



Comparative Study of Sugars, Organic Acids and *trans*-Resveratrol in Red and White Grapes Grown in Denizli Region, Turkey

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Grape berry ripening includes a broad range of physical and biochemical processes. In this study, compare the changes in organic acid, sugar and *trans*-resveratrol content during at four different ripening stages (lag phase, veraison, maturity and late harvest) between red (Çalkarasi and Shiraz) and white (Sultana and Round Seedless) grapes grown in Denizli region were investigated. Analysis was carried out using the high performance liquid chromatography-diode array detector (HPLC-DAD). Glucose and fructose content was increased between lag phase stages and late harvest stages. Tartaric and malic acid content of all cultivars decreased throughout the maturation period. A continuous decrease in *trans*-resveratrol content in all varieties was observed during ripening.

Keywords: Grape, Organic acids, *trans*-Resveratrol, HPLC, Ripening.

INTRODUCTION

A grape is a non-climacteric fruit that grows on the perennial and deciduous woody vines of the genus *Vitis*. There are about 60 species of *Vitis*, which are mainly found in the temperate zones of the northern hemisphere and almost equally distributed between America and Asia¹. Grape skin colour, which is mainly decided by the composition and content of phenolic substances², plays an important role in determining fruit quality and the market value of table grapes, as well as wine and juice quality.

Grape ripening involves number of physical and biochemical modifications that begin during veraison and end with berry maturity³. Ripening changes are not simultaneously. Each compound evolves differently and its synthesis is influenced by climate and growing location⁴. Veraison marks the beginning of ripening in grape and a lot of events are initiated in this phase, like a change in skin colour, berry softening, sugar accumulating and organic acid decline^{5,6}. Grapes develop the properties intrinsic to the cultivar to which they belong during ripening. The ripening process depends on many factors and it determines grape quality at harvest.

Grape organoleptic quality greatly depends on the content and composition of sugars and organic acids⁷. Despite of lower contents of organic acids in comparison with sugars, organic acids have important effects on grape quality. Namely, organic acids enhance grape flavour and help to improve mouth-feel of grape⁸. The balance between sweetness and acidity is an

important quality criterion of consumer acceptance. The predominant sugars in berries are glucose and fructose in most genotypes and tartaric and malic acids typically account for > 90 % of total acids^{9,10}.

Besides sugars and organic acids, which have been investigated for the last few decades, phenolic compounds are extremely important constituents of grapes¹¹. Phenolic compounds, especially flavonoids and stilbenes have been recognized as being responsible for several beneficial physiological effects owing to their potent antioxidant and anti-inflammatory properties¹².

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) has been identified as the major active compound of the stilbene phytoalexins¹³. It exists as *trans* and *cis* isomers, both of which are present in biological materials in wide concentration range. Grapevine and wine is an important dietary source of resveratrol¹⁴. In grapevines (*Vitis vinifera*), resveratrol was synthesized in response to biotic and abiotic stress, such as fungal infection¹⁵, ultra violet light exposure¹⁶, ozone stress¹⁷, anoxic treatment¹⁸ and wounding¹⁵. Since the resveratrol was mainly accumulated in the grape berry skin and seeds¹⁹, much attention had been paid on the changes of resveratrol during grape berry development.

The object of this study was to determine and compare the changes of sugars, organic acid and *trans*-resveratrol content between red and white skinned grapes at four different ripening stages (lag phase, veraison, maturity and late harvest) applying chromatographic methods.

EXPERIMENTAL

Two varieties of red grape were selected, Çalkarasi and Shiraz, which were cultivated on two adjoining vineyards. These varieties were selected because Çalkarasi is the main and more extensively cultivated red grape variety in the Denizli region and Shiraz is an Iranian variety used as new complementary variety in Denizli region for the winemaking. Sultana and Round seedless varieties were selected as white grape. The Sultana is a "white" (pale green), oval seedless grape variety also called the Sultanina or Thompson Seedless (United States) and it represents over 50 % of the overall production in the Denizli region.

All of grape varieties were grown in vineyards at 870 m above sea level in the Çal area, location in the province of Denizli (Western Turkey). The area is characterized by a favourable climate for grape cultivation. Four samplings were carried out for each cultivar with grapes harvested on berry development stages basis: July 2nd (Lag phase), July 21th (Veraison), August 29th (Maturity) and September 11th (Late harvest) of the 2013 crop year. The sampling of all varieties in the four stages was performed using the same procedure by randomly picking clusters from the top, central and bottom part of plant. Çalkarasi samples were collected from 13-year-old vineyards (2.3 ha) and Shiraz samples 4 year-old vineyards (0.9 ha). Sultana samples were collected from 15-year-old vineyards (3.1 ha) and Round seedless samples 7 year-old vineyards (1.2 ha). The distance between the rows was 1.5 m and the distances between the grape vine canes 2.0 m. Ten kilograms of the grapes was collected from 20 to 25 plants for each variety and berry ripening stages. Two hundred berries per experimental unit were randomly collected from within clusters.

All reagents used were analytical or HPLC grade. Standard *trans*-resveratrol (Catalog No: R5010) and organic acid standards (L-tartaric, L-malic, citric acid) were provided by Sigma Aldrich Co. (Sigma-Aldrich Chemie, Steinheim). Methanol, acetonitrile and glacial acetic acid were obtained from Merck Milipore (Darmstadt, Germany).

Determination of Sugars by HPLC: Undamaged and disease-free berries were snipped from clusters. 10 g of berries was mixed with 10 mL of 80 % methanol [methanol:water (8:2)] and homogenized with a lab blender (Stomacher 400, Seward Medical, London, UK) for 3 min. Then the mixture was filtered through a filter paper (Munktell 67N grade 400 × 400 mm, Germany); the filtrate was diluted with 10 mL 80 % methanol [methanol:water (8:2)] Then the methanol was removed by rotary evaporation under vacuum at 40 °C. The final mixture was filtered through a 0.45 mm membrane filter before 20 µL injections.

Sugars were analyzed using a liquid chromatography pump (Shimadzu, Model LC-20AT-VP) with a refractive index detector (Shimadzu, Model RID-10A) and a degasser (Shimadzu, Model DGU 14A). The column temperature was set at 55 °C by a column oven (Shimadzu, CTO-20A). The injection volume was 20 µL. Sugar content were expressed as gram per liter of juice. A Bio Rad Aminex HPX-87 ion exclusion column (300 mm × 7.8 mm) with a guard column

cartridge (Bio Rad Micro-Guard column (30 mm × 4.6 mm) was used for sugar analysis. The mobile phase was distilled water and the flow rate was 0.6 mL/min. Lab Solution Chromatography software (Shimadzu, Japan) data system was used to integrate peak areas according to external standard solution calibrations.

Determination of organic acids by HPLC: Standard solutions and grape juices samples were filtered through a 0.45 mm millipore membrane filter (PTFE Sartorius, SM16555Q, Germany) and then 20 µL aliquots of samples or standards were injected into the HPLC.

For organic acid analysis chromatographic method, slightly modified from Evans *et al.*²⁰, Lamikanra *et al.*²¹, Perez *et al.*²² were used. Liquid chromatography pump (Shimadzu, Model LC-20AT-VP) with a photodiode array detector (Shimadzu, Model SPD-M20A-UV/VIS) and a degasser (Shimadzu, Model DGU 14A) were used. Integration and data storage were performed with Lab Solution Chromatography software (Shimadzu, Japan). The organic acids were eluted isocratically using a Bio Rad Aminex HPX-87 ion exclusion column (300 mm × 7.8 mm) preceded by a Cation H Bio Rad Micro-Guard column (30 mm × 4.6 mm) and a column oven (Shimadzu, CTO-20A) set at 25 °C. Organic acids were detected at 214 nm. The mobile phase (0.01 N H₂SO₄) was filtered through a 0.45 mm Millipore membrane-filter (PTFE Sartorius, SM16555Q, Germany) and degassed in vacuum. Mobile phase was used at a ow rate of 0.6 mL/min.

Determination of *trans*-resveratrol: The procedure for the extraction of phenolic compounds was carried out as described by Jeandet *et al.*²³ and involved different parts of the grapes including skins, seeds and pulp. Several modifications have been carried out and included lower temperature (25 °C) and time (1.5 h). The extraction method was conducted as follows:

Fresh grapes (10 g) from each cultivar was crushed in a mortar so that a very concentrated juice was obtained. The juice was mixed with 60 mL 90 % methanol and transferred into a 250 mL Erlenmeyer flask and stirred on a magnetic stirrer for 30 min. The product was centrifuged at 4000 rpm for 2 min, the supernatant poured into a test tube, wrapped with aluminum foil and stored in a fridge at 4 °C. Deposited material was removed, crushed again in a mortar, mixed with 60 mL 90 % methanol and subjected to the same procedure. This was repeated for the third time in the low-sunlight environment as possible as. The final extract, a mixture of three supernatants, was filled up to 200 mL with methanol and stored in a fridge (4 °C) until further analysis.

HPLC conditions of *trans*-resveratrol analysis: Liquid chromatography pump (Shimadzu, Model LC-20AT-VP) with a photodiode array detector (Shimadzu, Model SPD-M20A-UVVIS) and a degasser (Shimadzu, Model DGU 14A) were used for the *trans*-resveratrol analysis. Integration and data storage were performed with LabSolution Chromatography software (Shimadzu). *trans*-Resveratrol were eluted gradiently using a Bio Rad Aminex HPX-87 ion exclusion column (300 mm × 7.8 mm) preceded by a Cation H Bio Rad Micro-Guard column (30 mm × 4.6 mm). The mobile-phase flux was 0.6 mL/min. The eluents were (A) acetonitrile (65 %) and (B)

ultra pure water (35 %). The column was thermostatically controlled to maintain a temperature of 30 °C by a column oven (Shimadzu, CTO-20A). Injection was made by means of a Hamilton micro injection with 20 µL fixed loop. The separation was conducted by using elution with solvent A from 0 to 18 min then from B 100 % to A 100 % in 1 min and from A 100 % to B 100 % in 6 min to re-establish the initial conditions, before the injection of another sample. The eluent was monitored at 310 nm.

Analytical characteristics of the HPLC method

Limits of detection: The detection limits for each sugar, organic acid and *trans*-resveratrol, based on a signal-to-noise ratio (S/N) of 3, were 0.2 g/L for glucose, 0.15 g/L sucrose, 0.3 g/L fructose, 0.005 g/L for tartaric acid, 0.035 g/L for malic acid, 0.040 g/L for citric acid and 0.032 mg/kg for *trans*-resveratrol.

Recovery: The reliability of the method was confirmed by two recovery experiments. All grape varieties were analyzed before and after the addition of known amounts of mixtures of the sugar, organic acids and *trans*-resveratrol and analyzed in the same way as the samples. Recoveries for Çalkarasi, Shiraz, Sultana and Round seedless grape variety varied between 96.7-99.9 %, 98.2-102.3 %, 95.1-105.7 % and 94.7-107.4 %, respectively.

Further determinations: The pH of the samples was measured using a pH meter (PL-700PV Gondo-Taiwan) equipped with an electrode²⁴. The pH meter was standardized by a two point method against buffer standards of pH 7 and pH 4. Grape samples from each treatment were ground in a blender (Waring, USA) and juice was used to determine the total soluble solids (% °Brix) using a digital refractometer (RFM340 Bellingham Stanley, UK). The machine was standardized using purified water before readings were taken. Titratable acidity (TA) was determined as g tartaric acid/100 mL using the method of AOAC²⁴.

Statistical analysis: The statistical analysis of the data was carried out by analysis of the variance (ANOVA) and the DUNCAN's multiple range test to show measurements which

can be considered statistically different. A significance level of $p < 0.05$ was used. All statistical analyses were performed using the SPSS statistics software package (version 16.0; IBM Corporation, NY, USA).

RESULTS AND DISCUSSION

The progress of grape berry ripening was evaluated primarily by the changes in total soluble solids (TSS), titratable acidity and pH values. These physical and chemical properties of grape berries obtained from different maturity stages are presented in Table-1. Significant increase for total soluble solids and pH degree existed in all grape varieties in relation to maturity stages. The increase in total soluble solids is related to sugar accumulation, since the majority of soluble solids in grapes are glucose and fructose²⁵. Total soluble solids (°Brix) increased during the maturity progress. Sultana grape has the highest amount (27.12 %) at the late harvest stage in the analyzed grapes. On the other hand, titratable acidity decreased considerably during ripening, varying from 25.8 to 4.6 g/L for red grape varieties, from 35.8 to 3.7 g/L for white grape varieties, considering green and over mature grapes, respectively. Simultaneously with this decrease the pH increased, reaching 4.06, 4.22 for late harvest stage red and white varieties, respectively. The reduction in acidity is attributed to dilution by the increase in the berries weight, oxidative respiration and contribution of cations associated with climatic conditions that contribute to the degradation of these compounds.

Significant ($p < 0.05$) variations in the concentrations of three key sugars namely fructose, glucose and sucrose were found in red and white grape varieties with regard to the four maturity stages (Table-2). Glucose and fructose were the predominant sugars in red and white grape berries. Glucose concentrations in red and white grapes at different ripening stages ranged from 88.23 to 4.50 g/L and from 81.83 to 2.61 g/L, respectively. Similarly, fructose concentrations ranged from 90.30 to 1.67 g/L and 80.87 to 1.23 g/L, respectively.

No statistically significant differences were detected among the red and the white varieties for fructose or glucose

TABLE-1
SOME PHYSICAL AND CHEMICAL PROPERTIES OF ÇALKARASI, SHIRAZ, SULTANA AND ROUNDLESS GRAPE BERRIES AT DIFFERENT RIPENING STAGES

Types of grapes	Varieties	Ripening stages	pH	°Brix	Titratable acidity (g/L)*
Red grapes	Çalkarasi	Lag phase	2.08 ± 0.01d	5.15 ± 0.01d	23.3 ± 0.15a
		Veraison	2.71 ± 0.02c	12.98 ± 0.02c	16.3 ± 0.05b
		Maturity	3.84 ± 0.02b	22.16 ± 0.04b	6.2 ± 0.02c
		Late harvest	4.06 ± 0.01a	24.58 ± 0.07a	4.6 ± 0.06c
	Shiraz	Lag phase	2.18 ± 0.02a	3.87 ± 0.06d	25.8 ± 0.14a
		Veraison	2.50 ± 0.01b	8.60 ± 0.02c	20.2 ± 0.02b
		Maturity	3.48 ± 0.01c	20.87 ± 0.04b	7.6 ± 0.04c
		Late harvest	3.86 ± 0.01c	25.24 ± 0.05a	7.2 ± 0.01c
White grapes	Sultana	Lag phase	2.16 ± 0.02d	5.05 ± 0.03d	31.6 ± 0.07a
		Veraison	2.40 ± 0.02c	11.39 ± 0.01c	26.6 ± 0.03b
		Maturity	3.81 ± 0.01b	23.28 ± 0.07b	5.3 ± 0.02c
		Late harvest	4.22 ± 0.01a	27.12 ± 0.11a	3.7 ± 0.01d
	Round seedless	Lag phase	2.11 ± 0.02d	4.19 ± 0.02d	35.8 ± 0.04a
		Veraison	2.46 ± 0.01c	9.42 ± 0.05c	28.3 ± 0.02b
		Maturity	3.60 ± 0.02b	20.81 ± 0.08b	5.9 ± 0.05c
		Late harvest	3.99 ± 0.02a	23.78 ± 0.10a	4.7 ± 0.01d

*Expressed as tartaric acid equivalents. Values as mean ± SD. Values within a column followed by the different letter are significant ($P < 0.05$).

TABLE-2
SUGAR, ORGANIC ACID AND *trans*-RESVERATROL CONCENTRATIONS OF RED AND WHITE GRAPE VARIETIES IN DIFFERENT BERRY DEVELOPMENT STAGES

Types of grapes	Varieties	Ripening stages	Glucose (g/L)	Fructose (g/L)	Sucrose (mg/L)	Tartaric acid (g/L)	Malic acid (g/L)	Citric acid (mg/L)	<i>trans</i> -Resveratrol (mg/kg)
Red grapes	Çalkarasi	Lag phase	4.50±0.01d	1.67±0.00d	90.40±0.09a	19.17±0.02a	2.49±0.03a	658.20±0.08a	6.22±0.03a
		Veraison	27.12±0.02c	20.62±0.05c	80.71±0.08b	14.69±0.01b	2.36±0.02b	492.25±0.03b	5.23±0.02b
		Maturity	82.31±0.09a	88.41±0.08a	15.80±0.07c	5.04±0.02c	2.13±0.01c	89.35±0.05c	1.98±0.02c
		Late harvest	78.43±0.07b	81.62±0.08b	4.51±0.01d	4.68±0.01d	1.06±0.01d	51.10±0.02d	1.06±0.01d
	Shiraz	Lag phase	5.12±0.01d	2.21±0.03d	84.81±0.11a	24.70±0.02a	3.20±0.02a	783.60±0.11a	7.25±0.02a
		Veraison	29.91±0.01c	22.54±0.05c	75.92±0.06b	17.95±0.01b	2.93±0.01b	563.45±0.04b	6.54±0.03b
		Maturity	88.23±0.09a	90.30±0.07a	10.90±0.04c	7.25±0.03c	2.73±0.01b	159.65±0.05c	2.62±0.01c
		Late harvest	83.71±0.08b	85.61±0.06b	5.14±0.01d	6.31±0.01d	1.69±0.02c	105.60±0.02d	1.73±0.01d
White grapes	Sultana	Lag phase	2.61±0.01d	1.23±0.01c	86.11±0.04a	22.66±0.17a	2.90±0.05a	727.90±0.09a	1.98±0.01a
		Veraison	27.30±0.06c	17.70±0.03b	74.90±0.04b	15.60±0.15b	2.78±0.04a	527.60±0.07b	1.73±0.01b
		Maturity	81.83±0.08a	79.72±0.07a	7.24±0.01c	5.38±0.05c	2.51±0.06b	116.10±0.05c	0.14±0.01c
		Late harvest	73.62±0.09b	78.20±0.08a	1.87±0.01d	4.95±0.07c	1.36±0.03c	72.30±0.04d	0.06±0.02d
	Round seedless	Lag phase	3.22±0.01d	1.48±0.01d	87.12±0.06a	21.25±0.16a	2.74±0.03a	706.50±0.15	1.79±0.01a
		Veraison	25.40±0.04c	18.11±0.01c	78.25±0.04b	14.12±0.08b	2.47±0.05b	511.20±0.08	1.61±0.02b
		Maturity	78.14±0.18a	80.87±0.07a	11.17±0.01c	5.08±0.06c	2.34±0.04b	107.05±0.13	0.17±0.01c
		Late harvest	71.73±0.05b	78.80±0.03b	2.14±0.01d	4.93±0.04c	1.29±0.02c	67.80±0.07	0.07±0.02d

Values as mean ± SD; Values within a column followed by the different letter are significant ($P < 0.05$).

content. However, the Shiraz variety had consistently higher glucose and fructose contents than the others at each different ripening stage. However, relative percentage of glucose content was drastically increased between veraison and maturity stages. Similar increasing trend was also observed for fructose content of both red and white grape varieties (Fig. 1a and 1b). On the other hand glucose and fructose concentration of all red and white varieties were slightly decreased between maturity and late harvest stages (Fig. 1a and 1b). With regard to sugars, sucrose was present in the lowest amounts for red and white grape varieties.

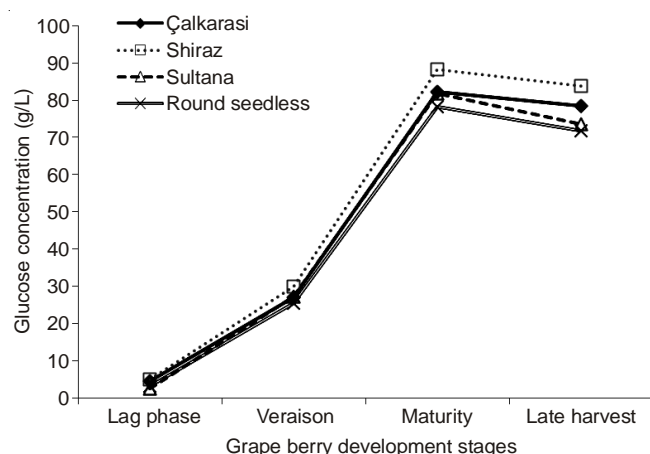


Fig. 1a. Changes of glucose content of grape varieties during ripening

Organic acids are principally employed to determine fruit maturity. The major organic acids accounting for total acids in red and white grape berries were found tartaric acid and malic acid. Citric acid was found to be in lower concentrations as compared to tartaric acid and malic acid. In both red and white grape varieties, tartaric acid was the most abundant acid. Tartaric acid, malic acid and citric acid concentrations of cultivars at different stages of berry maturation are also shown in Table-2. The contents of tartaric acid and malic acid depended

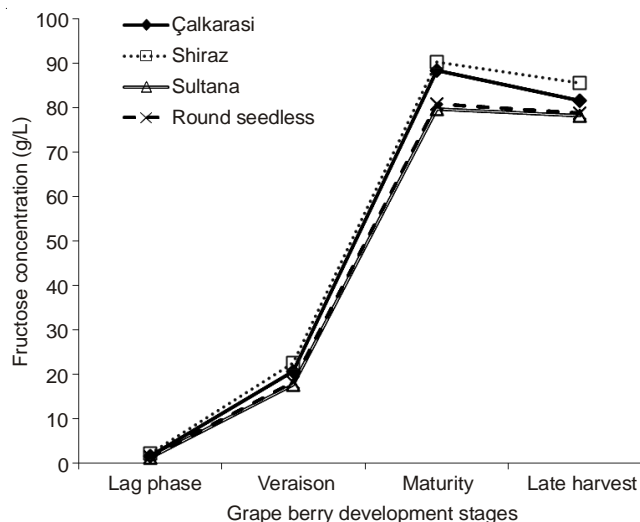


Fig. 1b. Changes of fructose content of grape varieties during ripening

largely upon genotype. Tartaric acid and malic acid content of all cultivars decreased gradually throughout the maturation period, inversely to sugar content. Tartaric acid varied from 24.70 to 4.68 g/L in red grapes and from 22.66 to 4.93 g/L in white grapes, while malic acid ranged from 3.20 to 1.06 g/L in red grapes and from 2.90 to 1.23 g/L in white grapes, respectively. Average tartaric acid content at maturity stage in grape berries (6.15 g/L in red grapes and 5.23 g/L in white grapes) was significantly higher than that of malic acid (2.43 g/L in red grapes and 2.42 in white grapes). However, relative percentage of tartaric acid concentrations in red and white grapes were drastically reduced between veraison and maturity stages (Fig. 2a). A significant reduction in the malic acid concentration of both red and white varieties was observed between maturity and late harvest stage (Fig. 2b). The reduction in acidity is attributed to dilution by the increase in the berries' weight, oxidative respiration and the contribution of cations, mainly potassium, associated with climatic conditions that contribute to the degradation of these compounds.

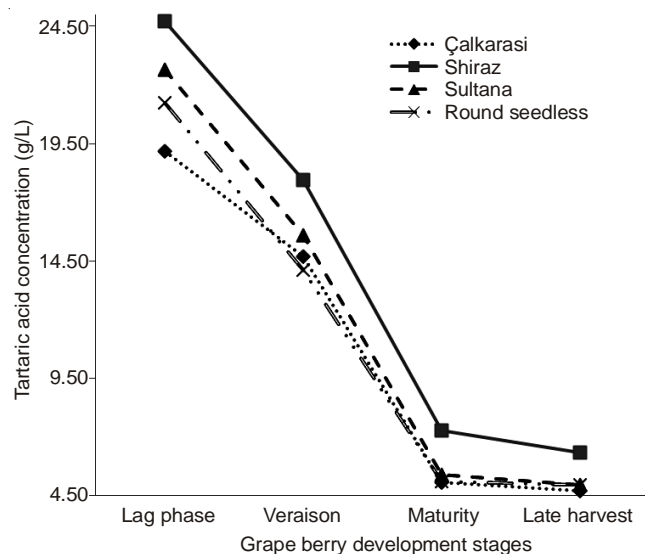


Fig. 2a. Changes of tartaric acid content of grape varieties during ripening

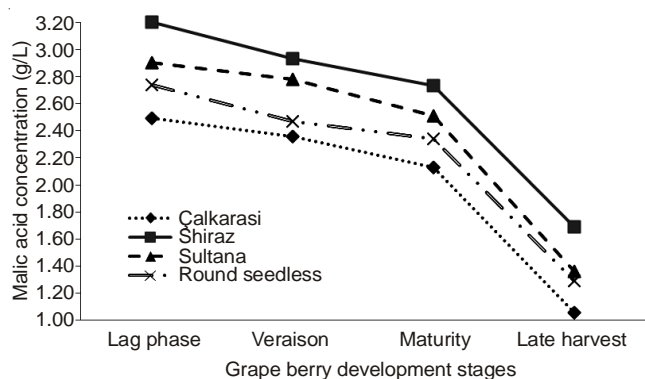
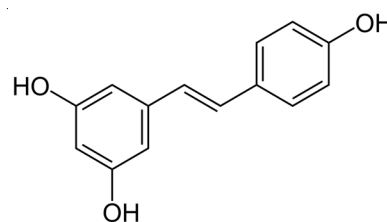
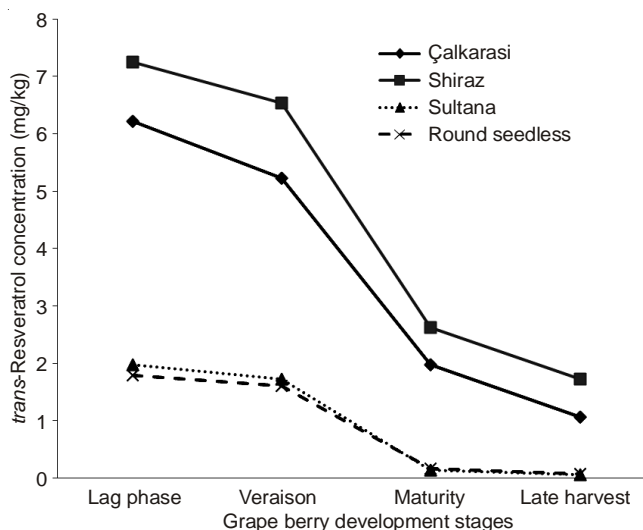


Fig. 2b. Changes of malic acid content of grape varieties during ripening

Chemical structure of *trans*-resveratrol was given in Fig. 3. The changes of *trans*-resveratrol in the grape berries of the two varieties of red-skinned grapes (Çalkarasi and Shiraz) and white skinned grapes (Sultana and Round seedless) that were studied during ripening. Analysis of *trans*-resveratrol evidenced a decreasing trend for all varieties from the lag phase stage to the late harvest stage. Besides, this analysis demonstrated significant differences in contents of *trans*-resveratrol between red and white skinned grapes (Fig. 4). *trans*-Resveratrol concentrations in red and white grapes at different ripening stages ranged from 7.25 to 1.06 mg/kg and from 1.98 to 0.06 mg/kg, respectively. Of all the cultivars, Shiraz showed by far the highest *trans*-resveratrol content in all sampling stages. A remarkable reduction in the *trans*-resveratrol content of all varieties was observed between veraison and maturity stage (Fig. 4). Moreover, there was a clear negative correlation between the *trans*-resveratrol content of grape berries and grape berry development stages.

Conclusion

In this study, organic acids, sugars and *trans*-resveratrol contents of the red and white grapes obtained from the Çal region have been examined. The results indicated that *trans*-resveratrol contents of red grapes (Çalkarasi and Shiraz) were significantly higher than white grapes. Shiraz variety had higher *trans*-resveratrol content compared to other all varieties.

Fig. 3. Chemical structure of *trans*-resveratrolFig. 4. Changes of *trans*-resveratrol concentrations of grape varieties during ripening

As expected, the concentrations of tartaric, malic acid and *trans*-resveratrol decreased with maturity. Quantitatively the major organic acid and sugar were found as tartaric acid and glucose in the all samples, respectively.

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