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Chromatographic (HPLC) Determinations of Organic Acids and Trans-Resveratrol in "Calkarasi" & "Shiraz" Grapes during Different Stages of Ripening

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Abstract

Ripening in grape is a highly organized and genetically programmed process involving changes in specific biochemical and physical attributes. In this paper, changes of trans-resveratrol and organic acid content during at four developmental time points (Lag phase, Veraison, Maturity and Late Harvest) of grape varieties of "Çalkarası" and "Shiraz" grown in Denizli region are analyzed and experimental results are presented. Liquid chromatographic (HPLC) method was used for the analysis of the contents of Organic acids and trans-resveratrol and examined compounds are thoroughly separated within 25 min. Tartaric acid and malic concentrations of Çalkarası and Shiraz varieties ranged from 19.17 g/L to 4.68 g/L; 2.49 g/L to 1.06 g/L; 24.70 g/L to 6.31 g/L and 3.20 g/L to 1.69 g/L, respectively. As the ripening increased, a sustained decrease in trans-resveratrol content was determined. "Shiraz" had the high trans-resveratrol content, showing a decrease of 76% from the stage of lag phase to stage of late harvest, while "Çalkarasi" produced the low trans-resveratrol concentration, showing a decrease of 82% from the first to last sampling.

Keywords: Grape; Shiraz; Calkarasi; Ripening; Organic Acids; Trans-Resveratrol; HPLC

Introduction

Grape (Vitisvinifera L.) cultivation is important beneficial farming enterprises all over the world. Turkey is one of the most leading countries in the world in terms of grape growing areas especially Aegean region [1]. Grape varieties are mainly categorized in three ways. They are defined as either table grapes, wine grapes and raisin [2]. Most of the production of grape is destined to wine-making [3]. The amount and composition of the phenolic substances present in the grape skin determines the grape skin color [4], which also has significant role for the quality of grape (market value of the table grapes, wine and juice).

Maturation of grape berry includes series of the physical and biochemical pathways from verasions to mature berries. Not only ripening but also climate, growing location and soil quality have important role in the synthesizing of polyphenolic compounds in the grape berry [5]. Consequently, the ripening process is affected from many factors, which have role in the determination of the grape quality at harvest.

It is possible to talk about different kinds of grape ripeness. Industrial ripeness refers to the optimum sugar / acid ratio in grape bulk while technological ripeness is expressed as the level at which the phenolic and aroma skin compounds reach the highest concentration [6].

The metabolism of fruit products are determined primarily by their chemical structure, which depends on factors such as the degree of organic acid, sugar and their phenolic substances. Organic acids, that have a significant effect on the unique flavor of the fruit, are important in terms of other quality properties such as stability, color and aroma [7]. High acidity adversely affects the taste of table grapes and complicates the wine processing [8].

Several factors such as variety, ripening level, climate, cultivation area and year can affect the chemical structure of grapes [9,10]. Usually malic and tartaric acid contents accumulate prior to veraison and show a significant decrease in malic acid concentration during maturation [11]. Even though grapes have the same genotype that is cultivated from various climate zones, have different organic acid contents [12]. There is a lower acid concentration in fruit maturity due to the increase in Malic Acid (MA) degradation, in the process of ripening of grapes in regions where steady warm weather conditions are observed [8].

Organic acids in wine obtained from grapes and those present in fresh grapes such as MA and tartaric acid (TA) that are the quantitatively important acids which are composed of 90-95% the total acidity in grapes and acetic, cis-aconitic, ascorbic, cinnamic, citric, isocitric, shicimic and succinic acids being present in smaller amounts [2,8,13]. The organic acids, metabolized by the fruit during the maturity, results a decrease in total acidity [10,14,15] and adulteration in fruit juices [16]. These organic acids are also used as indicators of wine alterations and/or spoilage [17] and as acidity regulators in foods [11,18,19].

Especially flavonoids and stilbenes from phenolic substances are responsible for various beneficial physiological effects due to their strong antioxidant and anti-inflammatory properties [20].

Trans-resveratrol which has two isomers: trans-resveratrol and

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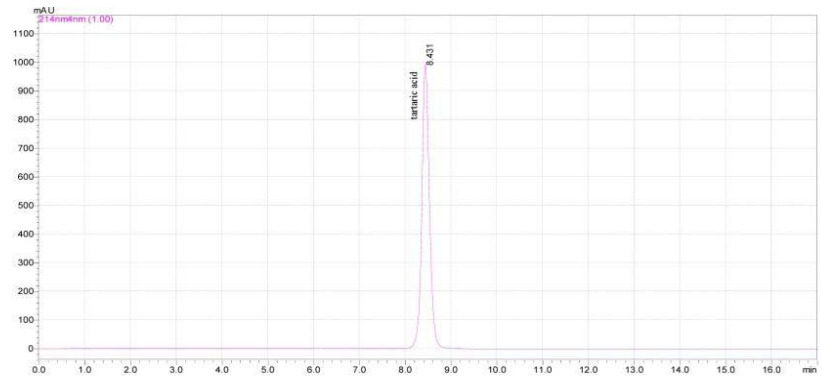

Figure 1: Typical chromatogram of a standard tartaric acid.

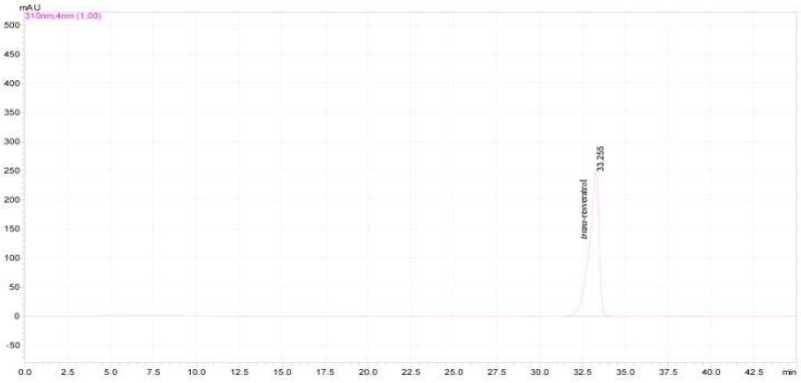

Figure 2: Typical chromatogram of a standard *trans*-resveratrol.

cis-resveratrol is a phenolic substances that is member of stilbenes. In the plant tissue they can be transformed to piceids (as a two stereoisomers: trans-piceid and cis-piceid) [21]. Trans-resveratrol is found largely in the skin of grape [22], and mainly in a glucosylated form [23]. This substance was also detected in lower abundance in grape seeds [24] and stems [25]. Generally, trans-resveratrol is known as phytoalexins which can be biosynthesized in grapevines as a defense to external invasions such as fungal diseases, abiotic stress and UV irradiation [26]. Cis-resveratrol is formed from trans-resveratrol which exposed to UV [27], is generally at lower concentrations and has frequently less biological activity than the trans-resveratrol. The amount of trans-resveratrol in grapes is adhering to several factors such as vintage, fungal infection, grape cultivar, environmental conditions, etc [28]. Gas chromatography, CG–MS, capillary electrophoresis and HPLC are used for the analysis of trans-resveratrol. Gas chromatography, CG–MS, capillary electrophoresis and HPLC have been utilized for the analysis of trans-resveratrol. However, HPLC is the preferred tectnique in terms of sample preparation, reliability, selectivity, sensitivity, and the cost and time of analysis.

To the best of our knowledge, there is no published research study about the changes of organic acids and trans-resveratrol content of Shiraz and Calkarası grape varieties at different maturation stages. The objective of this research is to determine tartaric acid, malic acid and trans-resveratrol content of Shiraz and Çalkarası grape varieties during different maturation stages of ripening (lag phase, veraison, maturity and late harvest) by using HPLC technique.

Materials and Methods

Materials

Sampling and preparation procedures: Çalkarası and Shiraz red grapes varieties were selected from the two adjoining vineyards during the study which are the main and more extensively cultivated varieties for the winemaking in the Denizli region. Both of the varieties of grapes were cultivated at the vineyards in the Çal area (Denizli province at the Western region of Turkey) which serves favorable climate for grape cultivation with the 870 meters from the sea level. Four sampling stages were determined depending on the harvest sessions considering the berry development as; July 2nd (Lag phase), July 21th (Veraison), August 29th (Maturity) and September 11th (Late harvest) of the 2013 crop year. The sampling of both varieties was performed with consistent procedure by randomly picking up clusters from the top, central and bottom part of plant. While Calkarasi vineyard has 13-year old (2.3 ha), Shiraz has only 4 year old vineyard (0.9 ha). The distance between the rows and grape vine canes were 1.5 m and 2.0 m, respectively. About, ten kilograms of the grapes were collected from 20 to 25 selected vines for each variety at the predetermined four ripening stages.

Standard solutions and reagents: HPLC grade methanol, acetonitrile and glacial acetic acid were supplied from Merck

Table 1: Some physical and chemical properties of Calkarasi and Shiraz grape berries at different ripening stages.

Types of Grape	Ripening Stages	pH	°Brix	TitratableAcidity(g/L)*
	Lag Phase	2,08±0,01d	5,15±0,01d	2,33±0,15a
Çalkarası	Veraison	2,71±0,02c	12,98±0,02c	1,63±0,05b
	Maturity	3,84±0,02b	22,16±0,04b	0,62±0,02c
	Late Harvest	4,06±0,01a	24,58±0,07a	0,46±0,06c
	Lag Phase	2,18±0,02a	3,87±0,06d	2,58±0,14a
Shiraz	Veraison	2,50±0,01b	8,60±0,02c	2,02±0,02b
	Maturity	3,48±0,01c	20,87±0,04b	0,76±0,04c
	Late Harvest	3,86±0,01c	25,24±0,05a	0.72±0,01c

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

(Darmstadt, Germany). Doubly distilled and deionised water was used in the experiments. Analytical-reagent grade standards of trans-resveratrol (Catolog no: R5010) and organic acid standards (L-tartaric, L-malic, citric acid) were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim-Germany) and stock-standard solutions of all standards were prepared in related mobile phases. Calibration curve was prepared with five different concentrations of each standard. Solutions used in the study were first sonicated and stored in dark glass flasks, in order to protect them from light, and then kept under refrigeration. Thus, five point calibration curves with the correlation coefficients of 0.999 based on the concentration (mg/ml for trans-resveratrol; g/L for organic acids) versus peak area (mAU) were prepared for trans-resveratrol, L-tartaric, L-malic and citric acid.

Methods

Determination of organic acids by HPLC: Following the centrifugation of grape juices at 5000 rpm for 10 min (Model 2-16, Sigma Bioblock Scientific), the supernatant was filtered through a 0.45 μm millipore membrane filter (PTFE Sartorius, SM16555Q, Germany). After filtration, aliquot of 20 μl clear supernatant was injected into the HPLC column for the quantitative determinations of trans-resveratrol, L-tartaric, L-malic and citric acid. In addition, FP 30/45 CA-S filters (Schleicher & Schuell, Darmstadt-Germany) with 0.45 μm (7 bar max) pore size were used for the filtration of samples prior to HPLC analysis. A typical chromatogram for the standard of tartaric acid is shown in (Figure 1).

The determination of organic acids was carried out isocratically with some modifications of chromatographic methods of Evans et al. [29], Lamikanra et al. [10], Perez et al. [15]. A liquid chromatography (Shimadzu Corporation, Kyoto, Japan) system consisting of a UV-VIS DAD detector set at 214 nm (Model SPD-M20 AVP, Shimadzu), a column oven (Model CTO-20ASVP, Shimadzu) set at 25 °C, a quadruple liquid chromatography pump (Model LC-20AT-VP, Shimadzu), a degasser (Model DGU 14A, Shimadzu) was used for the analysis. Data acquisition and data integration were done using the Lab Solution Chromatography software (Shimadzu, Japan). A syringe (Hamilton Co., Reno, NV, USA) was used for the injection of the sample (20 μL) into the HPLC. Additionally, a Bio Rad Aminex HPX-87 ion exclusion column (300 x 7.8 mm^2) was used for the separation of compounds. The column was preceded by a cationic exchanger (Bio-Rad Micro-Guard Cation H cartridge (30 x 4.6 mm^2)

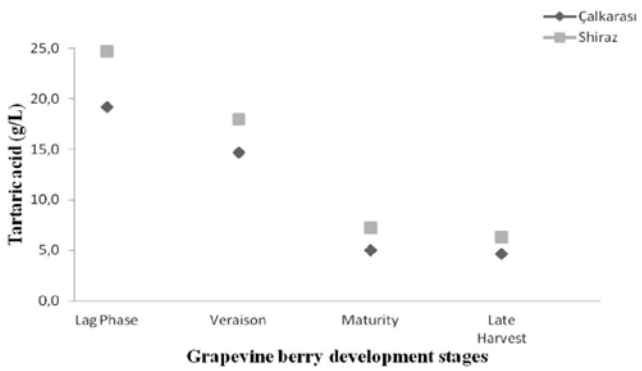

Figure 3: Changes of tartaric acid content of Calkarasi and Shiraz varieties during ripening.

at a constant temperature set at 25 °C with a column oven (Shimadzu, CTO-20A). 0.01 N H_2SO_4 was used as a mobile phase, which was and degassed before used. The flow rate of the mobile phase was constant at 0.6 mL/min during the analysis.

Determination of trans-resveratrol: A slightly modified method, which the temperature decreased to 25°C and extraction time decreased to 1.5 h, of Jeandet et al. [30], was used for the extraction of phenolic compounds. Whole parts of grapes including pulp, skins and seeds were used in the procedure. Ten grams of fresh grapes was crushed in a mortar and 60 mL 90% methanol was added to the concentrated juice in an Erlenmeyer flask. Then, the mixture was homogenized in laboratory blender for 30 min. Later, the supernatant was poured into an aluminum foil wrapped test tube following the centrifugation of the product at 4000 rpm for 2 min in order to separate unsolved solid material. Two series of extraction procedure were performed in order to ensure to extract whole soluble and extractable components remaining in the deposited material separated by the centrifugation. The final extract, consisted of a mixture of three supernatants, filled up to 200 mL with methanol and stored at 4°C until the further analysis.

HPLC conditions of trans-resveratrol analysis: A liquid chromatography (Shimadzu Corporation, Kyoto, Japan) system consisting of a UV-VIS DAD detector (Shimadzu Model SPD-M20A-UV-VIS) set at 310 nm for the determination of trans-resveratrol was used to achieve the column eluate monitoring, a column oven (Shimadzu CTO-20A) set at 30°C, a quadruple liquid chromatography

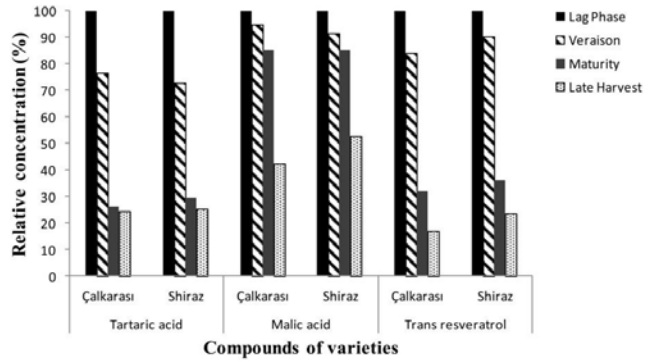

Figure 4: Relative concentration of tartaric acid, malic acid and *trans*-resveratrol in the grape extracts of Calkarasi and Shiraz variety in different grape berry development stages.

pump (LC20AD, Shimadzu), a degasser (Shimadzu, DGU-14A) and a Shimadzu Software Program (LabSolution Chromatography software) was used for the integration and analysis. A syringe (Hamilton Co., Reno, NV, USA) was used for the injection of the sample (20 μL) into the HPLC. Additionally, Bio Rad Aminex HPX-87H ion exclusion column (300×7.8 mm^2) was used in the HPLC system. Mobile phase, used in the HPLC system, was gradient and 65% acetonitrile (Pump A) and 35% püre water (Pump B) with the flow rate of 0.6 ml/min. 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. Typical chromatogram of a standard trans-resveratrol is given in (Figure 2).

Analytical characteristic of the HPLC method: Detection Limit (sensitivity), Recovery (effectiveness of the method-working range) and Precision (coefficient of variation) of the method were determined.

Further determinations (Brix, pH, total acidity): Method of Association of Official Analytical Chemists (1990) was used for the analysis of total soluble solid (% ˚Brix), pH and titratable acidity (as g tartaric acid/100 ml). Firstly, grape samples were ground in a blender (Waring, USA) and juice was used to determine the total soluble solids (% ˚Brix) (Digital refractometer, RFM340 Bellingham Stanley, UK), pH (pH meter, PL-700PV Gondo-Taiwan) and titratable acidity.

Statistical analysis

"Statistical Analysis Systems" of SAS® software (1985) was used for the statistical analysis of all data. Data means were compared using least significant difference test, when analysis of variance (ANOVA) showed a significant effect ($P < 0.05$). All statistical analyses were performed using the SPSS statistics software package (version 16.0; IBM Corporation, NY, USA).

Results and Discussion

Detection limit, Recovery and Precision

The detection limit for tartaric acid, malic acid, citric acid and trans-resveratrol, based on "S/N" (signal/noise) of 3 (Li & Chen, 2000), were 0.005 g/L, 0.035 g/L, 0.040 g/L and 0.032 mg/kg, respectively. The reliability of the method was confirmed by recovery experiments.

Standard addition procedure was used for the recovery test. Thus, known concentrations of tartaric acid, malic acid, citric acid and trans-resveratrol standards were added to the samples. In each addition level, six determinations were realized. The average percentage recoveries of tartaric acid, malic acid, citric acid and trans-resveratrol in grapes were found to be 98.18%, 99.5%, 98.32% and 97.21%, respectively.

The method precision was evaluated using the same reagents and apparatus under the same experimental conditions with six determinations of the same grape sample. In addition, intra- and inter-day tests were applied for the calculation of precision and the results were expressed as relative standard deviation (RSD,%). The evidence of good precision for HPLC is low RSD value that determined 0.73% for tartaric acid, 0.49% for malic acid, 0.62% for citric acid and 1.04% for trans-resveratrol in our study. In addition, the low RSD value also shows non-variability of the data.

The pH, total soluble solids and titratable acidity values of Çalkarasi and Shiraz grape berries obtained from different ripening stages are given in (Table 1). Significant increase for total soluble solids and pH degree existed in selected Çalkarası and Shiraz grape in relation to ripening stages. The vast majority of the soluble solids in the grape are glucose and fructose. For this reason, the increase in total soluble solids with maturation is due to the increase in sugars [31,32]. As the maturity progressed, total soluble solids of Çalkarası and Shiraz grapes increased and reached the highest amounts as 24.58% and 25.24%, respectively at the late harvest stage. On the other hand, Titratable Acidity (TA) decreased considerably during ripening, considering green and over mature grapes, respectively. However, decrement between maturity and late harvest stages was not found significant both for Çalkarası and Shiraz grapes. As the maturity progressed, pH values of Çalkarasi and Shiraz grapes decreased and reached the highest amounts as 4.06 and 3.86, respectively at the late harvest stage. In contrast, decrement between maturity and late harvest stages was not found significant for Shiraz grape. As shown in (Table 1), increase of pH and decrease of acidity are in compatible. As known, pH and acidity values are used for the assessment of the optimal time of harvest. In addition, these results are consistent with the fact that malic and tartaric acids are synthesized by the leaves and the green berries, and are used as an energy source in cellular respiration and in the process of fruit ripening (Table 1).

Effect of ripening on the organic acid content

Organic acid and trans-resveratrol concentrations of Çalkarası and Shiraz varieties in different berry development stages were given in (Table 2). As seen from (Table 2), tartaric and malic acids are the most abundant acids both in Çalkarası and Shiraz grapes. In grape berry, malic and tartaric acids are the principal organic acids, and commonly these acids accumulate before veraison, followed by a strong decline during berry ripening. On the other hand, citric acid is quite low in both grape varieties compared to tartaric and malic acid.

As shown in (Figure 3), tartaric acid content of Çalkarası and Shiraz grape varieties decreased significantly from lag phase (for Çalkarası and Shiraz respectively, 19.17 g/L; 24.70 g/L) to late harvest stage (for Çalkarası and Shiraz respectively, 4.68 g/L; 6.31 g/L). This decrease is thought to result mainly from the increase in berry volume (dilution) and conversion of free acids to salt forms [33] (Figure 3).

Table 2: Organic acid and trans-resveratrol concentrations of Calkarasi and Shiraz varieties in different berry development stages.

Types of Grape	Ripening Stages	Tartaric acid (g/L)	Malic acid (g/L)	Citric acid (mg/L)	Trans-resveratrol (mg/kg)
Çalkarası	Lag Phase	19,17±0,02a	2,49±0,03a	658,20±0,08a	6,22±0,03a
	Veraison	14,69±0,01b	2,36±0,02b	492,25±0,03b	5,23±0,02b
	Maturity	5,04±0,02c	2,13±0,01c	89,35±0,05c	1,98±0,02c
	Late Harvest	4,68±0,01d	1,06±0,01d	51,10±0,02d	1,06±0,01d
Shiraz	Lag Phase	24,70±0,02a	3,20±0,02a	783,60±0,11a	7,25±0,02a
	Veraison	17,95±0,01b	2,93±0,01b	563,45±0,04b	6,54±0,03b
	Maturity	7,25±0,03c	2,73±0,01b	159,65±0,05c	2,62±0,01c
	Late Harvest	6,31±0,01d	1,69±0,02c	105,60±0,02d	1,73±0,01d

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

Data on organic acids indicated that the relative percentage of tartaric acid decreased from 100% in lag phase stage to 24.41% for Calkarasi and 25.55% Shiraz variety in late harvest stage (Figure 4). However, relative percentage of tartaric acid was drastically reduced between veraison and maturity stages. Similar decreasing trend was also observed for malic acid content of both Calkarasi and Shiraz grape varieties (Figure 4). Malic acid content of two grape varieties decreased from lag phase (for Çalkarası and Shiraz respectively, 2.49 g/L; 3.20 g/L) to late harvest stage (for Calkarasi and Shiraz respectively, 1.06 g/L; 1.69 g/L). Because malic acid is metabolized in the process of respiration during the ripening stage [33,34]. Relative percentage of malic acid decreased from 100% in first sampling to 42.57%, 52.81% in last sampling for Çalkarası and Shiraz variety, respectively (Figure 4). A remarkable point is that significant decrease in the malic acid concentration of both Çalkarası and Shiraz varieties was observed between maturity and late harvest stage. In addition, highly significant differences between ripening stages of Calkarasi and Shiraz grapes were determined for tartaric, malic and citric acids. However, decrement between veraison and maturity stages was not found significant for Shiraz grape. In addition, tartaric and malic acid concentrations of Shiraz grape variety was always higher than Çalkarası grape for the same grape berry development stages.

Effect of ripening on the trans-resveratrol content

The changes in trans-resveratrol concentration in the two different red grapes cultivars (Çalkarası and Shiraz) studied during maturation were presented in (Figure 4). The results obtained from the trans-resveratrol analysis showed that there was a decrement tendency to lag phase stage to the late harvest stage in both grape varieties. Of both varieties, Shiraz variety demonstrated the highest trans-resveratrol concentration in all developmental stages, ranging from 7.25 mg/kg recorded on lag phase stage to 1.73 mg/kg recorded at the late harvest grade, resulting in a decrease of 76% during the ripening stages. Similar data (82%) were found in ripe fresh berry of Calkarasi variety. Consequently, there was a significantly inverse correlation between the trans-resveratrol content of grapes and their developmental stages.

Conclusion

Results clearly indicated that tartaric, malic, citric acid and trans-resveratrol were more in the unripe berries before skin colored (veraison stage) compared overripe berries (late harvest stage). Besides, when compared with the Çalkarası variety, Shiraz had higher organic acid and trans-resveratrol. There is a highly significant difference between ripening stages and tartaric, malic, citric acids and trans-resveratrol. Organic acid and trans-resveratrol concentrations of Çalkarası and Shiraz grape varieties were at the highest level at lag phase.

As the maturity progressed, concentrations of tartaric, malic citric acid and trans-resveratrol of Çalkarası and Shiraz grapes descended the lowest levels in the late harvest stage. Of all maturation stages, high decrement in tartaric acid, malic acid, citric acid and trans-resveratrol concentrations between veraison and maturity stages are most noticeable. This study will help to determine the stage in which the grape should be harvested depending on the intended use of the grape and to accelerate the improvement of quality in grapevines.

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