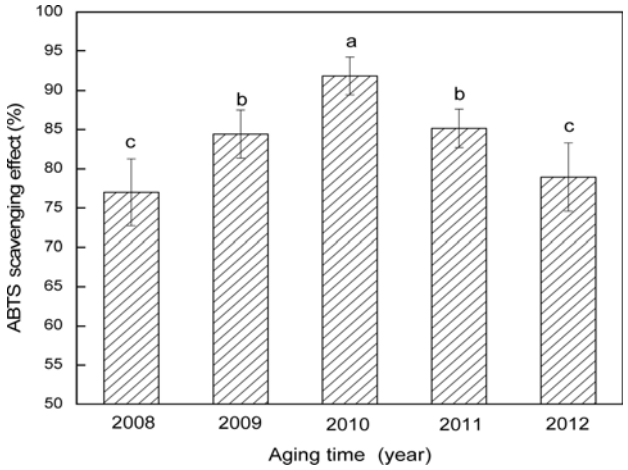

Fig. 2. Scavenging activities against ABTS free radicals of pomelo wine at different aging times.

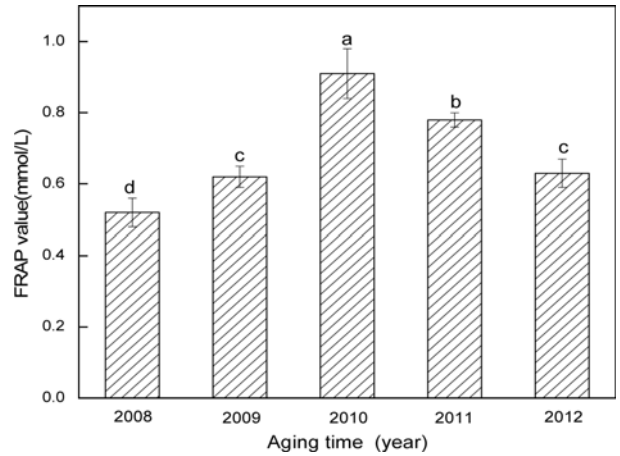

Fig. 3. Reducing power of pomelo wine at different aging times. 1) Error bars indicate SD of triplicate experiments. 2) Different letters represent significant differences among groups (p<0.05); ns=not significantly different

Table 3. TFC and TPC of pomelo wine at different aging times[1)]

Index (μg/mL)	2008	2009	2010	2011	2012
TPC	118.67±7.02[d]	143.67±5.69[c]	220.0±6.24[a]	184.33±4.04[b]	138.67±8.51[c]
TFC	33.33±3.51[c]	42.33±3.51[b]	56.67±4.04[a]	48.67±4.16[b]	42.70±1.80[b]

[1)]Mean±standard error. Different letters represent significant differences among groups ($p<0.05$).

Table 4. Correlation coefficients for antioxidant activity, TFC, and TPC values of wine samples[1)]

Correlation	TFC	DPPH	ABTS	FRAP
TPC	0.42 ($p<0.05$)	0.73 ($p<0.05$)	0.93 ($p<0.01$)	0.92 ($p<0.01$)
TFC		0.49 ($p<0.05$)	0.47 ($p<0.05$)	0.35 ($p<0.05$)
DPPH			0.70 ($p<0.01$)	0.63 ($p<0.01$)
ABTS				0.88 ($p<0.01$)

[1)]Correlation is significant at $p< 0.01$. Statistical significant at $p<0.05$

concentrations. Correlations between antioxidant activity values and TFC and TPC values were also analyzed. Correlation coefficients for antioxidant activity, TFC, and TPC values in different wine samples are shown in Table 2. TPC, TFC, and DPPH radical scavenging, ABTS radical scavenging, and Fe^{3+} reduction activities differed among wine samples. The TPC value was significantly ($p<0.05$) correlated with DPPH radical scavenging, ABTS radical scavenging, and the Fe^{3+} reduction activities.

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