

Article

Changes in Bioactive Compounds, Antioxidant Activity and Nutritional Quality of Blood Orange Cultivars at Different Storage Temperatures

Fariborz Habibi^{1,2}, Asghar Ramezani¹, Fabián Guillén², Salvador Castillo², María Serrano³ and Daniel Valero^{2,*}

¹ Department of Horticultural Science, School of Agriculture, Shiraz University, Shiraz, Iran.; fariborz_h659@yahoo.com (F.H.); ramezani@shirazu.ac.ir (A.R.)

² Department of Food Technology, University Miguel Hernández. Ctra. Beniel km. 3.2, 03312, Orihuela, Alicante, Spain; fabian.guillen@umh.es (F.G.); scastillo@umh.es (S.C.)

³ Department of Applied Biology, University Miguel Hernández. Ctra. Beniel km. 3.2, 03312, Orihuela, Alicante, Spain; m.serrano@umh.es (M.S.)

* Correspondence: daniel.valero@umh.es; Tel.: +34-96-674-9743 (D.V.)

Abstract: The changes in nutritional quality, bioactive compounds and antioxidant enzymes in the juice of four blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored during 6 months at 2 and 5 °C plus 2 days at 20 °C for shelf life were studied. Sucrose was the sugar found at higher concentration and decreased during storage for all cultivars, as did glucose and fructose. Organic acids decreased at both temperatures and the highest content was found in 'Sanguinello', especially the major (citric acid) and ascorbic acid. Total phenolics content (TPC), total anthocyanins (TAC), and the individual (cyanidin 3-glucoside and cyanidin 3-(6"-malonylglucoside)) increased for all cultivars, the 'Sanguinello' having the higher concentrations. Antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were higher also in 'Sanguinello' and increased during storage. Overall, these results together with the sensory analysis suggest that 'Sanguinello' would be the best cultivar for prolonged storage.

Keywords: organic acids; sugars; anthocyanins; antioxidant enzymes; ascorbic acid

1. Introduction

Blood oranges (*Citrus sinensis* L. Osbeck) are one of four groups within the sweet orange species. The main different of blood orange fruit with other oranges is the synthesis of anthocyanins pigment in the flesh and sometimes in the peel [1]. The most important commercial cultivars of blood oranges are 'Moro', 'Tarocco', 'Sanguinello' and 'Sanguine' [2] and are the result of a spontaneous bud mutation [3]. Blood oranges are consumed as fresh fruit due to bioactive compounds such as anthocyanins, ascorbic acid, hydroxycinnamic acids and flavonoids [4]. Anthocyanins content has been considered an important quality component due to their attributed antioxidant activity with impact in some diseases including anti-inflammatory, anticancer, and antidiabetic [5-9]. In blood oranges, 10 anthocyanins have been identified [6, 10] although the major anthocyanins in the flesh are cyanidin 3-glucoside and cyanidin 3-(6"-malonylglucoside) [1, 11]. Free anthocyanins are synthesized via the flavonoid pathway, and occur at the cytoplasmic face of the endoplasmic reticulum and further transported to the vacuole in order to preventing the oxidation and to act as pigment [12].

Blood orange cultivars exhibit different levels of pigmentation under similar growing conditions. For example, 'Moro' has the highest pigmentation followed by 'Sanguinello' and 'Tarocco', and therefore, genetic background can be considered as main factor for range of anthocyanin levels [12]. Besides cultivar type, some factors including cultural practices, soil characteristics, region of cultivation, climate condition, environmental condition, physiological factors, maturity stage, and harvesting time can affect anthocyanin accumulation in blood oranges [3, 12]. In addition, blood oranges need a wide day/night temperature range for obtaining high anthocyanin concentration in the flesh [13], and in subtropical or tropical climates the commercial production is limited due to very low or lack of anthocyanin concentration at commercial maturity [11].

Blood oranges are exceptional fruit since, unlike the non-climacteric fruit, they can increase the internal quality after harvest by synthesis of anthocyanins during cold storage [1]. It has been reported that cold temperature below 6 °C can induce anthocyanin synthesis in blood oranges fruit during storage due to the activation of enzymes involved in the phenylpropanoid pathway including phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR), and UDP-glucose flavonoid glucosyl transferase (UFGT) [14]. Therefore, cold storage can be used as a simple technology for enhancing anthocyanin accumulation in blood oranges that are poorly pigmented in aforesaid climates [15].

Since blood orange cultivars have distinct pomological, physiological and biochemical characteristics, consequently, it is possible to find different storability and postharvest physiological behaviour. Several researches have shown a relationship between anthocyanin synthesis in blood oranges and low moderate temperature of storage [11, 15, 16, 17]. However, information about prolonged storage of blood orange cultivars stored at temperatures below 6 °C has been poorly afforded. Additionally, changes in bioactive compounds, antioxidant activity and nutritional quality of blood orange can give very useful information for finding the best storage period and preservation with the highest quality of each cultivar for the market. Therefore, the objective of this study was to compare the behaviour of four blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored at 2 or 5 °C during 180 days by establishing (1) the optimal storage period to maintain the higher content of bioactive compounds (total phenolics, total anthocyanins and ascorbic acid), (2) the efficiency of the temperatures for enhancing the major individual anthocyanins concentration (3) the sugars and organic acids of each cultivar to cold temperatures, and (4) the maximum storability of the cultivars at the two temperatures.

2. Materials and Methods

2.1. Plant material and storage conditions

Cultivars of blood oranges, 'Moro', 'Tarocco', 'Sanguinello' and 'Sanguine' were harvested from commercial citrus orchard of Dashtenaz company in Sari (36.5659° N, 53.0586° E), Mazandaran province, Iran, at mid-January 2018 and immediately transported to the postharvest laboratory. In this area of Iran, the climate is subtropical and the 4 cultivars reach commercial maturity in January and were harvested at the same time and according to TSS/TA ratio. Trees were seven years-old and grafted on 'C-35' citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) rootstock. All trees grown under same conditions and cultural practices. Fruit were selected based on uniformity of size and checked for no defects or rind injuries for each cultivar and disinfected with 2 % NaOCl solution and rinsed with distilled water. Fruit were divided into sets of three replicates of five fruit for each

replicate and placed in polyethylene bag containing 16 holes, and stored 180 days at 2 or 5 °C and 90 % relative humidity (RH). Parameters were measured after 0, 30, 60, 90, 120, 150 and 180 days of cold storage plus 2 days at 20 °C (shelf life). For each cultivar, sampling date and replicate, the juice from the 5 fruits was squeezed and half used for total phenolics, total anthocyanins, and antioxidant activity determinations, and the other half was freeze-dried (FD-5003-BT, Tehran, Iran) for individual sugars and organic acids, individual anthocyanins and antioxidant enzyme activities measurements.

2.2. Bioactive compounds and antioxidant activity

Total anthocyanin concentration (TAC) was measured based on pH differential method [15]. Fruit juice were diluted (1:4 dilution) with potassium chloride (KCl) buffer as pH 1.0 and sodium acetate (C₂H₃NaO₂) buffer as pH 4.5. Absorbance measured at 510 and 700 nm with a microplate spectrophotometer (Epoch, USA) and TAC was reported as mg L⁻¹. For measurement of total phenolic content (TPC), 32 µL of fruit juice was mixed with 900 µl of 2% sodium carbonate, and after 3 min, 180 µL of 50% Folin-Ciocalteu was added and placed for 30 min in a dark place. Different concentrations of gallic acid used for preparation of standard curve. Samples and standard were measured at 750 nm and results reported as mg gallic acid equivalents (GAE) L⁻¹ [18].

Total antioxidant activity (TAA) was measured by adding 100 µl of juice to 1 mL 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.1 mM) and 1 mL Tris-HCl (pH=7.5) buffer and maintained in darkness for 30 minutes. Absorbance was measured at 517 nm and TAA reported as percentage with the following equation [19]:

$$\text{TAA (\%)} = \left[1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right] \times 100$$

2.4. High performance liquid chromatography (HPLC) analyses

2.4.1. Individual anthocyanin

For individual anthocyanins, 0.5 g of lyophilized flesh were grounded and mixed with 10 mL of methanol/formic acid/water (79:1:20, v/v/v), centrifuged at 10500 ×g for 10 min and the supernatant was filtered through a 0.45 µm syringe filter and 20 µL injected to HPLC. HPLC was equipped with a Luna C18 column (25 cm × 0.46 cm i.d., 5 µm particle size; Phenomenex, Macclesfield, UK) with a C18 security guard (4.0 × 3.0 mm) cartridge system (Phenomenex, Macclesfield, UK). Mobile phases were formic acid (99:1, v/v) (phase A) and acetonitrile (phase B), with a flow rate of 1 mL min⁻¹. The gradient started with 8% of solvent B, reaching 15% at 25 min, 22% at 55 min, and 40% at 60 min, which was maintained up to 60 min. Anthocyanins (cyanidin 3-glucoside and cyanidin 3-(6"-malonylglucoside)) were detected at 520 nm and quantified by known standards and were expressed as mg L⁻¹ [20].

2.4.2. Individual sugars and organic acids

In order to quantify organic acids and sugars, 0.5 g of lyophilized blood orange flesh were grounded and mixed with 10 mL of 50 mmol L⁻¹ phosphate buffer (pH=7.8). Mixtures were centrifuged at 10500 ×g for 10 min and one mL of the extract was filtered through a 0.45 µm syringe filter and 10 µL was injected to HPLC (Hewlett-Packard HPLC Series 1100). The elution system consisted of 0.1% phosphoric acid running isocratically with a flow rate of 0.5 ml min⁻¹. Organic acids were detected by absorbance at 210 nm and sugars by refractive index detector. Individual organic acids (citric acid, ascorbic acid, malic acid, oxalic acid and succinic acid) were quantified by

standard curve of pure organic acids and reported as mg 100 g⁻¹ with the exception of citric acid that was g 100 g⁻¹. Standard curves of pure sugars were used for quantification of individual sugars (sucrose, glucose and fructose) and expressed as g 100 g⁻¹ [20]. Sugars and organic acids standards were purchased from Sigma-Aldrich (Sigma-Aldrich, Madrid, Spain).

2.5. Enzyme activities assay

The protocol for enzyme activities was the same that for flavedo tissue (21). In brief, enzyme activities of flesh were evaluated spectrophotometrically. Catalase (CAT) and peroxidase (POD) activities were measured at 240 and 470 nm, respectively [22]. Ascorbate peroxidase (APX) activity was assessed at 290 nm [23]. Superoxide dismutase (SOD) activity was determined at 560 nm [24]. Phenylalanine ammonia-lyase (PAL) activity was determined by evaluation of the absorbance of trans-cinnamic acid at 290 nm [25]. Polyphenol oxidase (PPO) activity was measured at 425 nm [26]. Total protein content of enzymes extract was measured according to the Bradford method [27]. Results for all specific enzyme activities were expressed as U mg⁻¹ protein.

2.6. Sensory quality evaluation

Descriptive sensory analysis tests of blood orange cultivars were evaluated by ten trained panellists (five men and five women) for market acceptability. In this case, fruit was peeled with knife and segments separated with hand, then placed in glass dishes with three-digit code. Each combination of segments prepared from five fruit of three replicates from each cultivar. Three scattered segments of fruit were tasted by panellists. Panellists evaluated sensory acceptability of fruit as edible quality by 9-point scale from 1 as the lowest edible quality and 9 as the best edible quality according to quantitative descriptive sensory analysis [28].

2.7. Statistical analysis

The experiment was conducted according to a completely randomized design (CRD) with three replicates. Data were analysed using three-factors (cultivar, temperature and storage time) analysis of variance (ANOVA). Data analyses were performed with SAS software package v. 9.4 for Windows. Mean comparisons were done by least significant difference (LSD) test ($P < 0.05$) with standard errors (SE) of means. Linear regressions were performed with SigmaPlot software v. 11.

3. Results

3.1. Sugars and organic acids

Individual sugars (sucrose, glucose and fructose) were affected in all cultivars during storage at both temperatures (Figure 1). Sucrose, glucose and fructose at 5 °C were 6.73%, 9.54% and 9.63% higher than 2 °C, respectively. The highest sucrose concentration was found in 'Moro' and 'Tarocco' cultivars at both temperatures, and lowest concentration of sucrose, glucose and fructose were observed in 'Sanguinello'. Sucrose increased in all cultivars and then decreased to the end of storage at both temperatures. With respect to glucose and fructose, these sugars diminished in all cultivars, especially at 2 °C. 'Sanguinello' showed the lowest levels of both sugars at the end of storage either at 2 or 5 °C.

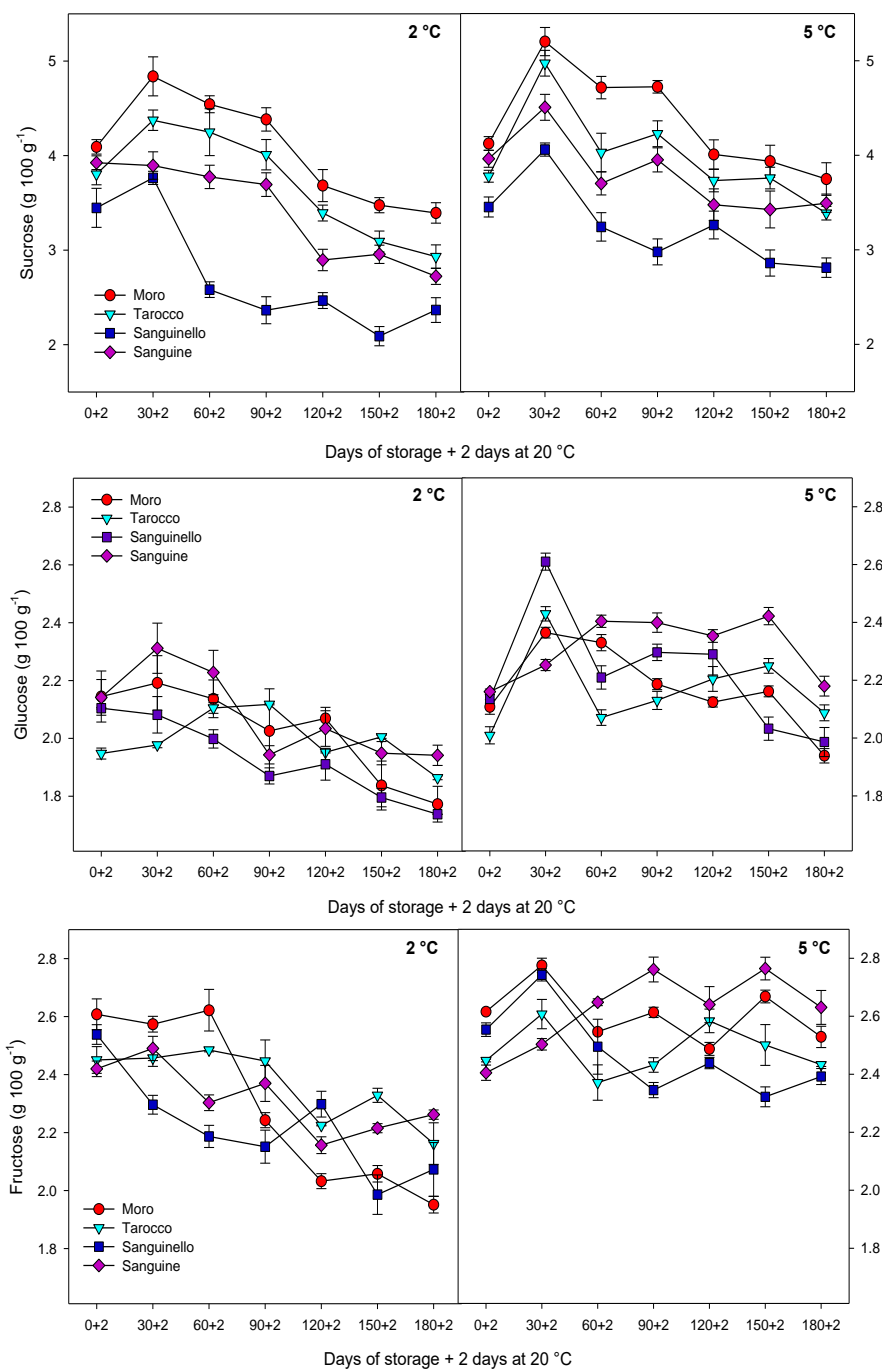


Figure 1. Changes in individual sugars sucrose, glucose and fructose of blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored at 2 or 5 °C during 180 days plus 2 days at 20 °C. Vertical bars represent \pm standard error (SE) of means. LSD ($p < 0.05$) values are 0.41, 0.21, and 0.37, for sucrose, glucose, fructose, respectively.

Individual organic acids (citric acid, malic acid, succinic acid and oxalic acid) of blood orange cultivars significantly were affected during storage at both temperatures. The major organic acid was citric acid followed by malic and succinic acids and lowest was oxalic acid (Figure 2).

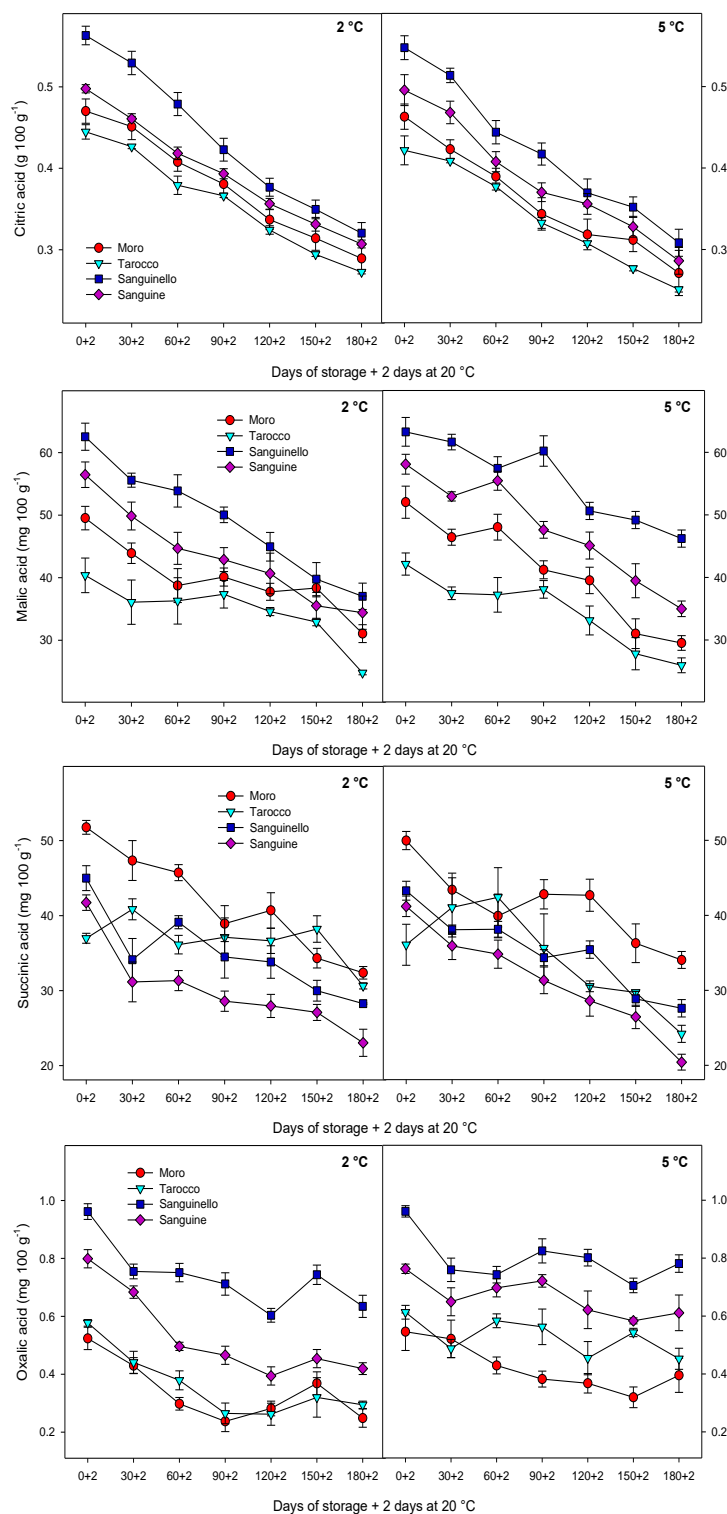


Figure 2. Changes in individual organic acids: citric, malic, succinic acid oxalic acids of blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored at 2 or 5 °C during 180 days plus 2 days at 20 °C. Vertical bars represent \pm standard error (SE) of means. LSD ($p < 0.05$) values 0.05, 4.11, 5.98 and 0.09, for citric, malic, succinic acid oxalic acids, respectively.

3.2. Bioactive compounds and antioxidant activity

Total anthocyanin concentration (TAC), and the major individual anthocyanins are shown in Figure 3.

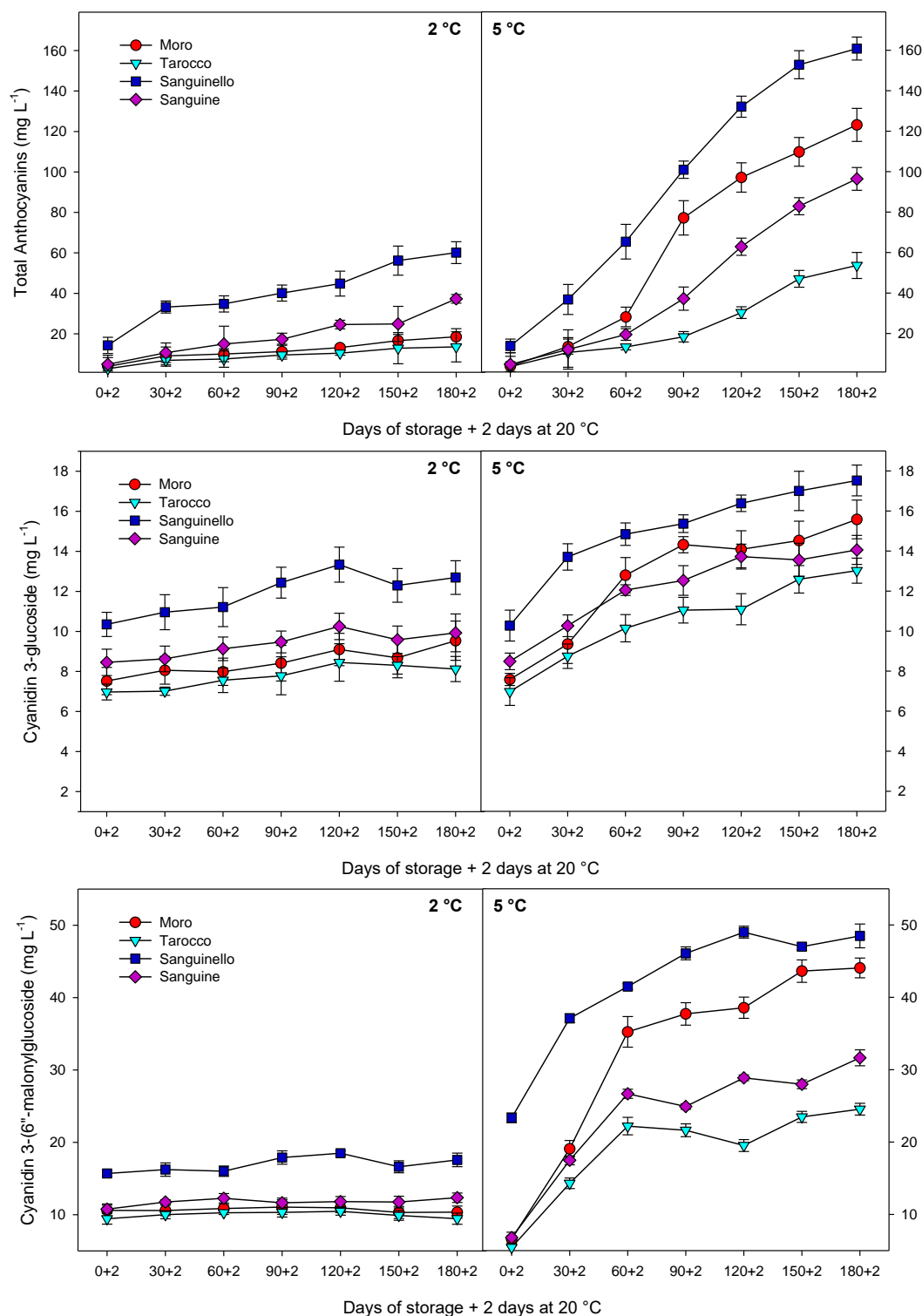


Figure 3. Changes in total anthocyanin concentration, cyanidin-3-glycoside and cyanidin 3-(6''-malonylglucoside) of blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored at 2 or 5 °C during 180 days plus 2 days at 20 °C. Vertical bars represent \pm standard error (SE) of means. LSD ($p < 0.05$) values are 3.2, 1.7, and 2.4, for total anthocyanin concentration, cyanidin-3-glycoside, and cyanidin 3-(6''-malonylglucoside), respectively.

For all cultivars a significant increase in TAC was observed during cold storage at both temperatures, the increase being enhanced at 5 °C (65% higher). Among cultivars, 'Sanguinello' reached the highest TAC (160.8 ± 5.6 mg L⁻¹) while 'Tarocco' showed the lowest (53.6 ± 6.4 mg L⁻¹) at

the end of storage at 5 °C. Two major individual anthocyanins were detected by HPLC-DAD for all cultivars: cyanidin 3-glucoside and cyanidin 3-(6''-malonylglucoside), which was found at higher concentration. For both, and similarly to TAC, levels were higher at 5 than at 2 °C and 'Sanguinello' reached the maxima contents for both cyanidin 3-glucoside and cyanidin 3-(6''-malonylglucoside) while 'Tarocco' had the lowest levels of these anthocyanins.

Total phenolic content (TPC) was affected by cultivars, storage and temperatures (Figure 4).

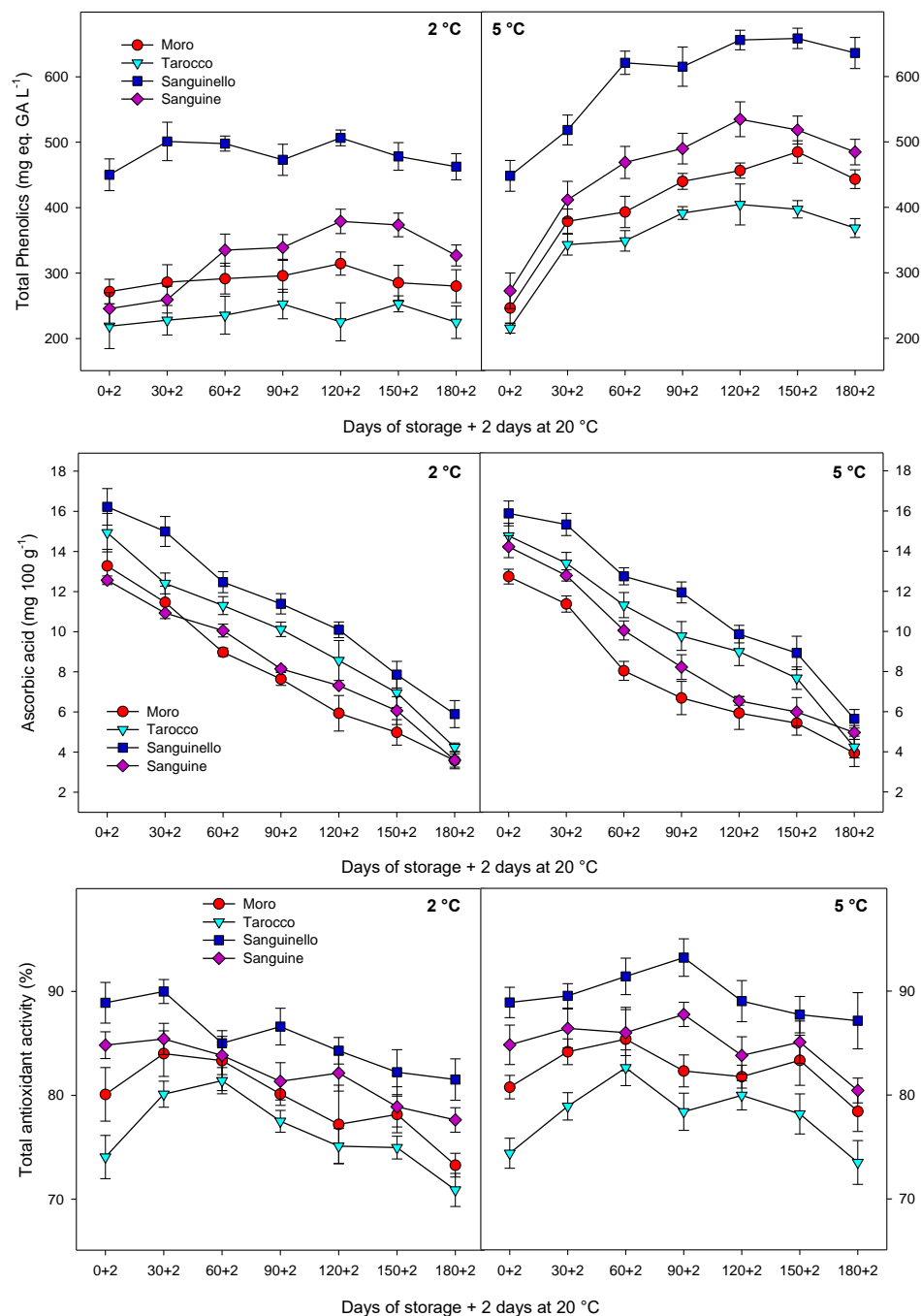


Figure 4. Changes in total phenolics, ascorbic acid and total antioxidant activity of blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored at 2 or 5 °C during 180 days plus 2 days at 20 °C. Vertical bars represent \pm standard error (SE) of means. LSD ($p < 0.05$) values are 31.55, 0.44 and 4.29, for total phenolics, ascorbic acid and total antioxidant activity, respectively.

TPC increased for all cultivars and then remained constant or decreased to the end of storage at both temperatures, the increase being enhanced at 5 °C (27% higher). Among cultivars, the highest

and the lowest TPC were observed for 'Sanguinello' and 'Tarocco' with concentrations of (636 ± 24) and 368 ± 14 mg eq, gallic acid L⁻¹, respectively at the end of storage.

Ascorbic acid content at harvest was different among cultivars, with 'Sanguinello' and 'Moro' having the highest and lowest concentrations, respectively (Figure 4). For all, ascorbic acid concentration was significantly reduced during storage at both 2 and 5 °C, although the final levels were higher in 'Sanguinello' at 2 (5.89 ± 0.68 mg 100 g⁻¹) and 5 °C (5.64 ± 0.46 mg 100 g⁻¹), respectively.

Total antioxidant activity (TAA) increased for all cultivars and then decreased to the end of storage at both temperatures (Figure 4), although TAA at 5 °C was higher than 2 °C. In addition, the reduction of TAA at 2 °C was greater than 5 °C during storage. The highest TAA was observed in 'Sanguinello' throughout storage at both temperatures and the lowest TAA was shown in 'Tarocco'.

3.3. Enzyme activities

Activities of antioxidant enzymes (CAT, APX and SOD) in the flesh were affected by cultivars, storage times and temperatures (Figure 5), although POD was not found in any cultivars. For all, the activity was higher at 5 than at 2 °C (12, 10 and 23 % higher for CAT, APX and SOD, respectively).

Activities of antioxidant enzymes increased up to 30 or 60 days and then decreased to the end of storage at both temperatures, this reduction being greater at 2 than 5 °C. Among cultivars, 'Sanguinello' had the highest level of antioxidant enzyme activities at both temperatures, while 'Moro' and 'Tarocco' showed the lower antioxidant enzyme activities at both storage conditions.

PAL activity in flesh of cultivars was affected at both temperatures during storage (Figure 6). PAL activity increased up to 120 days in all cultivars at 5 °C and then decreased to the end of storage. Among cultivars, 'Sanguinello' showed the highest PAL activity and 'Tarocco' the lowest during storage at both temperatures, although PAL activity at 5 °C was 14 % higher than at 2 °C. PPO activity increased during storage at both temperatures, although the activity was very low for all cultivars during cold storage (Figure 6). 'Moro' had the highest PPO activity during cold storage, especially at the lowest temperature.

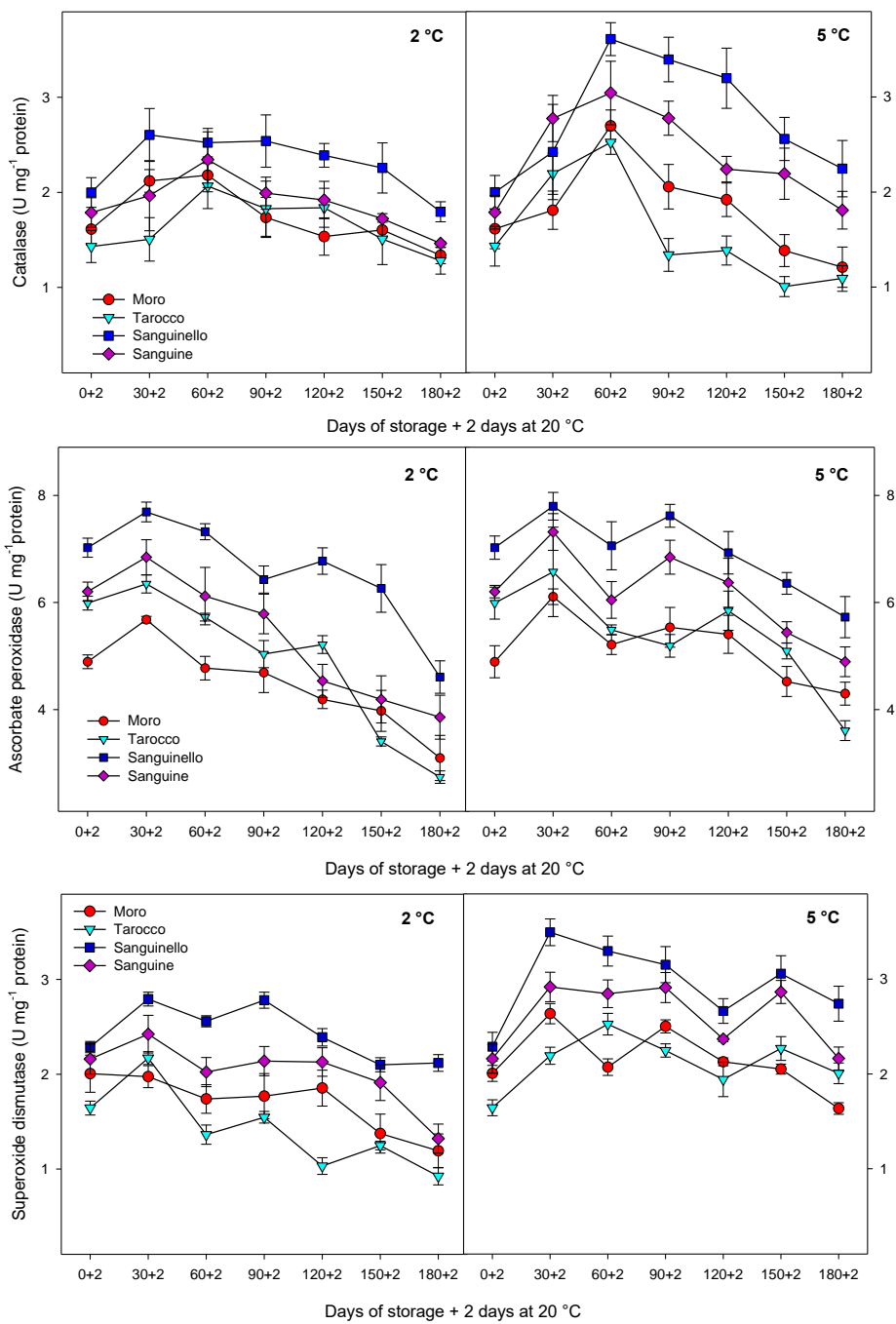


Figure 5. Changes in catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) enzyme activities of blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored at 2 or 5 °C during 180 days plus 2 days at 20 °C. Vertical bars represent \pm standard error (SE) of means. LSD ($p < 0.05$) values are 0.69, 1.04 and 0.30, for CAT, APX, SOD, respectively.

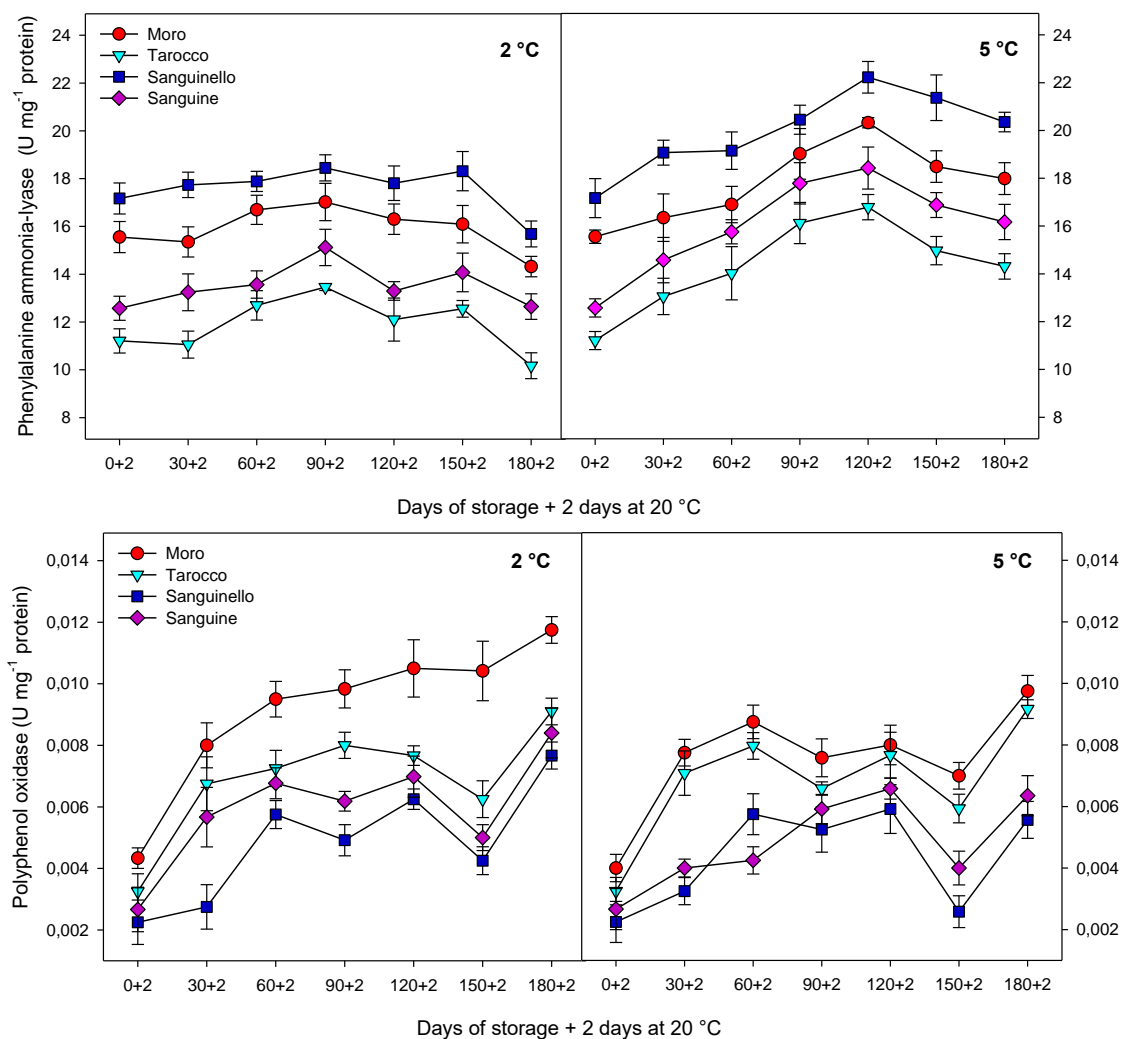


Figure 6. Changes in phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) enzyme activities of blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored at 2 or 5 °C during 180 days plus 2 days at 20 °C. Vertical bars represent \pm standard error (SE) of means. LSD ($p < 0.05$) values are 1.91 and 0.005, for PAL and PPO, respectively.

3.4. Sensory quality

Edible quality of blood orange cultivars was analysed during storage at both temperatures. The highest edible quality was scored immediately after harvest (9 points for both 2 and 5 °C). For all cultivars, edible quality was always higher at 5 than at 2 °C although it decreased at both temperatures. At the end of storage, 'Moro' and 'Tarocco' showed the lower scores at 2 °C (3.1 ± 0.12 and 2.1 ± 0.15 , respectively). 'Sanguinello' was evaluated as the best cultivar at 5 °C, with scores of 6.3 ± 0.18 compared with those obtained at 2 °C (4.2 ± 0.15).

4. Discussion

Information about changes in nutritional, bioactive compounds and antioxidant activity of blood orange cultivars can provide a comprehensive insight for assessment the best storage period as a result of maintaining the highest fruit quality during long-term cold storage. In this paper, a comparative study of 4 blood orange cultivars ('Moro', 'Tarocco', 'Sanguinello' and 'Sanguine') stored for 180 days at 2 or 5 °C was carried out.

Citrus fruit after harvest and during cold storage needs to provide energy by catabolism of organic acids concomitant sugars as substrates for maintaining cellular metabolism. Accumulation and degradation of sugars and organic acids occur through glycolysis and Krebs cycle. On the other hand, the reduction of organic acids and sugars in the flesh may be affected by biosynthesis and catabolism [29]. Therefore, different cellular metabolism and catabolism of organic acids and sugars concentrations in citrus fruit is crucial to assessment citrus fruit postharvest life at prolonged storage. In this study, the major sugars of blood orange cultivars were sucrose, glucose, and fructose, which levels at harvest were different depending on cultivar, but for all the concentration of sugars were reduced during storage at both 2 and 5 °C. Differences on initial individual sugars concentration among cultivars was probably due to different activity of sucrose synthase and sucrose phosphate synthase or invertases [30]. In addition, sucrose metabolism depends on β -fructosidase and α -glucosidase activities which lead to the formation of fructose and glucose, respectively [31]. Furthermore, individual sugars increased at initial sampling time and then decreased at both temperatures. The reduction of sucrose, glucose, and fructose was probably due to senescence or sugars consumption in respiratory process for ATP production during long-term storage [1]. Another mechanism for sugars reduction is carbohydrate transport from flesh to pericarp or carbohydrate redistribution that might be occurred in pumelo cultivars [29]. These authors hypothesized that pericarp has a direct contact with the surrounding storage air and fruit carry out cellular respiration due to adequate oxygen supply and consumes substrates by aforementioned mechanism.

Besides sugars content, organic acids are important components of citrus fruit juice and their concentration depends on the fruit species and cultivars [31, 32]. In our study, the main organic acids were citric, malic, succinic and oxalic acids, which concentrations were different among blood orange cultivars, although for all decreased during cold storage at both temperatures. Citric and malic acids were the main organic acids in blood oranges, respectively, which concentrations being higher in 'Sanguinello' and lower in 'Tarocco'. The different organic acids content of cultivars, especially citric acid at initial sampling time, could be related to H⁺-ATPase pump on the vacuolar membrane which can provide a large influx of H⁺ within tonoplast. This proposed mechanism can accumulate additional citric acid in citrus fruit [31]. The reduction of organic acids during cold storage is probably due to their consumption as main substrates for energy production, providing carbon skeletons for the synthesis of phenolic compounds and also synthesis of sugars from organic acids [1]. In addition, reduction of organic acids could be related to fruit senescence after prolonged storage. In this study, reduction of organic acids at 2 was greater than 5 °C and might be related to alcoholic fermentation at lower temperature [13].

Among the citrus species, blood oranges accumulate anthocyanin pigments which are considered as quality index [1, 12]. Low temperature can stimulate the synthesis of anthocyanins in blood oranges during storage [16], mainly due to activation of the phenylpropanoid pathway [17].

In this study, all cultivars had a pale red colour at harvest except 'Sanguinello' and TAC increased during cold storage, the enhancement of TAC at 5 °C being significantly higher than at 2 °C. Accordingly, TAC increased in 'Moro', 'Tarocco', 'Sanguinello' and 'Sanguine' up to 31-fold, 14-fold, 11-fold and 20-fold, respectively at 5 °C, while at 2 °C remained almost unchanged. Anthocyanin synthesis in blood oranges during cold storage depends on the activation of the enzymes involved in phenylpropanoid metabolism. Therefore, activation of these enzymes can be induced by low temperatures. For example, the expression of structural genes involved in

phenylpropanoid biosynthesis pathway including PAL, chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR), and UDP-glucose flavonoid glucosyl transferase (UFGT) at 4 °C were higher than in 'Tarocco' cultivar stored at 25 °C, suggesting that low temperature strongly induced transcriptome of gene expression [14]. In addition, it has been reported that expression levels of most genes involved in the phenylpropanoid pathway were higher in blood orange than in blond one [11]. In addition, TAC rose a 500 % and a 19 % in 'Tarocco' and Moro', respectively, stored at 8 °C after 86 days in comparison with concentration at harvest [16]. Moreover, TAC in 'Tarocco' cultivar raised up 87 % at 4 °C after 70 days of storage in comparison with initial levels [17]. Therefore, anthocyanins can increase under moderate cold temperatures, as occurred at 5 °C for all cultivars, and especially for 'Sanguinello' which reached the maximum anthocyanins levels after 180 days plus 2 days at 20 °C, that is 11-fold increase. In this study, cyanidin 3-glucoside and cyanidin 3-(6"-malonylglucoside) were two main anthocyanins in the 4 blood orange cultivars, the latter being found at higher concentrations, and both showed the same trend as TAC during storage at both temperatures for all cultivars. Previous studies suggested that metabolic pathways involved in anthocyanins biosynthesis in blood oranges could have been partially inhibited at very low temperatures, such as below 3 °C [11]. A possible explanation could be that for all cultivars, PAL activity was higher at 5 than at 2 °C as did the anthocyanins content. In fact, a positive close relationship was found between PAL activity and anthocyanin accumulation ($R^2=0.65-0.79$). The anthocyanins content followed the order 'Sanguinello' > 'Moro' > 'Sanguine' > 'Tarocco', as did the PAL activity.

On the other hand, stability and degradation of the anthocyanin molecule depends on PPO and POD activities during cold storage [12]. However, in this study POD activity was not detected in the flesh of any cultivar. In addition, PPO activity was very low for all cultivars during storage and without significant differences between the two temperatures. This low activity was probably attributable to the acidic conditions due to the high content of organic acids of blood oranges fruit [33].

In this study, TPC increased at both temperatures and then remained constant or decreased to the end of storage, although concentrations were higher at 5 °C. As occurred with TAC, the changes of phenolic compounds could be attributed to PAL activity since a close relationship was found ($R^2=0.67-0.72$). Similarly, TAA increased for all cultivars and then decreased to the end of storage, the reduction being higher at 2 °C. However, ascorbic acid content was drastically reduced over storage for all cultivars and probably does not contribute to TAA in blood oranges. These results would confirm previous reports in which there was a positive correlation between TAC and TPC with TAA [15]. Cold temperatures can induce the accumulation of reactive oxygen species (ROS) and fruit use enzymatic and non-enzymatic antioxidant systems to scavenge the ROS generated at low temperatures [34]. Antioxidant systems including CAT, APX and SOD activities can scavenge ROS. In this study, antioxidant enzyme activities increased up to 30 or 60 days and then decreased to the end of storage at both temperatures. In addition, non-enzymatic antioxidants including ascorbic acid and phenolic compounds can act as another mechanism for scavenging ROS [35]. In this study, the reduction of ascorbic acid at 2 °C was probably due to overproduction of ROS and then ascorbic acid was used as an electron donor to neutralize ROS during storage [1].

Blood oranges are non-climacteric fruit with low metabolic activities, but biochemical changes can influence largely on fruit quality during long-term cold storage [1]. Citrus fruit taste depends on sugars and organic acids, which are reduced at higher rates compared with sugars and therefore

flavour changes after long-term storage [28]. In this study, cultivar and storage temperature affected the acceptability of blood oranges, and in many cases sub-tropical crops such as citrus fruit stored at lower than optimal temperatures induce undesirable changes [36]. Overall, panellists gave the higher scores to 'Sanguinello' which could be attributed to a better balance between sugars and acids than the other cultivars together with the enhancement of anthocyanins. Sugars are considered as the main soluble components in the flesh of citrus fruit and are responsible for sweetness of the juice. Since fructose and glucose are 80% and 60% sweeter than sucrose, respectively, therefore, fruit taste depends on the proportions of sucrose, glucose and fructose [35]. Sensory evaluation revealed that the lowest edible quality for all blood orange cultivars that 2 °C, which could be related to a fermentative metabolism that can produce some compounds that reduce the edible quality and occurrence of off-flavours [28]. For example, ethanol, acetaldehyde, furaneol and polyvinylguaiacol are related to the off-flavours in citrus fruit at cold temperature. In addition, changes in the volatile and non-volatile components at suboptimal temperature can produce non-volatile flavour compounds that had a negative effect on citrus fruit taste including putrescine and limonin as reported in sweet orange cultivars [37].

5. Conclusions

This is the first comparative study on the changes in bioactive compounds, antioxidant activity and nutritional quality of four blood orange cultivars at different storage temperatures. For all cultivars, the temperature of 2 °C could not be appropriated for long-term storage compared with 5 °C. At this temperature, bioactive compounds such as TAC, TPC and individual anthocyanins cyanidin 3-glucoside and cyanidin 3-(6"-malonylglucoside) were enhanced, which is consistent with the increased PAL activity. Among cultivars, 'Sanguinello' was the best one since quality parameters was better retained during the 6 months of storage. Based on these results, we propose a moderate cold temperature after harvest can increase bioactive compounds, especially anthocyanin and phenolic compounds, which are related to the human-health.

Author Contributions: Conceptualization, F.H. and D.V.; methodology, F.H.; software, F.H. and D.V.; validation, D.V., M.S., F.G., A.R. and S.C.; formal analysis, F.H.; investigation, F.H.; resources, D.V.; data curation, D.V., M.S., and F.G., A.R.; writing—original draft preparation, F.H.; writing—review and editing, D.V., M.S., and F.G.; visualization, D.V. and M.S.; supervision, D.V.; project administration, D.V.; funding acquisition, A.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: We would like to thanks to Shiraz University Research Council for financial support and University Miguel Hernández (UMH) for providing the sabbatical opportunity to Fariborz Habibi and members of postharvest group of fruit and vegetables for HPLC technical assistance. Also, we thank Dashtenaz company, for providing blood orange cultivars.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Habibi, F.; Ramezani, A.; Guillén, F.; Serrano, M.; Valero, D. Blood oranges maintain bioactive compounds and nutritional quality by postharvest treatments with γ -aminobutyric acid, methyl jasmonate or methyl salicylate during cold storage. *Food Chem.* **2020**, *306*, 125634. <https://doi.org/10.1016/j.foodchem.2019.125634>
2. Molinu, M.G.; Dore, A.; Palma, A.; D'Aquino, S.; Azara, E.; Rodov, V.; D'hallewin, G. Effect of superatmospheric oxygen storage on the content of phytonutrients in 'Sanguinello Comune' blood orange. *Postharvest Biol. Technol.* **2016**, *112*, 24-30. <https://doi.org/10.1016/j.postharvbio.2015.09.037>
3. Butelli, E.; Licciardello, C.; Zhang, Y.; Liu, J.; Mackay, S.; Bailey, P.; Reforgiato-Recupero, G.; Martin, C. Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges. *The Plant Cell* **2012**, *24*, 1242-1255. <https://doi.org/10.1105/tpc.111.095232>
4. Fallico, B.; Ballistreri, G.; Arena, E.; Brighina, S.; Rapisarda, P. Bioactive compounds in blood oranges (*Citrus sinensis* (L.) Osbeck): Level and intake. *Food Chem.* **2017**, *215*, 67-75. <https://doi.org/10.1016/j.foodchem.2016.07.142>
5. Arena, E.; Fallico, B.; Maccarone, E. Evaluation of antioxidant capacity of blood orange juices as influenced by constituents, concentration process and storage. *Food Chem.* **2001**, *74*, 423-427. [https://doi.org/10.1016/S0308-8146\(01\)00125-X](https://doi.org/10.1016/S0308-8146(01)00125-X)
6. Cebadera-Miranda, L.; Domínguez, L.; Dias, M.I.; Barros, L.; Ferreira, I.C.; Igual, M.; Martínez-Navarrete, N.; Fernández-Ruiz, V.; Morales, P.; Cámara, M. Sanguinello and Tarocco (*Citrus sinensis* [L.] Osbeck): Bioactive compounds and colour appearance of blood oranges. *Food Chem.* **2019**, *270*, 395-402. <https://doi.org/10.1016/j.foodchem.2018.07.094>
7. Kelebek, H.; Canbas, A.; Selli, S. Determination of phenolic composition and antioxidant capacity of blood orange juices obtained from cvs. Moro and Sanguinello (*Citrus sinensis* (L.) Osbeck) grown in Turkey. *Food Chem.* **2008**, *107*, 1710-1716. <http://doi.org/10.1016/j.foodchem.2007.10.004>
8. McDougall, G.J. Phenolic-enriched foods: Sources and processing for enhanced health benefits. *Proc Nutr Soc.* **2017**, *76*, 163-171. <http://doi.org/10.1017/S0029665116000835>
9. Mueller, D.; Jung, K.; Winter, M.; Rogoll, D.; Melcher, R.; Kulozik, U.; Schwarz, K.; Richling, E. Encapsulation of anthocyanins from bilberries—Effects on bioavailability and intestinal accessibility in humans. *Food Chem.* **2018**, *248*, 217-224. <http://doi.org/10.1016/j.foodchem.2017.12.058>
10. Hillebrand, S.; Schwarz, M.; Winterhalter, P. Characterization of anthocyanins and pyranoanthocyanins from blood orange [*Citrus sinensis* (L.) Osbeck] juice. *J. Agric. Food Chem.* **2004**, *52*, 7331-7338. <https://doi.org/10.1021/jf0487957>
11. Carmona, L.; Alquézar, B.; Marques, V.V.; Peña, L. Anthocyanin biosynthesis and accumulation in blood oranges during postharvest storage at different low temperatures. *Food chemistry* **2017**, *237*, 7-14. <https://doi.org/10.1016/j.foodchem.2017.05.076>
12. Lo Piero, A.R. The state of the art in biosynthesis of anthocyanins and its regulation in pigmented sweet oranges [(*Citrus sinensis*) L. Osbeck]. *J. Agric. Food Chem.* **2015**, *63*, 4031-4041. <https://doi.org/10.1021/acs.jafc.5b01123>
13. Rapisarda, P.; Bianco, M.L.; Pannuzzo, P.; Timpanaro, N. Effect of cold storage on vitamin C, phenolics and antioxidant activity of five orange genotypes [*Citrus sinensis* (L.) Osbeck]. *Postharvest Biol. Technol.* **2008**, *49*, 348-354. <https://doi.org/10.1016/j.postharvbio.2008.02.002>

14. Lo Piero, A.R.; Puglisi, I.; Rapisarda, P.; Petrone, G. Anthocyanins accumulation and related gene expression in red orange fruit induced by low temperature storage. *J. Agric. Food Chem.* **2005**, *53*, 9083-9088. <https://doi.org/10.1021/jf051609s>
15. Habibi, F.; Ramezani, A. Vacuum infiltration of putrescine enhances bioactive compounds and maintains quality of blood orange during cold storage. *Food Chem.* **2017**, *227*, 1-8. <https://doi.org/10.1016/j.foodchem.2017.01.057>
16. Rapisarda, P.; Bellomo, S.E.; Intelisano, S. Storage temperature effects on blood orange fruit quality. *J. Agric. Food Chem.* **2001**, *49*, 3230-3235. <https://doi.org/10.1021/jf010032l>
17. Pannitteri, C.; Continella, A.; Cicero, L.L.; Gentile, A.; La Malfa, S.; Sperlinga, E.; Napoli, E.; Strano, T.; Ruberto, G.; Siracusa, L. Influence of postharvest treatments on qualitative and chemical parameters of Tarocco blood orange fruits to be used for fresh chilled juice. *Food Chem.* **2017**, *230*, 441-447. <https://doi.org/10.1016/j.foodchem.2017.03.041>
18. Meyers, K.J.; Watkins, C.B.; Pritts, M.P.; Liu, R.H. Antioxidant and antiproliferative activities of strawberries. *J. Agric. Food Chem.* **2003**, *51*, 6887-6892 <https://doi.org/10.1021/jf034506n>
19. Brand-Williams, W.; Cuvelier, M.-E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT- Food Sci. Technol.* **1995**, *28*, 25-30. [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5)
20. Martinez-Espla, A.; Zapata, P.J.; Valero, D.; García-Viguera, C.; Castillo, S.; Serrano, M. Preharvest application of oxalic acid increased fruit size, bioactive compounds, and antioxidant capacity in sweet cherry cultivars (*Prunus avium* L.). *J. Agric. Food Chem.* **2014**, *62*, 3432-3437. <https://doi.org/10.1021/jf500224g>
21. Habibi, F.; Ramezani, A.; Rahemi, M.; Eshghi, S.; Guillén, F.; Serrano, M.; Valero, D. Postharvest treatments with γ -aminobutyric acid, methyl jasmonate, or methyl salicylate enhance chilling tolerance of blood orange fruit at prolonged cold storage. *J. Sci. Food Agric.* **2019**, *99*, 6408-6417. <https://doi.org/10.1002/jsfa.9920>
22. Chance, B.; Maehly, A. Assay of catalases and peroxidases. *Methods in Enzymol.* **1955**, *2*, 764-775. [https://doi.org/10.1016/S0076-6879\(55\)02300-8](https://doi.org/10.1016/S0076-6879(55)02300-8)
23. Nakano, Y.; Asada, K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and cell physiol.* **1981**, *22*, 867-880. <https://doi.org/10.1093/oxfordjournals.pcp.a076232>
24. Beauchamp, C.; Fridovich, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **1971**, *44*, 276-287. [https://doi.org/10.1016/0003-2697\(71\)90370-8](https://doi.org/10.1016/0003-2697(71)90370-8)
25. Liu, Q.; Xi, Z.; Gao, J.; Meng, Y.; Lin, S.; Zhang, Z. Effects of exogenous 24-epibrassinolide to control grey mould and maintain postharvest quality of table grapes. *Int. J. Food Sci. Tech.* **2016**, *51*, 1236-1243. <https://doi.org/10.1111/ijfs.13066>
26. Silva, C.R.d.; Koblitz, M.G.B. Partial characterization and inactivation of peroxidases and polyphenol-oxidases of umbu-cajá (*Spondias* spp.). *Food Sci. Technol.* **2010**, *30*, 790-796. <https://doi.org/10.1590/S0101-20612010000300035>
27. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
28. Khorram, F.; Ramezani, A.; Hosseini, S.M.H. Shellac, gelatin and Persian gum as alternative coating for orange fruit. *Sci. Hortic.* **2017**, *225*, 22-28. <https://doi.org/10.1016/j.scienta.2017.06.045>
29. Sun, X.-H.; Xiong, J.-J.; Zhu, A.-D.; Zhang, L.; Ma, Q.-L.; Xu, J.; Cheng, Y.-J.; Deng, X.-X. Sugars and organic acids changes in pericarp and endocarp tissues of pumelo fruit during postharvest storage. *Sci. Hortic.* **2012**, *142*, 112-117. <https://doi.org/10.1016/j.scienta.2012.05.009>

30. Zhou, Y.; He, W.; Zheng, W.; Tan, Q.; Xie, Z.; Zheng, C.; Hu, C. Fruit sugar and organic acid were significantly related to fruit Mg of six citrus cultivars. *Food Chem.* **2018**, *259*, 278-285. <https://doi.org/10.1016/j.foodchem.2018.03.102>
31. Asencio, A.D.; Serrano, M.; García-Martínez, S.; Pretel, M.T. Organic acids, sugars, antioxidant activity, sensorial and other fruit characteristics of nine traditional Spanish Citrus fruits. *Eur. Food Res. Technol.* **2018**, *244*, 1497-1508. <https://doi.org/10.1007/s00217-018-3064-x>
32. Lado, J.; Gambetta, G.; Zacarias, L. Key determinants of citrus fruit quality: Metabolites and main changes during maturation. *Sci. Hortic.* **2018**, *233*, 238-248. <https://doi.org/10.1016/j.scienta.2018.01.055>
33. Catalano, A.E.; Ingallinera, B.; Todaro, A.; Rapisarda, P.; Spagna, G. Degradative enzymatic activities in fresh-cut blood-orange slices during chilled-storage. *Int. J. Food Sci. Tech.* **2009**, *44*, 1041-1049. <https://doi.org/10.1111/j.1365-2621.2008.01878.x>
34. Sayyari, M.; Babalar, M.; Kalantari, S.; Martínez-Romero, D.; Guillén, F.; Serrano, M.; Valero, D. Vapour treatments with methyl salicylate or methyl jasmonate alleviated chilling injury and enhanced antioxidant potential during postharvest storage of pomegranates. *Food Chem.* **2011**, *124*, 964-970. <https://doi.org/10.1016/j.foodchem.2010.07.036>
35. Valero, D.; Serrano, M. *Postharvest biology and technology for preserving fruit quality*; CRC press: **2010**. Taylor & Francis-CRC Press, Boca Raton, Florida, USA.
36. Brizzolara, S.; Manganaris, G.A.; Fotopoulos, V.; Watkins, C.B.; Tonutti, P. Primary metabolism in fresh fruits during storage. *Front. Plant Sci.* **2020**, *11*, 1-16. <https://doi.org/10.3389/fpls.2020.00080>
37. Fabroni, S.; Amenta, M.; Timpanaro, N.; Todaro, A.; Rapisarda, P. Change in taste-altering non-volatile components of blood and common orange fruit during cold storage. *Food Res. Int.* **2020**, *131*, 108916. <https://doi.org/10.1016/j.foodres.2019.108916>