STUDIA UBB CHEMIA, LXX, 4, 2025 (p. 219-232)
(RECOMMENDED CITATION)
DOI:10.24193/subbchem.2025.4.12

HPLC/DAD ANALYSIS OF VITAMIN C AND ANTIOXIDANT CAPACITY DETERMINATION OF *VITIS VINIFERA L.* GRAPES DURING RIPENING

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ABSTRACT. This study aims to determine the vitamin C (ascorbic acid, AA) content and antioxidant capacity in five varieties of grapes (skin, pulp and seeds). A simple, fast and sensitive High–Performance Liquid Chromatography/Diode Array Detection (HPLC-DAD) method was used for the determination of ascorbic acid in grapes (*Vitis vinifera L*.) samples from Murfatlar vineyard. Total antioxidant capacity was determined through photochemiluminiscence method as ACL (Antioxidant capacity of lipid soluble substances). The LOD was 0.40 µg/mL for the HPLC-DAD method. The advantages of the method are using of small amounts of samples and reagents, short analysis time and minimum steps for sample preparation. Antioxidant capacity of *Vitis vinifera L*. grapes alcoholic extract ranged between 0.02 and 7.42 mmoles equivalent TROLOX/100g product. After statistical analysis (ANOVA), a significant correlation between the ascorbic acid concentration and the antioxidant capacity was observed in peel, pulp, and seeds during ripening. Due to the high content of ascorbic acid and antioxidant capacity, future analysis should focus on the determination of other bioactive substances in grapes.

Keywords: *Ascorbic acid, grapes, peel, pulp, seed, HPLC-DAD, antioxidant capacity*

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INTRODUCTION

The grapes are cultivated all over the world and the most important species is *Vitis vinifera L.* from Europe and from which are derived all major varieties of table grapes and wine. The grapes contain a lot of nutrients, especially polyphenols, which are important due to their role as natural antioxidants [1]. Antioxidants are used in the prevention and treatment of cancer [2], inflammatory [3,4], cardiovascular [5,6] and neurodegenerative diseases [7-10]. There are also trace elements (reducing and oxidizing agents) essential for carrying out the chemical reactions required for cell multiplication (Se, Ni, Cr, I, Zn, Cu, Mn, F, V, Co) which give the grape some original nutritional qualities. Its content is enriched with many organic acids found in grape berries (malic, tartaric, citric, isocitric, ascorbic, caffeic, oxalic, succinic, lactic, fumaric, etc.), water (80% of the fresh fruit weight) and inorganic compounds (K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, SO_4^{2-}, PO_4^{3-}) [11].

One interesting point with grapes is that they accumulate very low levels of ascorbic acid, in comparison with other fruits. This may be due to the fact that grape berry tissues transform AA to tartaric acid. The level varies depending on the maturity degree of grapes. Ascorbic acid (more abundant in the grape berries exposed to sunlight) varies between 4 and 10 mg/100g [11-15].

Also, the grapes contain fats (0.16 g/100g), proteins (0.72 g/100g), carbohydrates (17 g/100g), vitamins (especially B complex which is well represented by vitamins B_1 – 0.069 mg/100g, B_3 – 0.188 mg/100g and B_5 – 0.05 mg/100g) [11]. In fact, fruits are an excellent type of food characterized by a low content of calories and a high amount of antioxidant substances, which are able to prevent a wide range of diseases. Therefore, fruits represent a major source of antioxidants. The content of phytochemical substances is influenced by numerous factors such as ripening time, genotype, cultivation techniques, and climatic conditions. Maturity stages are another important factor that influences the compositional quality of the fruit. During fruit ripening, several biochemical, physiological and structural modifications happen, and these changes determine the fruit quality attributes. In fact, harvesting at the proper maturity stage is essential for optimum quality and often for the maintenance of this quality after harvest and storage [16].

There is a controversial debate in the literature about the influence of ascorbic acid on the antioxidant capacity of fruits or vegetables. It is also known that fruits generally contain more antioxidants and most of these proved to be phenolic compounds and anthocyanins [16].

Many analytical methods [17-23] have been proposed for the determination of AA in different fruits: peroxidase-mimetic colorimetric biosensors [24], a food-grade Fe β Cyclodextrin nanozymes [25] or a smartphone-

based colorimetric sensor using Zn/Co bimetallic organic framework-derived nanozymes [26]. However, the preferred method by far for analyzing AA is HPLC with ultraviolet (UV) or diode array (DAD) detection [27-32].

There are many methods (*in vitro* and *in vivo*) to determine antioxidant capacity: TEAC determination, seric malondialdehyde determination and low and total seric glutathione determination [33-39]. In this paper total antioxidant capacity of grape aqua-alcoholic solution was estimated using Chemiluminescence method in lipid samples (ACL), according to Analytic Jena procedure, Germany, using PHOTOCHEM coupled to PC apparatus [40-42].

The grapes varieties studied in this paper were from Murfatlar village. Harvested grapes in vineyard Murfatlar were used to obtain local Romanian wine, a sort of wine that has won many medals in international competitions [43]. In relation to the importance of phytochemicals and antioxidant power concerning the functional aspect of grape fruits, the aim of this work was to evaluate the changes of vitamin C content and antioxidant capacity during ripening. ANOVA, Tukey post hoc test and Pearson correlation (r) analysis were performed to assess the relationship between ascorbic acid content and antioxidant capacity across different ripening stages.

RESULTS AND DISCUSSION

Ascorbic acid has multiple biological functions, and it is believed that the role of AA in disease prevention is due to its ability to scavenge free radicals in the biological systems [17]. Despite the fact that the grapes contain a small amount of AA, this gives the grape some original nutritional qualities such as microelements and the other antioxidants.

For ascorbic acid determination, the procedure described by ASRO, SR EN 14130 [44] was optimized under laboratory conditions. First, to determine the wavelength detection, a sample of ascorbic acid solution, with a concentration of 0.4 mg/mL, was injected in the HPLC-DAD. The absorption spectrum was recorded against the blank (mobile phase), in a 1 cm cuvette, figure 1.

Under the described chromatographic (HPLC-DAD) conditions, ascorbic acid was eluted at 6.54±0.01 min, (Figure 2).

For the linearity and limit of detection, solutions of the standards of six different concentrations were analyzed and the calibration curves ($y = a + bx$) were constructed by plotting the peak area ratios (y) of analyte versus the respective concentrations. Figure 3 shows the calibration curve performed within the working range: 0.5- 15 µg /mL**.**

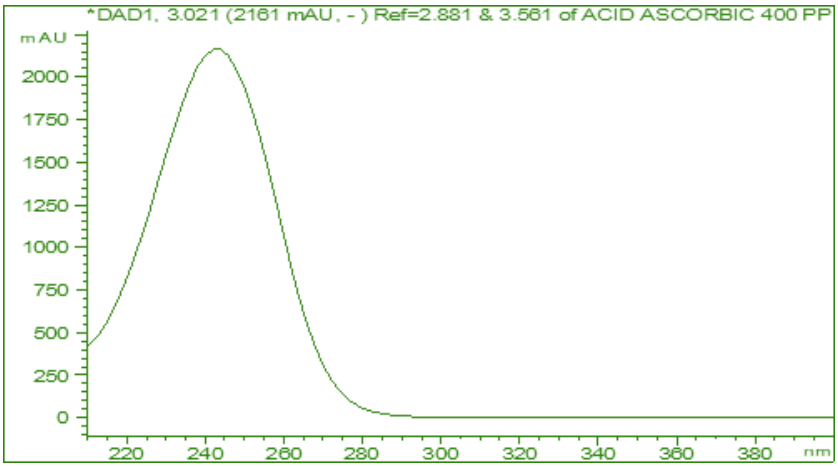


Figure 1. The absorption spectrum of standard ascorbic acid solution

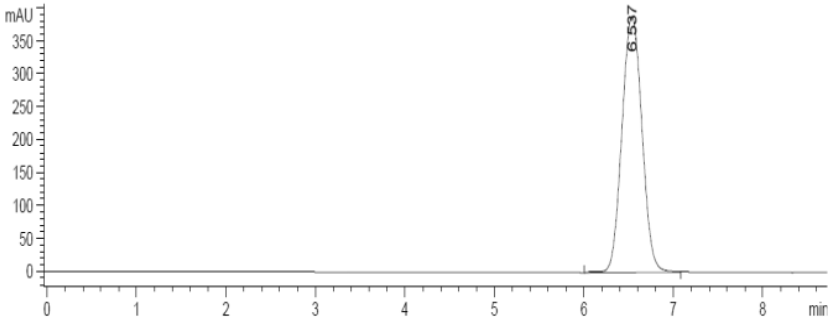


Figure 2. The chromatogram of standard ascorbic acid solution of concentration 15 µg/mL

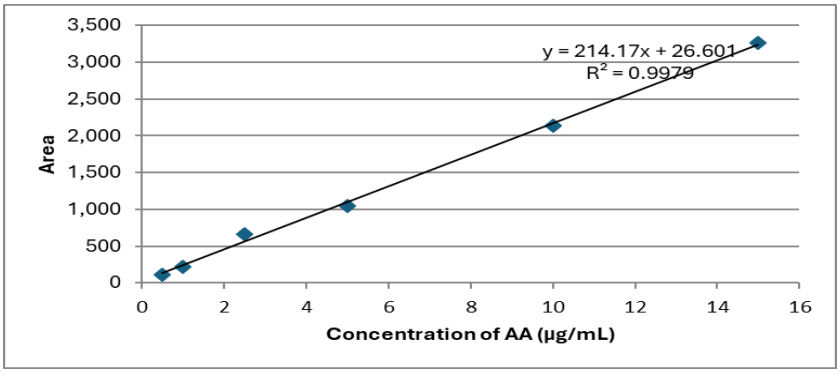


Figure 3. Calibration curve for AA determination within the working range: 0.5- 15 µg /mL

The linear dynamic range and the limit of detection (LOD) of the HPLC-DAD method were determined. It was observed a good linearity of the method response in the 0.5-15 µg/mL concentration range with R^2 = 0.9979. The equation curve registered was: A = 214.2c – 26.6. The LOD was 0.40 µg/mL for the HPLC with DAD detection. 6.54±0.01 min (values are given as means ± standard deviations (n = 3)) of the retention time was also registered.

For the precision of the method, every sample solution of the same concentration on the calibration curve was injected at least three times to determine the relative standard deviation (RSD) values for peak areas. The RSD values for peak areas ranged from 0.77% to 1.59% for standard solutions of ascorbic acid. The obtained RSD values (below 5%) presented in Table 1 indicated an excellent repeatability and precision of the proposed method [45].

The concentration of ascorbic acid was determined in five varieties of grape (*Vitis vinifera L.)* samples from Murfatlar vineyard (Murfatlar village, Constanta) using HPLC-DAD.

Table 1. The relative standard deviation (RSD) values for peak areas

Acid ascorbic concentration (µg/ mL)	RSD% (Area)
0.5	1.37
1	1.59
2.5	1.04
5	1.18
10	0.98
15	0.77

A typical chromatogram of a pulp white grape sample is shown in Figure 4. The concentration of ascorbic acid was determined in three different parts of the grape: peel, pulp and seed which were collected during ripening, at the beginning of the ripening period (unripe), in the middle (almost ripe) and before the harvest (ripe).

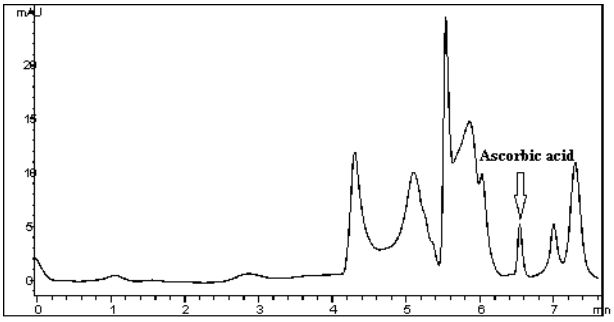


Figure 4. Representative HPLC-DAD chromatogram for a variety of white grape pulp sample

The results of the analyzed samples were summarized in figure 5.

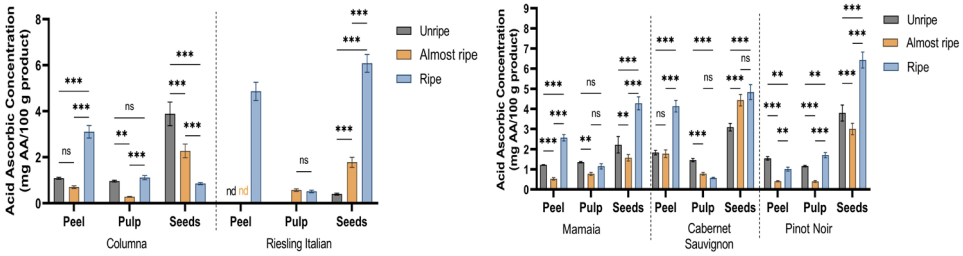


Figure 5. The acid ascorbic content of grapes on three different dates of ripening period: (a) White grapes (Columna, Riesling Italian). (b) Red grapes (Mamaia, Cabernet Sauvignon, Pinot Noir).
Bars show mean ± standard deviation (n = 3 replicates, nd: not detected). Pairwise differences among ripening stages within *Vitis Vinifera* varieties × vegetal material were tested by two-way ANOVA with Tukey post-hoc test (ns: $p \geq 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

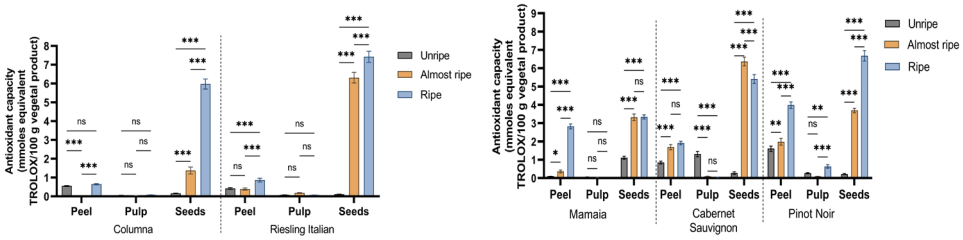
The highest content of ascorbic acid was found in seeds from most of the varieties (white and red grapes) while the lowest content was found in the pulp (between 0 and 4.13 mgAA/100g) of the grapes samples. The peel contains amounts of ascorbic acid ranging from 0 to 4.86 mgAA/100g whereas in the seeds the concentration reaches up to 6.43 mgAA/100g product. The results obtained in ripe grapes for vitamin C are comparable to those obtained by High Performance Liquid Chromatography/Electrospray Ionization-Mass Spectrometry [46].

Statistical analysis (Figure 5) confirmed that these differences were significant for most comparisons across ripening stages ($p < 0.05$), particularly in seeds and peel, while in pulp the variation with maturity was generally not significant.

The antioxidants capacities (mmoles equivalent TROLOX/100 g vegetal product) of white and red grapes on three different dates of the ripening period from Murfatlar vineyard were presented in figure 6 and were estimated using the Chemiluminescence method in lipid samples (ACL).

The antioxidant capacity of grape samples varied both with the type of vegetal material and the ripening stage. In white grape cultivars (Figure 6 (a)), the highest antioxidant capacity was consistently recorded in the seeds, reaching values above 6 mmol Trolox equivalents/100 g product in the ripe stage, whereas the peel and pulp exhibited markedly lower activities, often

below 2 mmol Trolox equivalents/100 g. A similar trend was observed in red grape cultivars (Figure 6 (b)), where the seeds showed the most pronounced antioxidant capacity, with significant increases during ripening ($p < 0.001$), while the pulp remained the poorest source of antioxidants across all stages.



(a) **(b)**

Figure 6. The antioxidant capacity of grapes at three different dates of ripening period: (a) White grapes (Columna, Riesling Italian). (b) Red grapes (Mamaia, Cabernet Sauvignon, Pinot Noir).
Bars show mean ± standard deviation (n = 3 replicates, nd: not detected). Pairwise differences among ripening stages within *Vitis Vinifera* varieties × vegetal material were tested by two-way ANOVA with Tukey post-hoc test (ns: $p \geq 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

It was found that the antioxidant activity of grapes not only depends on the content of ascorbic acid, but also on other bioactive compounds. In the analysed samples, the antioxidant capacity was generally higher in those with increased ascorbic acid content, both in white and red grape varieties. Therefore, a significant correlation between the ascorbic acid concentration and the antioxidant capacity was observed in peel, pulp, and seeds during ripening (Figures 7, 8), which is in agreement with the findings of Aldina Kesic et al. [33].

In white grapes, the correlation between ascorbic acid content and antioxidant capacity is consistently strong throughout berry development, although the direction of association varied depending on the vegetal material. In the unripe stage, highly significant and very strong positive correlations were observed across all vegetal materials, with *r* values ranging between 0.955 and 0.959. At the almost ripe stage, ascorbic acid showed a very strong positive correlation with antioxidant capacity in the pulp ($r = 0.960$), a very strong negative correlation in the peel ($r = -0.989$), and a weak, non-significant association in the seeds ($r = -0.338$). In the ripe stage, ascorbic acid was very strongly and positively correlated with antioxidant capacity in the peel and seeds ($r = 0.924 - 0.983$), whereas in the pulp it was very strongly and negatively correlated ($r = -0.977$).

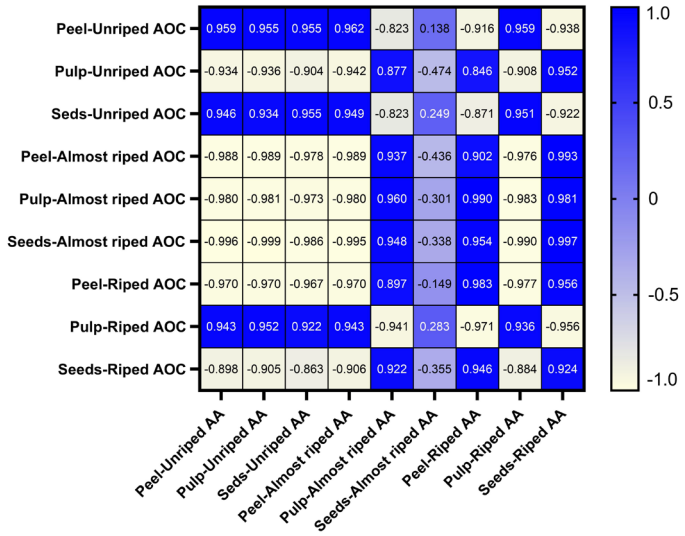


Figure 7. Heatmap for correlation between between the acid ascorbic acid content and antioxidant capacity in the peel, pulp and seed of ripe grape for white grapes. Blue indicates strong positive correlations (r = 1), while yellow signifies strong negative correlations (r = -1). |r| > 0.811 statistically significant at $p < 0.05$.

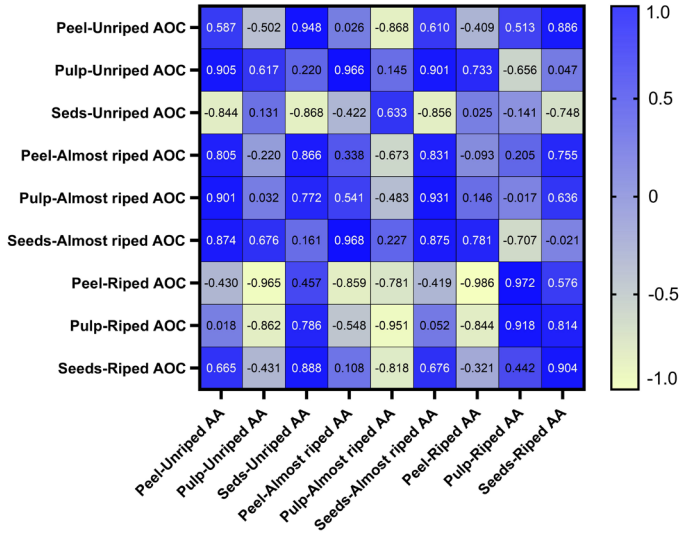


Figure 8. Heatmap for correlation between between the acid ascorbic acid content and antioxidant capacity in the peel, pulp and seed of ripe grape for red grapes. Blue indicates strong positive correlations (r = 1), while yellow signifies strong negative correlations (r = -1). |r| > 0.602 statistically significant at $p < 0.05$.

In red grapes, the correlation between ascorbic acid content and antioxidant capacity varied depending on the vegetal material and the ripening stage. In the unripe stage, the correlation was weak and not statistically significant in the peel and pulp (r = 0.587- 0.617), whereas in the seeds it was very strong and highly significant, but negative (r = -0.868). At the almost ripe stage, ascorbic acid showed weak and non-significant correlations with antioxidant capacity in the peel (r = 0.338) and pulp (r = -0.483), while in the seeds it was very strongly and positively correlated (r = 0.875). In the ripe stage, ascorbic acid was very strongly and negatively correlated with antioxidant capacity in the peel (r = -0.986), whereas in the pulp and seeds it was very strongly and positively correlated (r = 0.904 - 0.918).

It can be observed that the ascorbic acid concentration and the antioxidant capacity in grapes increased during the ripening process. But Tavarini et al (2008) reported that the accumulation of AA during ripening depends on the type of fruit and that when fruits become overripe, the ascorbic acid content declines with the degradation of fruit tissues. They found that the AA content increased with ripening in apricot, peach and papaya, but decreased in apple and mango [16]. Similarly, our results showed an increase in ascorbic acid content with grape ripening.

According to literature data, the antioxidant capacity is generally higher in seeds compared to the peel of grapes. Lachman et al. analyzed grapes from five vineyards in the Czech Republic and found average antioxidant capacity values of 6.27% in peel and 27.21% in seeds, based on 25 samples [48]. This aligns with our findings, where seeds consistently exhibited higher antioxidant activity than peel, showing that seeds represent the major contributors to the antioxidant potential of grapes.

CONCLUSIONS

The parameters of HPLC-DAD method for the determination of ascorbic acid in samples of grapes were developed for linearity, precision and for low detection limits values, as well. The method provides a rapid and sensitive means of determining ascorbic acid in grape samples from Murfatlar vineyard. The method described is very simple, involves little sample preparation, small amounts of sample and reagents and is fast since the total analysis time does not exceed 10 minutes. The results obtained using the HPLC-DAD method are comparable to the results obtained by other researchers.

The ascorbic acid and the antioxidant capacity of grapes depend on the varieties of grapes and on the harvesting time. It can observe that the ascorbic acid concentration and antioxidant capacity in grapes increased with ripening grapes. A significant correlation between the ascorbic acid concentration and the antioxidant capacity was observed in peel, pulp, and seeds during ripening.

EXPERIMENTAL SECTION

Materials

Standard of ascorbic acid was purchased from Merck (Darmstadt, Germany). HPLC-grade methanol, acetic acid, metaphosphoric acid and trisodium phosphate were purchased from Fluka (Buchs, Switzerland). All chemicals were of analytical grade (purity > 98%) and were used without further purification. 6-hydroxy-2,5,7,8-tetramethylchroman-2–carboxylic acid, as standard substance TROLOX, was purchased from Merck.

All the solutions were made up of ultrapure water from a Milli-Q Elix3 system. Solvents were filtered through 0.2-μm membranes (Millipore, Bedford, MA, USA) and degassed before use.

Standard solution

The stock solution of ascorbic acid was prepared at a concentration of 1mg/mL by weighing accurately the appropriate mass using a Metler Toledo analytical balance (±0.0001 g accuracy). The stock solution was diluted with acidified water for the preparation of working solutions in a concentration range from 0.5 to 15 μg/mL ascorbic acid that were stored in the dark at a temperature of 4^0 C. Standard substance TROLOX, a derivative of vitamin E, is Hoffman-LaRoche's trade name for 6-hydroxy-2,5,7,8-tetramethylchroman-2–carboxylic acid and was used for the antioxidant capacity. This standard solution was diluted to prepare plot calibration curve for 6 concentrations (0.5, 1, 1.5, 2, 2.5 and 3 nmols TROLOX).

SAMPLES PREPARATION

Samples

The methods were applied to the analysis of real samples including five grape varieties from Murfatlar vineyard (Murfatlar town, Constanta country) collected during the ripening period. Three red grape varieties: Pinot Noir, Cabernet Sauvignon and Mamaia and two white grape varieties: Columna, Riesling Italian were chosen for analysis. The grapes were carefully separated into peel, pulp and seeds; they peeled them with the help of a scalpel.

Acid Ascorbic determination

For the chromatographic determinations, extraction was performed on the day of analysis, by homogenizing (using a Bosh blender) three fruit portions of 5 g each. Thus, three biological replicates were obtained. The homogenized material (peel, pulp or seeds) was dissolved in 25 mL of metaphosphoric acid 20 g/L in a volumetric flask. Samples were adjusted to pH 2.5 to 2.8 with trisodium phosphate 200g/L. The homogenate was sonicated by a Bransonics cleaner and then centrifuged at 4 ^{0}C and 5000 rpm for 10 min, using a Hettich Universal 320 centrifuge (Andreas Hettich GmbH & Co. KG). The supernatant was filtered through 0.45-µm filters (Chromafil Xtra PFTE-45/25 particle size of 0.45 mm), The samples were kept in dark colored vials and analyzed immediately after extraction.

Antioxidant capacity determination

For the antioxidant capacity, 5 g of homogenized dried material (peel, pulp or seeds) was left in contact with 50 mL methanol 98% in brown recipients in the dark in order to obtain the extracts. The mixture was strongly shaken three times every day. After seven days of contact time 5 mL alcoholic extract, previously filtered, were collected in glass recipients and stored at 4 ^{0}C. 5 µL, 10 µL or 20 µL of samples were analyzed after extraction. For determination each extract was diluted with ethanol.

INSTRUMENTATION

HPLC-DAD analysis

The identification and quantification of ascorbic acid was carried out using an HPLC Agilent 1200 system with the following components: quaternary pomp, DAD, thermostat, degassing system, autosampler. The autosampler temperature was set to 4 ^{0}C. The separation of the analyte was carried out on a Zorbax XDB C18, 250 mm × 4.6 mm; 5 µm column (Phenomenex, SUA). The mobile phase consisted of (A – 95 %) 0.1% acetic acid in HPLC-grade water and (B – 5 %) methanol, eluted in isocratic conditions. The injected volume sample was 20 µL at a flow rate of 0.25 mL min^{-1}. Detection using a DAD was performed at a specific wavelength of 245 nm for ascorbic acid [44]. The analysis time was 10 minutes. The results were obtained by comparison with the standard. The results were the mean values from three replicates of the same sample.

Antioxidant capacity analysis

The total antioxidant activity of grapes aqua-alcoholic solution was estimated using the Chemiluminescence method in lipid samples (ACL). This was performed according to Analytic Jena procedure, Germany, using PHOTOCHEM coupled to PC apparatus [28]. The extern source of light was represented by a Hg lamp carried with phosphorus, with highest energy at the wavelength λ= 351 nm and the free radicals source was H_2O_2. The antioxidant activity was calculated using the calibration curve and was expressed as Trolox equivalents. This reaction takes place in the presence of a light quant registered by the detector (photomultiplicator) [40-42]. The results were expressed in units of TROLOX (mmols).

Statistical analysis

All experiments were carried out in triplicate, and the results are expressed as mean ± standard deviation (SD). Prior to inferential analyses, the normality of data distribution was assessed using the Shapiro–Wilk test. The statistical analysis was conducted with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences between samples were evaluated by two-way analysis of variance (ANOVA), followed by Tukey post hoc test, with statistical significance set at $p < 0.05$. In addition, the Pearson correlation (r) analysis was performed to assess the relationship between the ascorbic acid content and the antioxidant capacity across different ripening stages.

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