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Profile of Bioactive Compounds in Orange Juice Related to the Combination of Different Scion/Rootstocks, Packaging and Storage

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Abstract: Citrus scion/rootstock combinations alter the concentration of bioactive compounds in orange juice. The shelf life of freshly squeezed juice can be maximized through packaging and storage. The profiles of ascorbic (AA), dehydroascorbic acid (DHAA), and phenolic compounds were analyzed in juices of four sweet orange scions, Sanguínea de Mombuca (SM), Rubi (R), Lue Gin Gong (LGG), and Valência Delta Seedless (VDS), grafted onto 'Rangpur' lime (RL) and 'Swingle' citrumelo (SC) rootstocks. The juices obtained from the combination of the 'Rubi' orange in both rootstocks stood out by their higher concentration of ascorbic acid (AA) and dehydroascorbic acid (DHAA). Overall, all SC-grafted scions showed higher AA and DHAA and some phenolic compound concentrations. In all combinations, phenolic compounds showed the highest concentrations in the juices at the time of fruit extraction and decreased during storage. Dark packaging provided higher bioactive compounds in juices stored for longer periods. These findings can contribute to the diversification of scion/rootstock cultivars in order to increase the variety of orchards by choosing the best combinations for pasteurized orange juice with higher nutritional value.

Keywords: *Citrus* spp.; ascorbic acid; cultivar diversification; phenolic compounds

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1. Introduction

Brazil is the world's largest producer of orange juice. The Brazilian fruit stands out for presenting characteristics such as color, aroma, flavor, and nutritional value, which provide high quality juice [1], and mainly for presenting low cost due to the large scale production process [2]. The orange juice, besides being pleasant to the palate, presents benefits to health because it is a source of bioactive compounds, such as vitamin C and phenolic compounds, among others [3].

The sustainability of citrus crops is a global concern [4,5]. Brazil is among the vulnerable regions that cultivate a reduced number of citrus genotypes, which leads to greater susceptibility to pests and diseases, as well as less economic competitiveness among growers [6]. Diversification of citrus cultivars is an approach adopted by growers to increase the variety and profitability of orchards and to adapt to climate changes. New scion/rootstock cultivar combinations are a constant need of the citrus growers and also

aim to satisfy the preferences of consumers, who are becoming increasingly demanding in relation to the quality attributes of the fruit and the orange juice consumed.

The cultivar Sanguínea de Mombuca (*Citrus sinensis* (L.) Osbeck) arose from spontaneous mutation and was subsequently selected and released by the Agronomic Institute of Campinas. This cultivar is a rich source of nutraceuticals, including the carotenoids β-carotene and lycopene that are responsible for the red color of the pulp [7,8]. The tree has early-ripening spherical-shaped medium-sized (140 g) fruits that contain 55% juice on average [8].

The cultivar Rubi originated in the active germplasm bank located in the city of Araras, São Paulo State, Brazil. The cultivar exhibits early fruit ripening, an average size of 172 g, annual production of up to 40 t/ha, and a juice yield of 49% [9]. Lue Gi Gong is a cold-tolerant Valencia-type cultivar, which is tolerant to citrus canker (*Xanthomonas axonopodis* Starr and Garces emend; Vauterin et al. pv. *citri* (Hasse) Dye) and shows late maturation of the fruit, which, under refrigeration conditions after harvest, can be preserved for longer than one month. The main limitations of this cultivar are its propensity to produce small fruits and its alternate bearing [10]. The Valência Delta Seedless cultivar originated from spontaneous bud mutation of the Valencia cultivar or by nucellar seedlings, with the Valencia cultivar as the genitor. It has tolerance to citrus canker, late ripening, and seedless fruits, but is also alternate bearing [11].

Grafting is a widely used technique in citrus farming [12] and, when performed with the use of proper rootstock, it can provide important improvements for the scion [13], such as juvenile period reduction, homogeneous tree architecture, pest and disease protection, water and nutrient absorption, tolerance to abiotic stress [14], and increased yield and fruit quality. Fruit size, juice quality, sugar and acid content, fruit skin color and thickness, and fruit ripening and production duration are also influenced by rootstock [15].

The 'Rangpur' lime (*Citrus limonia* Osbeck) tree is a natural hybrid of *Citrus medica* L. and mandarin (*Citrus reticulata* Blanco) and is suggested to be native to India [16]. In Brazil, the rootstock of 'Rangpur' lime has previously been used in citrus orchards due to its vigor, drought tolerance, high yield, precocity, and early fruit maturation [17]. Although it is tolerant to *Citrus* tristeza virus (CTV), it is susceptible to *Citrus exocortis* viroid (CEVd) and *Citrus* sudden death-associated virus (SCDaV) [18].

'Swingle' (*P. trifoliata* (L.) Raf × *C. paradisi* Macf.) is the most cultivated citrumelo in the world. It is among the main rootstocks used for diversification of orange groves, providing scions with high quality fruits, high juice yield, greater soluble solids content and yield, and lower scion vigor. This cultivar is ideal for semi-dense planting in cooler locations [1]. It is resistant to *Citrus* sudden death-associated virus and decline [19].

Citrus is one of the most important fruit crops widely investigated for its bioactive composition and its health benefits [20]. The bioactive compounds present in the fruits prevent the oxidative damage of cells by detoxifying the free radicals, thus minimizing the incidence of various diseases [21].

Significant advancements have been made to study the composition, content, and health-promoting activities of citrus fruits' bioactive compounds [22]. However, new studies should be addressed to identify the traditional and new cultivar variations and contents of bioactive compounds. This information can help to select bioactive-rich cultivars for food formulations. Moreover, precise identification of the bioactive-rich growth stage of citrus fruits processing into juice suitable for consumption is necessary [3]. Post-harvest processing can induce changes in the levels of several primary and secondary metabolites, including storage of fruits and derivatives. In the world, there is a continuous increase in the search for packaged foods and beverages that maintain the nutritional and phytochemical characteristics, including the composition of compounds with antioxidant properties. The food industry has been looking for ways to preserve quality, because it is known that during processing and storage, nutritional and sensory changes can occur, which are a limiting factor in determining the shelf life of the juice [23,24].

Nowadays, there is an increasing demand for nutritious food and many attempts have been made to maximize the retention of nutrients during storage as much as during the processing [24]. The shelf life of freshly squeezed juice can be maximized through packaging and storage.

Vitamin C or L-ascorbic acid is a water-soluble unstable vitamin that has been used as an important marker or indicator of fruit juice quality [23]. The vitamin C content in orange juice can be different depending on the raw material and the processing conditions. Vitamin C and bioactive flavonoids play an important role in oranges to scavenge free radicals and to prevent some diseases [24]. The study was performed by UHPLC (ultra-high performance liquid chromatography) and aimed to evaluate the degradation of vitamin C, as well as the content of phenolic compounds in orange juice stored in different packages for longer periods and from different combinations of scion/rootstock cultivar combinations.

2. Materials and Methods

2.1. Experimental Area Characterization

The experiment was conducted at the São Manuel Experimental Farm, School of Agriculture, São Paulo State University (FCA UNESP), Brazil (22°44′28″ S, 48°34′37″ W) located at an altitude of 740 m a.s.l. According to the Köppen–Geiger climate classification, the climate of the area is Cwa, or warm temperate (mesothermal) and humid, and the average temperature of the warmest month is approximately 22 °C [25]. The soil is classified as a sandy-textured Latossolo Vermelho distroférrico according to the Brazilian system of soil classification [26], that is, a dystrophic Typic Hapludox [27].

2.2. Plant Material and Crop Management

A replicated trial was performed in two consecutive harvest seasons (2019–2020) in a non-irrigated orchard of trees of three and four years of age, respectively. The trees were planted with 6 m spacing between rows and 4 m spacing between trees (i.e., 416 trees/ha).

The sweet orange scion cultivars Sanguinea de Mombuca (SM), Rubi (R), Lue Gin Gong (LGG), and Valencia Delta Seedless (VDS) were used, grafted on the rootstocks of 'Rangpur' lime (RL) and 'Swingle' citrumelo (SC) trees (Figure 1).

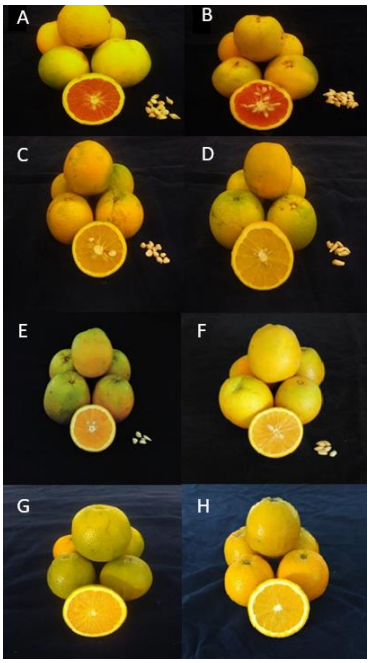

Figure 1. Fruits of sweet orange scion cultivars grafted onto two rootstocks: SM/RL (**A**); SM/SC (**B**); R/RL (**C**); R/SC (**D**), LGG/RL (**E**); LGG/SC (**F**); VDS/RL (**G**); VDS/SC (**H**).

The experimental area was prepared based on soil analysis and orange crop recommendations, using ploughing, sorting, and liming. The trees received the standard management practices recommended for citrus orchards.

2.3. Treatments and Experimental Design

The treatments consisted of eight scion/rootstock combinations: 'Sanguínea de Mombuca'/'Rangpur' lime (SM/RL); 'Sanguínea de Mombuca'/'Swingle' citrumelo (SM/SC); 'Rubi'/'Rangpur' lime (R/RL); 'Rubi'/'Swingle' citrumelo (R/SC); 'Lue Gim Gong'/'Rangpur' lime (LGG/RL); 'Lue Gim Gong'/'Swingle' citrumelo (LGG/SC); 'Valência Delta Seedless'/'Rangpur' lime (VDS/RL); 'Valência Delta Seedless'/'Swingle' citrumelo (VDS/SC).

The experimental design was completely randomized, considered separately for each scion/rootstock combination, using five replicates. Each replicate consisted of three trees per experimental plot, with guard trees external to the trial.

A split-plot design, with rootstocks (RL and SC) considered as plots and storage (transparent and dark bottles) as subplots, was used to determine the contents of phenolic compounds and ascorbic and dehydroascorbic acids in juices.

2.4. Fruit Harvesting and Sample Preparation

The harvest was performed when the fruits reached the ripeness index or *ratio* (soluble solids/titratable acidity) between 8.5 and 10. The Rubi and SM cultivars are early ripening and their fruits were harvested at 246 and 244 days after anthesis, respectively. The late ripening cultivars LGG and VDS were harvested at 402 and 406 days after anthesis, respectively.

After harvesting, the preparation of whole juices took place in the Beverage Laboratory of the Horticulture Department of FCA/UNESP. Juices were extracted using a semi-industrial juicer and after were pasteurized. Then, the pasteurization units (PU) were calculated. The calculation for PU units was performed using the method described by Peña et al. [28].

$$TL = (T_{obs} - T_{ref})/Z$$

where

TL is lethal rate;
T_{obs} is observed temperature;
T_{ref} is reference temperature;
Z is interval of temperature (causes a variation of 10 times in the speed of destruction).

To reach the desired PU, the counting of the PU was initiated when the juice samples (scion/rootstock combinations) reached 70 °C (reference temperature). At 70 °C, the temperature was recorded every minute using a digital thermometer and the number of PU was calculated.

During this process, the temperature was constantly balanced at around 75 °C. Upon reaching the desired PU (50 PU), cooling took place using a serpentine heat exchanger (chiller). The cooling lasted approximately 30 s, at which time the juice samples reached a temperature below 70 °C (reference temperature).

The juices were packaged in 300 mL transparent and dark polyethylene terephthalate bottles, covered with aluminium foil, and sealed by inverting the rotation cap. Subsequently, the samples were stored in a refrigerator at 4 °C for 0, 7, 14, 21, 28, and 35 days.

2.5. The Content of Ascorbic Acid (AA) and Dehydroascorbic Acid (DHA) in Juices

The determination of ascorbic and dehydroascorbic acid in juices was performed by UHPLC (ultra-high performance liquid chromatography). The methodology for extracting and quantifying ascorbic acid and dehydroascorbic acid in the samples was performed according to the methodology described by Spínola et al. [29], adapted.

Weekly (0, 7, 14, 21, 28, and 35 days of storage), juice samples stored in transparent bottles were evaluated. On day thirty-five the analysis of the juice stored in dark bottles was also performed. The evaluation of the ascorbic acid and dehydroascorbic acid content during the whole storage period was carried out only for juices packaged in transparent bottles, since this is the predominant packaging in the commercialization of this type of juice in Brazil.

Samples were diluted in Milli-Q water (1:9), filtered (PTFE, 0.45 µm, Hydrophilic, MA, USA), and injected (20 µL) into a CLUE system (Ultimate 3000 BioRS, Dionex-Thermo Fisher Scientific Inc., Fair Lawn, NJ, USA) equipped with a diode array detector (DAD), Ace 5 C18 4.6 mm × 250 mm column (Advanced Chromatography Technologies, Aberdeen, UK) at 25 °C. The flow was 0.8 mL min^{-1} for 17 min and the reading was performed at 245 nm. The substances were identified by comparing their retention times and the areas under the curves were determined and compared with standard curves of ascorbic acid ($y = 2300.3x - 1.1376$ $r^2 = 0.99$) and dehydroascorbic acid ($y = 9.1003x - 1.0026$ $r^2 = 0.99$), with purity ≥ 95% (Sigma-Aldrich, Saint Louis, MO, USA). Results were expressed as mg of ascorbic acid 100 mL^{-1} of juice.

2.6. Profile of Phenolic Compounds

The profile of the phenolic compounds in juices from the scion/rootstock combinations was carried out in UHPLC (ultra-high performance liquid chromatography, Sigma-Aldrich, São Paulo, Brazil). The separation, identification, and quantification of these compounds was according to the method described by Natividade et al. [30], adapted. The analysis was performed on the juice preparation day (day 0), and after thirty-five (35) days of storage.

The juices were filtered (PTFE, 0.45 µm, Millipore, MA, USA) and injected (20 µL) into a CLUE system (Ultimate 3000 BioRS, Dionex-Thermo Fisher Scientific Inc., USA) equipped with a cluster array detector diode (DAD), Luna® 2.5 µm C18 column (2) HST 2.0 × 50 mm (Phenomenex®, Torrance, CA, USA). The run temperature was 39 °C and the flow rate was 0.6 mL/min. The mobile phase consisted of 0.85% phosphoric acid solution (solvent A) and 100% acetonitrile (solvent B). The gradient used was: 0–2.5 min: 4% B; 2.5–7.5 min: 8% B; 7.5–15 min: 12% B; 15–18 min: 15% B; 18–20 min: 20% B; 20–21 min: 25% B; 21–22 min: 35% B; 22–24 min: 65% B; 24–25 min: 65% B; 25–25.5 min 35% B; 25.5–26 min: 0%; 26–27 min: 0% B. The absorbance was measured at 280 nm, 320 nm, 360 nm, and 520 nm using a UV-Vis spectrophotometer. Calibration curves were prepared with commercial standards (hesperidin, naringerin, caffeic acid, chlorogenic acid, *p*-coumaric acid, trans-ferulic acid, and synaptic acid, Sigma-Aldrich) and, based on their retention times, compound quantification was performed. Data were expressed as mg/L. All analysis was performed in triplicate.

2.7. Statistical Analysis

Two-year data of the evaluated variables were analysed as repeated measures. Analysis of variance (ANOVA) was performed with a significance level of 5% and differences between means were determined by Tukey's test, using the Sisvar program (Lavras, MG, Brazil). Analyses were performed in triplicate. Regression analysis was used for weekly assessments of ascorbic and dehydroascorbic acid content in juices stored in transparent bottles.

Principal component analysis (PCA) was performed [31] using XLSTAT version 2019.4.1 (Addinsoft, New York, NY, USA) to obtain a better visualization and explanation of the variability in the evaluated variables.

3. Results and Discussion

3.1. Ascorbic Acid (AA) and Dehydroascorbic Acid (DHAA) Concentration of Orange Juice According to Rootstock, Storage Time, and Packaging

There was a significant effect of the rootstock (R) and storage (S) interaction on DHAA concentration for the SM cultivar. The cultivar R did not have any interaction and the cultivars LGG and VDS showed interaction on AA and DHAA concentration (Table 1).

Table 1. F-values, degree of freedom (DF), and coefficient of variation (CV) values of AA and DHAA concentrations in juices from different scion/rootstock combinations after 35 days of storage.

		SM		R		LGG		VDS	
	DF	**AA**	**DHAA**	**AA**	**DHAA**	**AA**	**DHAA**	**AA**	**DHAA**
Block		0.228 ^{ns}	2.552 ^{ns}	4.663 ^{ns}	0.659 ^{ns}	12.102 ^{ns}	3.642 ^{ns}	1.441 ^{ns}	0.027 ^{ns}
R	1	1414.012 **	329.793 **	15.067 ^{ns}	173.077 **	805.399 **	1072.488 **	51.630 *	637.588 **
S	2	1324.995 **	294.266 **	2745.036 **	45.851 **	5833.517 **	213.374 **	4512.925 **	256.593 **
R x S	2	0.245 ^{ns}	14.201 **	0.103 ^{ns}	2.397 ^{ns}	61.421 **	48.364 **	518.471 **	24.911 **
CV (%)		2.13	3.76	1.33	3.23	1.57	2.45	0.93	2.72
CV (%)		2.36	2.73	2.5	3.05	1.41	2.71	1.87	2.46
Mean		375.35	155.58	368.32	428.96	326.35	189.65	280.6	242.31

** = statistically different at 1%; * = statistically different at 5%; ^{ns} = do not differ statistically by the F test p <0.05. R—rootstock; S—storage; AA—ascorbic acid; DHAA—dehydroascorbic acid; SM—Sanguínea de Mombuca; R—Rubi; LGG—Lue Gin Gong; VDS—Valência Delta Seedless.

The cultivar R showed no difference between rootstocks in AA concentration. The highest concentrations were found in juices at the time of fruit extraction; for stored juices there was no difference. The R/SC combination provided the highest concentrations of DHAA. Juices at the time of fruit extraction and stored in transparent bottles had the highest concentrations (Table 2).

In the SM/SC combination, the highest concentrations were found in juices stored in transparent bottles, followed by dark bottles and juices at the time of fruit extraction. Regarding the rootstock, the SM/SC combination showed the highest concentrations compared with the SM/RL combination, with no difference between the juices at the time of extraction and storage (Table 2).

There was no difference between rootstocks for AA concentration in the LGG cultivar. The highest concentrations were obtained in juices at the time of fruit extraction, regardless of packaging (Table 2). Evaluation of AA concentration in the rootstocks indicated that the LGG/RL combination showed the highest concentrations in non-stored and stored juice (Table 2).

The VDS/RL and VDS/SC combinations showed the highest concentrations in juices at the time of fruit extraction, followed by those stored in dark and transparent bottles (Table 2). The VDS/SC combination showed the highest concentrations in juices stored in transparent bottles. The VDS/RL combination presented the highest AA concentrations in juices at the time of fruit extraction and stored in dark bottles. In the VDS/SC combination, the highest concentrations were obtained in juices stored in transparent bottles (Table 2).

The harvest quality and optimal citrus harvest time are based on the SS concentration and TA and their relationship (the ripeness index (RI) or ratio (SS:AT)). The RI or ratio represents the balance between the sugar and the organic acid concentration in the fruit; it is associated with juice taste and is widely used in the orange juice industry as an indicator of ripening and fruit quality [1].

AA concentration varied according to scion and harvest season, although the average data from the two harvest seasons were evaluated together. This may have been influenced by weather conditions, since rainfall occurred from March onwards and temperatures remained high, resulting in an increase in concentration due to water loss in the fruits. There were periods of severe drought during the assessment period. Environmental factors, such as irradiation and stress, can stimulate the expression of genes involved in AA production [32].

Table 2. Ascorbic acid and dehydroascorbic acid concentrations in juices from Rubi, Sanguínea de Mombuca, Lue Gin Gong, and Valencia Delta Seedless cultivars with different rootstock combinations submitted to 35 days of storage.

Rubi				
AA (mg/L)				
RL	**SC**	**0**	**35**	**35D**
363.83 a *	372.80 a	595.47 a	253.33 b	356.14 b
DHAA (mg/L)				
RL	**SC**	**0**	**35**	**35D**
386.06 b	471.87 a	458.59 a	439.58 a	388.71 b
Sanguínea de Mombuca				
AA (mg/L)				
RL	**SC**	**0**	**35**	**35D**
304.21 b	446.37 a	527.61 a	296.35 b	302.07 b
DHAA (mg/L)				
	RL	SC		
0	101.94 Bb *	147.12 Ac		
35	151.38 Ab	216.28 Aa		
35D	138.28 Ab	178.46 Ab		
Lue Gin Gong				
	AA (mg/L)		**DHAA (mg/L)**	
	RL	**SC**	**RL**	**SC**
0	516.74 Aa *	463.41 Ba	137.66 Bb	299.11 Ab
35	244.34 Ac	194.70 Bc	184.82 Ba	319.07 Aa
35D	320.72 Ab	218.18 Bb	139.10 Bb	181.09 Ac
Valencia Delta Seedless				
	AA (mg/L)		**DHAA (mg/L)**	
	RL	**SC**	**RL**	**SC**
0	460.31 Aa *	410.04 Ba	266.89 Ba	299.11 Ab
35	98.32 Bc	201.07 Ac	213.99 Ba	319.07 Aa
35D	296.49 Ab	217.35 Bb	168.54 Bb	226.27 Ac

* Means followed by the same letter, lower case letter in the column (storage) and upper case letter in the row (rootstock), do not differ statistically, Tukey test at 5% probability level. AA—ascorbic acid; DHAA—dehydroascorbic acid; RL—'Rangpur' lime; SC—'Swingle' citrumelo; 0 = juice not stored; 35 = juice stored in transparent bottle for 35 days; 35D = juice stored in dark bottle for 35 days.

The sum of AA and DHAA concentrations in descending order for the scions evaluated were: R (797.28 mg/L), SM (530.93 mg/L), VDS (522.91 mg/L), and LGG (516.00 mg/L) (Table 1). The 'Rubi' orange stood out for presenting the highest concentrations of ascorbic acid (AA) in both rootstocks and the SM/SC combination for the highest DHAA concentration. The main hypothesis for these results is that AA and DHAA acids are genotype dependent variables. Moreover, the cultivars R and SM are classified as early maturing, in which less climatic variations occurred during the fruit ripening. In the late ripening cultivars LGG and VDS, the fruit remained longer in the field, having the influence of greater climatic changes that had an effect on the ripening period of the fruit.

In these cultivars, the fruit harvest occurred after a long dry season and higher temperatures, favoring a higher concentration of AA and DHAA. On the contrary, the harvest of the early maturing cultivars occurred after a period of high rainfall, which favored an increase in the mass of the fruits, as well as the dilution of the organic acids, decreasing the concentration of AA and DHAA. Acid concentration can be influenced by growing

conditions, climate, and even fruit size, which is influenced by the characteristics that the rootstock and scion can play in the fruit mass. The higher the fruit yield, the greater the dilution of organic acids, carbohydrates, and vitamins, thus decreasing the concentration of AA and DHAA [3,4]. Orange is a rich source of AA, which has several biological functions related to the immune system, collagen formation, iron absorption, nitrosamine inhibition, and antioxidant activity, therefore the SM and R scions, which have higher AA concentrations, are relevant [33]. The vitamin C concentrations in citrus juices are different from each other depending on processing conditions and raw material, such as 38 mg 100 g^{-1} for grapefruit juice, 46 mg 100 g^{-1} for lemon juice, 50 mg 100 g^{-1} for orange juice, and 31 mg 100 g^{-1} for mandarin juice [24]. The results obtained in this experiment confirm this report, as there were variations in AA and DHAA concentrations in each scion/rootstock cultivar combination evaluated.

In general, all SC-grafted scions showed higher AA and DHAA concentrations. The effect of rootstock on fruit quality can be attributed to several factors such as nutrient absorption and transport, compatibility, hormonal signaling, and gene expression [4]. The rootstock plays an important role in fruit ripening because it can accelerate or delay citrus tree development [1].

'Swingle' citrumelo is among the main rootstocks used in the diversification of orange groves because it provides scions with high-quality fruits with high juice yield and SS concentrations [34,35]. Scions on trifoliate orange rootstocks and their hybrids, as SC, produced better-quality fruits than those on other commonly used rootstocks. This has been well documented, but the genetic factors affecting fruit quality through the interaction between the scion and rootstock remain unclear. The results obtained by Hu et al. [15] demonstrated consistent correlations with the fruit quality of four 'Daya' mandarin cultivars grafted onto *Poncirus trifoliata* rootstocks related to the differential gene expression of small RNAs.

AA biosynthesis is generated from d-glucose, with nucleotides and sugars as intermediates, and the SC rootstock presents lower tolerance to water deficit, limiting the photosynthetic capacity of the tree, which may explain these results [32]. Photosynthesis, temperature, and light exposure can affect AA synthesis and production [36].

The concentration of acids can be influenced by growing conditions, climatic changes, and the size of the fruit, which is influenced by the characteristics that the rootstock and scion can play in the mass of the fruit [37]. The higher the fruit yield, the greater the dilution of organic acids, carbohydrates, and vitamins [38], thus decreasing the concentration of these compounds. The highest concentrations were observed in the juices at the time of fruit extraction, differing from those subjected to storage (Table 2). Similar results were reported by Nakilcioğlutaş and Ötleş [24], who, when evaluating the degradation of vitamin C in citrus juices, observed that the lowest vitamin C losses were found in non-stored juices. The lowest losses were recorded in juices stored at 4 °C.

Vitamin C (L-ascorbic acid) is a water-soluble and highly unstable vitamin. Vitamin C is often considered a nutrient quality indicator undergoing the processing and storage of foods, since it is seen that other nutrients are well preserved [39].

Data showed that the AA concentration in all scion/rootstock combinations had a negative linear effect, i.e., the concentrations decreased throughout the storage period. Differently, for DHAA concentrations, for SM and R cultivars, both the scion/rootstock combinations presented a positive quadratic effect. The cultivars LGG and VDS, grafted onto two rootstocks, showed a cubic effect during storage. This means that, for these combinations, there was no regular pattern of response (Figure 2). This result can be attributed to the late ripening of these cultivars, with physiological responses under constraining environments.

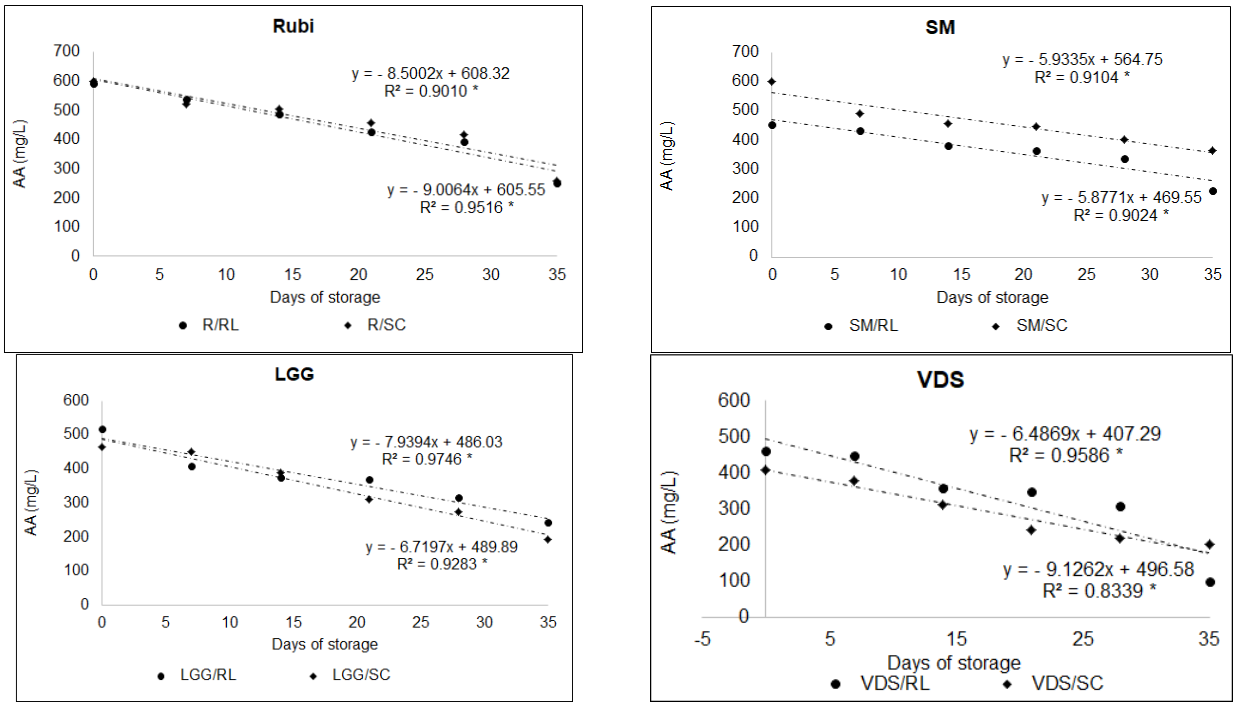

Figure 2. Ascorbic acid concentration (mg/L) in juices of Sanguínea de Mombuca (SM), Rubi (R), Lue Gin Gong (LGG), and Valencia Delta Seedless (VDS) sweet orange cultivars with different rootstock combinations subjected to 0, 7, 14, 21, 28, and 35 days of storage. RL—'Ranpur' lime; SC—'Swingle' citrumelo.

The decrease in AA concentrations occurs from the moment of processing and continues during the storage period of the juices. During storage, numerous deteriorating reactions occur, causing the degradation of AA, consequently leading to changes in the taste, color, texture, and appearance of the juice [23]. These results were confirmed in all scion/rootstock combinations evaluated, in which the concentrations of flavonoids, hesperidin, and naringerin decreased during the storage period.

Vitamin C in citrus juices is generally easily oxidized and therefore is lost in storage. There are many variables that affect this oxidation process such as light exposure, dissolved oxygen level, storage temperature, and presence of sugar and metal ions [39]. During storage, L-ascorbic acid oxidizes to dehydroascorbic acid (DHAA). This does not cause the loss of vitamin C, because DHAA can be converted back to ascorbic acid [40]. However, DHAA is easily hydrolyzed to 2,3-diketogulonic acid (DKGA) due to being highly unstable. DKGA has no biological activity [23]. These oxidation stages have been found to be particularly sensitive to oxygen availability, long-term heat treatment in the presence of oxygen, and exposure to light [40]. These reports were the main hypothesis to explain why, in some raw materials, juices stored in transparent bottles had higher concentrations of AA.

The variation in DHAA concentration observed in this experiment can occur in response to AA oxidation (Figure 3). AA degradation occurs during the storage of citrus juices and is mainly due to oxidation caused by storage temperature, light, and the presence of oxygen [24]. Due to this oxidation, AA is transformed into DHAA, which may explain the higher concentration of DHAA in stored juices. Wibowo et al. [23] studied the changes in acids, sugars, oxygen, and vitamin C due to the storage of orange juice and also observed that during storage, mainly due to the presence of oxygen, there were variations in the concentration of DHAA.

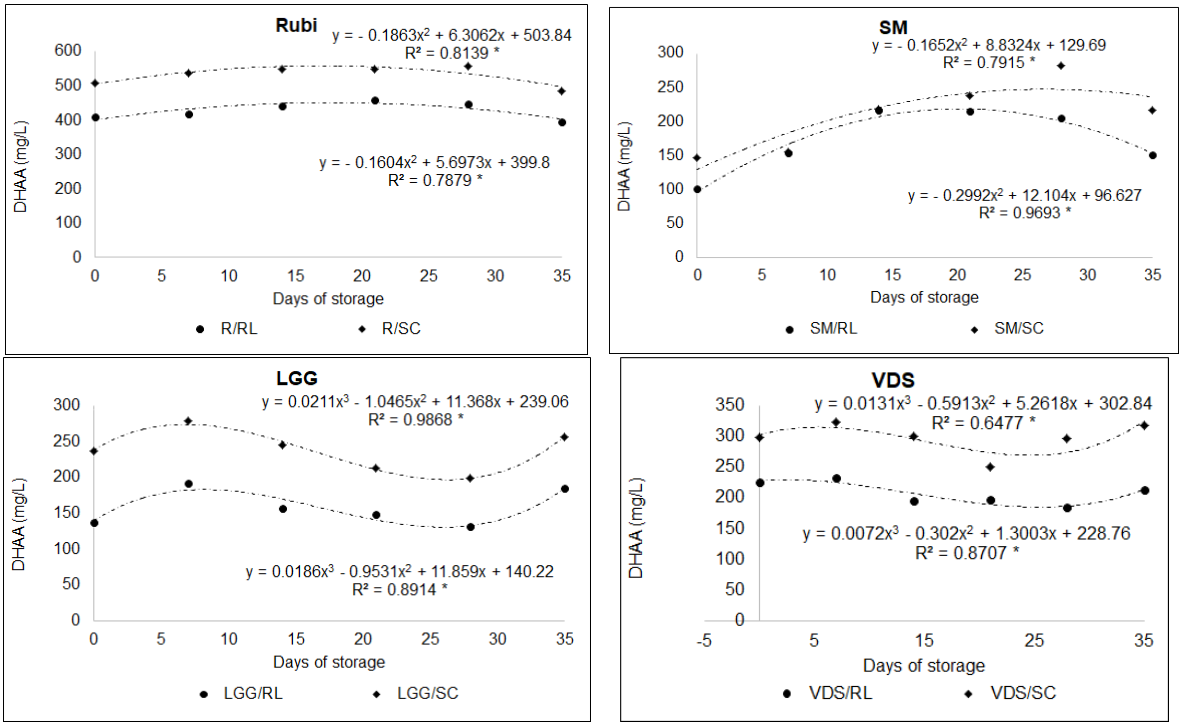

Figure 3. Dehydroascorbic acid concentration (mg/L) in juices from Sanguínea de Mombuca (SM), Rubi (Rubi), Lue Gin Gong (LGG), and Valencia Delta Seedless (VDS) sweet orange cultivars with different rootstock combinations submitted to 0, 7, 14, 21, 28, and 35 days of storage. RL—'Ranpur' lime; SC—'Swingle' citrumelo.

Principal component analysis (PCA) allowed demonstrating the concentration of AA and DHAA for each cultivar combined with the RL and SC rootstocks. In this study, PCA was applied to evaluate the concentration of AA and DHAA in response to juice storage and packaging. Variability was explained by two principal components, PC1 and PC2, for all cultivars. The cv. SM, accounting for 50.95% and 49.05%; cv. Rubi, responsible for 69.62% and 30.08%; cv. LGG, accounting for 50.52% and 49.42%; cv. VDS, responsible for 60.25% and 30.34% of the variation in the data (Figure 4).

The PCA demonstrated that the SM, LGG, and VDS sweet orange cultivars showed similar results in the first (PC1-DHAA) and second principal component (PC2-AA) and the cultivar R presented AA and DHAA in the first principal component (PC1).

Overall, the PCA allowed concluding that during juice storage there was an inverse relationship between AA and DHAA. It was also possible to identify that the highest AA concentrations occurred in juices at the time of fruit extraction, as well as the highest DHAA concentrations occurred in stored juices.

Despite the difference in the response of the cultivars, it is important to point out that all scion/rootstock combinations showed optimal levels of vitamin C (AA + DHAA) (Table 2), which is, according to Stinko et al. [37], around 529 mg L^{-1}. The degradation of AA and DHAA is more associated with the presence of oxygen than with light, since oxygen determines the rate of oxidative degradation of the compounds [24]. Oxygen is usually incorporated into the juice during preparation, processing, and storage and can pass through the package by diffusion process [41].

Studies that aim to quantify the vitamin C concentration before and after degradation are important, since vitamin C is a reliable indicator of the nutritional value and quality deterioration of the processed juice [42].

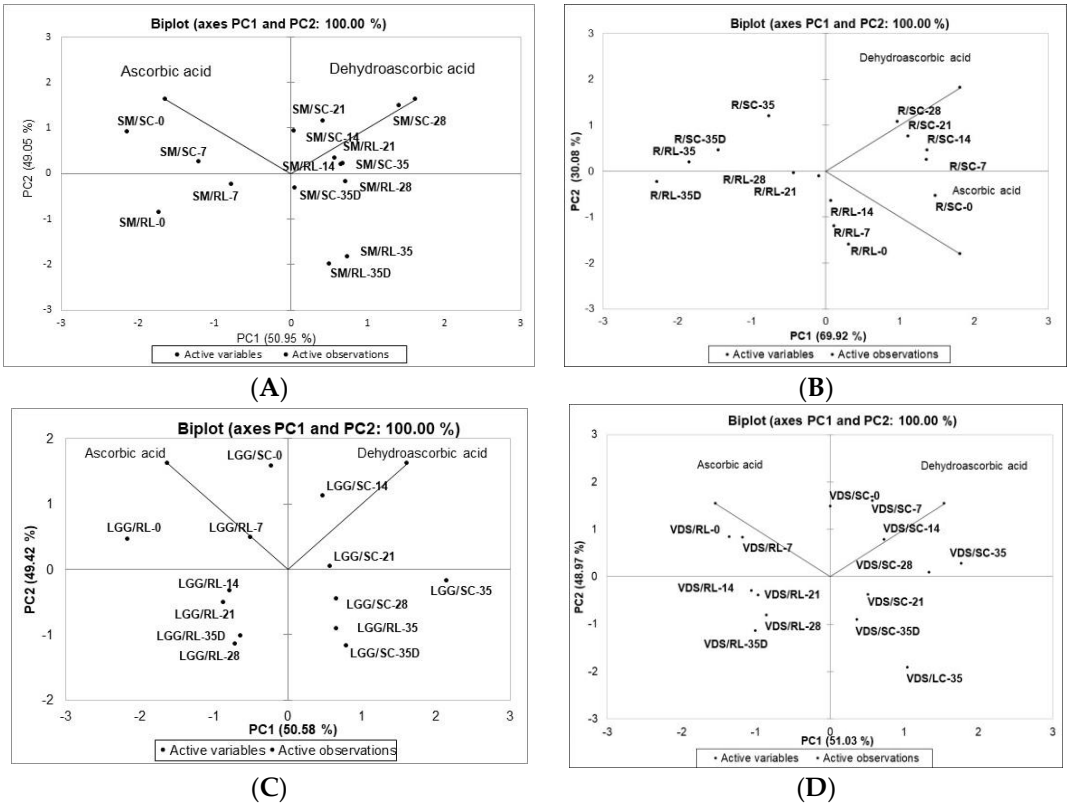

Figure 4. Principal component analysis (PCA) of AA and DHAA in juices from different scion/rootstock combinations subjected to 0, 7, 14, 21, 28, and 35 days of storage in transparent bottle and dark bottle (35D). (**A**) SM—Sanguínea de Mombuca; (**B**) R—Rubi; (**C**) LGG—Lue Gin Gong; (**D**) VDS—Valencia Delta Seedless; RL—'Rangpur' lime; CS—'Swingle' citrumelo.

3.2. Phenolic Compound Concentration of Orange Juice According to Rootstock, Storage Time, and Packaging

There was a significant effect of the rootstock and storage interaction for hesperidin, naringerin, caffeic acid, *p*-coumaric acid, and synaptic acid in the SM cultivar. The same result was observed for hesperidin, caffeic acid, chlorogenic acid, trans-ferulic acid, and synaptic acid in cultivar R and for hesperidin, naringerin, caffeic acid, and chlorogenic acid in 'LGG' orange and in cultivar VDS for hesperidin and synaptic acid. The results observed for each of the cultivars showed that there were variations in the concentrations of these compounds over the storage period of the orange juice. These variables are also genotype dependent (Table 3).

Regardless of the rootstock combination, SM scion had the highest concentrations of hesperidin, naringerin, caffeic acid, and *p*-coumaric acid in juices at the time of fruit extraction, except for synaptic acid, which had the highest concentrations in juices stored in dark bottles (Table 4). The degradation of phenolic compounds over time may occur due to storage conditions, processing, bottling, temperature, and exposure to light [43]. These variables can lead to the oxidation process and consequently the loss of the compounds [44], which may explain the higher concentration of the compounds at the time of juice extraction.

The hesperidin and caffeic acid had similar performances; for the combinations SM/RL and SM/SC, the highest losses occurred when the juice was stored in transparent bottles (Table 4). Naringerin showed different concentrations between the scion/rootstock combinations. The scion/rootstock combinations did not have differences in coumaric acid concentration between stored juices. Caro et al. [45], studying the concentrations of flavonoids in stored citrus juices, also reported a significant increase in some bioactive compounds during storage.

Table 3. F-values, degree of freedom (DF), and coefficient of variation (CV) of phenolic compounds in juices from different scion/rootstock combinations subjected to 35 days of storage.

				Sanguínea de Mombuca				
FV	**GL**	**Hesperidin**	**Naringerin**	**Cafffeic Acid**	**Chlorogenic Acid**	***p*-Coumaric**	**Ferulic Acid**	**Sinaptic Acid**
Block	2	5.295 ^{ns}	0.47 ^{ns}	61.00 **	0.717 ^{ns}	1.00 ^{ns}	0.33 ^{ns}	2.71 ^{ns}
RS	1	1357.05 **	27.84 *	7921.00 **	167.84 **	90.75 **	12.00 ^{ns}	43.75 *
S	2	3628.31 **	2238.00 **	569.71 **	492.23 **	650.67 **	470.86 **	132.72 **
RS x S	2	56.87 **	23.23 **	208.00 **	2.08 ^{ns}	32.00 **	3.43 ^{ns}	108.14 **
CV (%)		0.36	3.48	0.51	3.3	4.58	4.56	1.53
CV (%)		1.61	2.04	1.36	3.41	2.8	2.46	2.78
Mean		46.82	0.3	0.5	0.81	0.18	0.18	0.82
				Rubi				
Block	2	0.21 ^{ns}	21.00 *	7.00 ^{ns}	10.34 ^{ns}	1.00 ^{ns}	19.00 *	0.18 ^{ns}
RS	1	6637.85 **	484.00 **	240.25 **	26.23 *	6.25 ^{ns}	484.00 **	6.49 ^{ns}
S	2	21,648.56 **	468.12 **	403.15 **	1549.72 **	522.47 **	490.75 **	409.30 **
RS x S	2	98.210 **	1.65 ^{ns}	37.25 **	8.56 **	0.12 ^{ns}	4.75 *	214.25 **
CV (%)		0.26	0.65	0.84	2.04	2.58	1.26	3.49
CV (%)		0.51	3.31	2.27	2.32	3.76	2.51	3.73
Mean		40.17	0.36	0.56	0.9	0.18	0.19	0.82
				Lue Gin Gong				
Block	2	0.10 ^{ns}	0.08 ^{ns}	3.86 ^{ns}	2.71 ^{ns}	1.00 ^{ns}	0.11 ^{ns}	3.00 ^{ns}
RS	1	34.83 *	9.31 ^{ns}	96.57 **	57.14 **	42.25 *	25.00 *	32.00 **
S	2	20.356.46 **	271.05 **	0.273 ^{ns}	43.19 **	76.00 **	7.54 **	2.17 ^{ns}
RS x S	2	77.03 **	18.85 **	17.55 **	78.23 **	4.00 ^{ns}	1.39 ^{ns}	0.66 ^{ns}
CV (%)		0.87	1.76	1.01	1.29	2.17	4.7	0.26
CV (%)		0.67	2.65	1.25	1.36	1.09	3.99	0.69
Mean		67.71	0.48	0.63	0.97	0.22	0.15	1.85
				Valência Delta Seedless				
Block	2	3.75 ^{ns}	1.00 ^{ns}	4.33 ^{ns}	0.01 ^{ns}	0.33 ^{ns}	1.00 ^{ns}	0.16 ^{ns}
RS	1	0.02 ^{ns}	2.15 ^{ns}	75.00 **	2.81 ^{ns}	6.75 ^{ns}	1.00 ^{ns}	565.12 **
S	2	15,922.90 **	340.98 **	7.72 **	73.32 **	6.32 *	1.00 ^{ns}	13.23 **
RS x S	2	135.27 **	0.12 ^{ns}	2.91 ^{ns}	0.95 ^{ns}	3.12 ^{ns}	1.00 ^{ns}	5.05 *
CV (%)		0.71	4.12	0.57	4.52	3.7	1.58	1.33
CV (%)		1.01	3.29	1.87	1.35	3.78	1.58	0.95
Mean		79.49	0.55	0.71	0.99	0.22	0.15	2.01

** = statistically different at 1%; * = statistically different at 5%; ^{ns} = not statistically different by F-test at <0.05. RS—rootstock; S—storage; CV—coefficient of variation.

The evaluation of the rootstocks showed that the highest hesperidin concentrations were observed in the non-stored juice in the VDS/SC combination. In stored juices, regardless of the storage, the highest concentrations were recorded in the VDS/RL combination. The highest concentrations of synaptic acid were observed in the VDS/RL combination, regardless of the storage. There was no statistical difference between scion/rootstock combinations for naringerin and, caffeic, *p*-coumaric and *trans*- ferulic acids concentrations (Table 5).

Juices stored in dark bottles had higher concentrations of caffeic acid and juices at the time of fruit extraction had higher concentrations of *p*-coumaric acid. *Trans*-ferulic acid showed no difference for storage (Table 5).

Table 4. Concentration of phenolic compounds (mg/L) in 'Rubi' and 'Sanguinea de Mombuca' juices with different combinations of rootstock subjected to 35 days of storage in transparent bottles and dark bottles (D).

Rubi										
	Hesperidin		**Caffeic Acid**		**Chlorogenic Acid**		**Ferulic Acid**		**Sinaptic Acid**	
	RL	**SC**	**RL**	**SC**	**RL**	**SC**	**RL**	**SC**	**RL**	**SC**
0	54.51 aB *	57.41 aA	0.69 aA	0.66 aB	0.62 cA	0.55 cB	0.22 aB	0.25 aA	0.76 bA	0.30 bB
35	31.77 bB	35.47 bA	0.42 cB	0.52 cA	0.90 bA	0.83 bB	0.15 bB	0.17 cA	0.89 aB	1.04 aA
35D	31.11 cB	33.74 cA	0.52 bB	0.56 bA	1.25 aA	1.26 aA	0.16 bB	0.18 bA	0.87 aB	1.07 aA
	Naringerin						***p*-Coumaric**			
	RL	**SC**	**0**	**35**	**35D**	**RL**	**SC**	**0**	**35**	**35D**
	0.35 b	0.38 a	0.48 a	0.33 b	0.28 c	0.19 a	0.18 a	0.26 a	0.14 b	0.15 b
Sanguinea de Mombuca										
	Hesperidin		**Naringerin**		**Caffeic Acid**		***p*-Coumaric**		**Sinaptic Acid**	
	RL	**SC**	**RL**	**SC**	**RL**	**SC**	**RL**	**SC**	**RL**	**SC**
0	72.35 aA *	64.06 aB	0.41 aB	0.44 aA	0.43 aB	0.62 aA	0.20 aB	0.27 aA	0.82 bA	0.57 bB
35	34.92 cA	35.02 cA	0.19 cB	0.24 bA	0.38 cB	0.43 cA	0.13 bB	0.16 bA	0.78 bB	0.92 aA
35D	37.61 bA	36.97 bA	0.24 bA	0.24 bA	0.41 bB	0.48 bA	0.14 bB	0.16 bA	0.90 aA	0.90 aA
	Chlorog						**Ferul**			
	RL	**SC**	**0**	**35**	**35D**	**RL**	**SC**	**0**	**35**	**35D**
	0.72 b	0.89 a	0.54 c	0.86 b	1.03 a	0.17 a	0.19 a	0.22 a	0.15 c	0.16 b

* Means followed by the same letter, lower case in the column (storage) and upper case in the row (rootstock), do not differ statistically, Tukey test at 5% probability level. RL—'Rangpur' lime; SC—'Swingle' citrumelo; 0 = non-stored juice; 35 = juice stored in transparent bottle for 35 days; 35D = juice stored in dark bottle for 35 days.

Table 5. Concentration of phenolic compounds (mg/L) in 'Lue Gin Gong' and 'Valencia Delta Seedless' juices with different combinations of rootstock submitted to 35 days of storage.

Lue Gin Gong														
				Hesperidin		**Naringerin**		**Caffeic Acid**		**Chlorogenic Acid**				
				RL	**SC**	**RL**	**SC**	**RL**	**SC**	**RL**	**SC**			
			0	99.24 aA	97.39 aB	0.61 aA	0.55 aB	0.62 aA	0.63 bA	1.00 aA	0.85 bB			
			35	48.83 cB	53.44 bA	0.43 bB	0.45 bA	0.61 bB	0.64 bA	0.96 bB	1.01 aA			
			35 E	52.61 bB	54.75 bA	0.42 bA	0.42 cA	0.61 bB	0.65 aA	0.99 aA	0.98 aA			
***p*-Coumaric Acid**						**Ferulic Acid**					**Sinaptic Acid**			
RL	**SC**	**0**	**35**	**35D**	**RL**	**SC**	**0**	**35**	**35D**	**RL**	**SC**	**0**	**35**	**35D**
0.21 a *	0.22 a	0.23 a	0.22 b	0.21 c	0.14 b	0.16 a	0.16 a	0.15 b	0.15 b	1.65 b	2.04 a	1.84 a	1.85 a	1.84 a
Valencia Delta Seedless														
		Hesperidin			**Sinaptic Acid**					**Chlorogenic Acid**				
		RL		**SC**	**RL**		**SC**			**RL**	**SC**	**0**	**35**	**35D**
0		122.80 aB		131.42 aA	2.20 aA		1.87 aB							
35		55.88 cA		52.83 bB	2.17 bA		1.85 aB			1.01 a	0.98 b	0.94 b	1.01 a	1.03 a
35E		59.84 bA		54.17 bB	2.17 bA		1.87 aB							
Naringerin										**Caffeic Acid**				
RL		**SC**		**0**	**35**		**35D**			**RL**	**SC**	**0**	**35**	**35D**
0.55 a *		0.54 a		0.70 a	0.46 b		0.47 b			0.72 a	0.71 a	0.73 a	0.70 b	0.72 a
***p*-Coumaric Acid**										**Ferulic Acid**				
RL		**SC**		**0**	**35**		**35D**			**RL**	**SC**	**0**	**35**	**35D**
0.23 a		0.22 a		0.20 a	0.21 b		0.22 b			0.15 a	0.15 a	0.15 a	0.15 a	0.15 a

* Means followed by the same letter, lower case in the column (storage) and upper case in the row (rootstock), do not differ statistically, Tukey test at 5% probability level. RL—'Rangpur' lime; SC—'Swingle' citrumelo; 0 = non-stored juice; 35 = juice stored in transparent bottle for 35 days; 35D = juice stored in dark bottle for 35 days.

Differences in the concentrations of phenolic compounds may occur according to the raw material, pasteurization methods, storage conditions, and temperature, as well as the specific compound. In a comparative study, in the juice sacs of ripened fruits, flavonone hesperidin was the dominant phenolic compound in lemon (2213 mg/kg DW) and oranges (1957 and 1975 mg/kg DW in Washington Navel and Tarocco, respectively), whereas flavonone narirutin was the most prevalent in grapefruit (292 mg/kg DW) [20].

The reactions of the degradation of phenolic compounds occur through hydroxylation, methylation, isoprenylation, dimerization, and glycosylation effects. The enzymes polyphenol oxidase (PPO), peroxidase (POD), pectinamethylesterase (PME), and phenylalanine ammonia lyase (PAL) can also catalyze the oxidation of phenolic compounds in the presence of oxygen, causing the formation of dark compounds and consequently contributing to the loss of juice quality [46].

The phenolic compound concentrations are highly influenced by the ripening stage and the cropping system, genotype and environmental, since they are secondary compounds that are produced by the plant under stress conditions [47].

Overall, scions grafted on SC rootstocks had a higher concentration of some phenolic compounds due to SC showing less tolerance to water deficit [48]. Trees under water stress showed increased secondary metabolism, mainly phenolics, terpenes, alkaloids, and cyanogenic glycosides [49] (Table 4). Some compounds and/or scion/rootstock combinations in the stored juices presented higher concentrations in transparent bottles and others in dark bottles. Similarly, Giuffrè et al. [50] reported that hesperidin is the main flavonoid in orange juice and that storage was responsible in decreasing the flavonoid concentration. Chlorogenic acid is one of the main components present in citrus fruits and it usually occurs in larger amounts, as in this study [51]. The authors also reported that, during juice storage, the concentrations of these acids may change and, consequently, there may be a decrease in flavonoids.

The result of the principal component analysis (PCA) allowed an overview of the phenolic compounds for each cultivar combined with the RL and SC rootstocks. PCA was applied in order to evaluate the performance of phenolic compounds in response to juice storage and packaging. For all cultivars, the variability was explained by two principal components, PC1 and PC2; the cv. SM accounting for 79.68% and 13.96%, cv. R accounting for 87.33% and 9.00%, cv. LGG accounting for 54.14% and 29.10%, and 'VDS' accounting for 60.25% and 30.34% of the variation in the data (Figure 5).

The PCA showed that all scion cultivars presented similar performances. The juices of the 'SM' and 'Rubi' oranges presented in the first principal component (PC1) the flavonoids hesperidin and naringerin and the phenolic acids caffeic, *p*-coumaric, and trans-ferulic and in the second principal component (PC2), the phenolic acids chlorogenic and synaptic. The juices of the cultivars LGG and VDS presented in the first principal component (PC1) the flavonoids hesperidin and naringerin and the phenolic acids caffeic, *p*-coumaric, trans-ferulic, and synaptic (Figure 5). For all cultivars, chlorogenic acid was inversely proportional to the other compounds present in PC1. This same compound was observed in larger concentrations in stored juices, transparent and dark bottles, and in practically all the scion/rootstock combinations. Chlorogenic acid is derived from most phenolic acids, especially caffeic, *p*-coumaric, and trans-ferulic acids [20].

Most scion/rootstock combinations showed the highest concentrations of these phenolic acids at the time of juice extraction, which can explain the higher concentrations of chlorogenic acid in stored juices. These acids are biosynthesized by hydroxylation of the coumaroyl ester of the chemical acid. This hydroxylation produces the ester of shikimic acid, which is converted to chlorogenic acid [52].

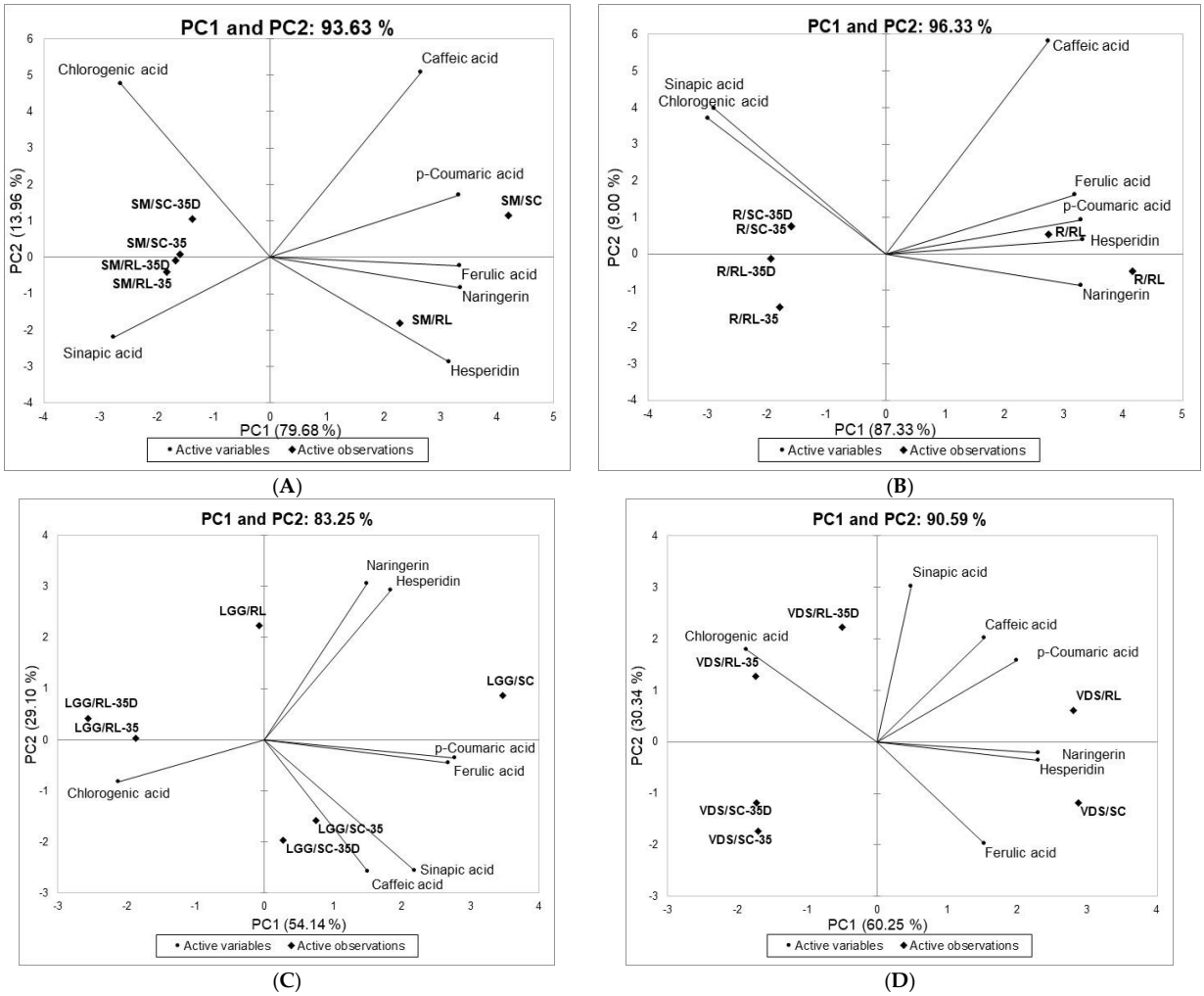

Figure 5. Principal component analysis (PCA) of phenolic compounds in juices from different scion/rootstock combinations subjected to 35 days of storage. (**A**) SM—Sanguínea de Mombuca; (**B**) R—Rubi; (**C**) LGG—Lue Gin Gong; (**D**) VDS— Valencia Delta Seedless; RL—'Rangpur' lime; CS—'Swingle' citrumelo; 0 = juice at the time of fruit extraction, 35 = juices stored for 35 days in transparent bottles; 35D = juices stored for 35 days in dark bottles.

The flavonoids hesperidin and naringerin also presented similar performances for all scion/rootstock combinations. The highest concentrations of these compounds were obtained in juices at the time of extraction, i.e., there was a decrease during storage (Figure 3). Zhang et al. [22] also concluded that flavonoid concentration decreased after juice storage. These authors hypothesized that the decrease in concentrations during storage is associated with the degradation of vitamin C and hydroxycinnamic acids, corroborating the results of the present study.

Summarizing, in all scion/rootstock combinations, the phenolic compounds hesperidin, naringerin, caffeic acid, *p*-coumaric acid, and trans-ferulic acid had the highest concentrations in the juices at the time of fruit extraction, so they decreased during storage. Hesperidin was the main flavonoid, as chlorogenic and synaptic acids were the main hydroxycinnamic acids. For stored juices, chlorogenic acid and synaptic acid were higher for most scion/rootstock combinations.

Both ascorbic acid and phenolic compounds are antioxidant substances that play an important role as indicators of juice quality. Information about their contents helps to add commercial and industrial value to orange juice. In general, the data showed that the contents varied with the scion/rootstock combinations and decreased during storage.

Significant studies have been made on the composition, content, and health-promoting activities of citrus juice bioactive compounds [20]. However, further investigations with new cultivars are needed to identify genetic variation and composition, due to these data may contribute to the selection of bioactive-rich citrus cultivars suitable for natural consumption and for processing into juice. These findings may also be useful in planning diversification of scion/rootstock combinations for new orchards by identifying genotypes best adapted to undesirable climatic conditions.

During the processing and storage of orange juice, nutritional and sensory changes may occur, which is a limiting factor for determining the shelf life of the juice. Studies that improve the processing and storage of orange juice, allowing the reduction of the degradation of bioactive compounds, have been increasingly requested. Choosing the best type of packaging and storage time to minimize nutritional losses in pasteurized orange juice is very important and should be the subject of future research considering the diversity of the raw material.

4. Conclusions

The juices obtained from the combination of the 'Rubi' orange in both rootstocks stood out by their higher concentration of AA and DHAA. However, all juice combinations evaluated showed optimal AA and DHAA concentrations in their composition and the highest AA concentrations were obtained in the juices at the time of extracting the fruit, without any storage, and were inversely proportional to the DHAA concentrations. With regard to combinations, the dark packaging provided a higher concentration of bioactive compounds in juices stored for longer periods. The data obtained from this study may provide additional contributions for choosing the best scion/rootstock combinations for processing orange juice with higher nutritional values and how to package and store orange juice.

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