Scientia Horticulturae 342 (2025) 114042

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Research Paper

Comparative study of rootstock effects on primary and secondary metabolites content in blood orange peel: Potential co-product perspectives

María Ángeles Forner-Giner [a], Manuel Ballesta-de los Santos [b,*], Pablo Melgarejo [b], Juan José Martínez-Nicolás [b], Roberto Gómez-Pérez [b], Alberto Continella [c], Pilar Legua [b]

[a] Valencian Institute of Agricultural Research (IVIA), 46113 Moncada, Spain
[b] Center for Agricultural and Environmental Research and Innovation (CIAGRO-UMH), Miguel Hernández University, Ctra. Beniel km 3.2, Orihuela 03312, Spain
[c] Department of Agriculture, Food and Environment, University of Catania, 95124 Catania, Italy

ARTICLE INFO

Keywords:
Anthocyanins
Blood orange
Orange peel
Co-products
Primary metabolites
Secondary metabolites

ABSTRACT

Blood oranges are important citrus crops worldwide due to their high productivity and high concentration of bioactive compounds. This study examined the effects of *Citrus macrophylla* and *Citrus reshni* rootstocks on fruit morphology, peel color, and primary and secondary metabolites in the varieties "Tarocco Ippolito", "Tarocco Lemso", "Tarocco Tapi", and "Tarocco Fondaconuovo". Significant differences were found in morphological parameters, peel color, and metabolites contents influenced by the rootstocks and varieties. "Tarocco Lemso" grafted onto *Citrus macrophylla* showed the highest values for fruit weight, peel weight, and caliber and exhibited the highest concentrations of amino acids by ^{1}H NMR, while "Tarocco Ippolito" onto *Citrus macrophylla* had high levels of total organic acids by ^{1}H NMR, and "Tarocco Lemso" grafted onto *Citrus macrophylla* displayed high levels of sugars by ^{1}H NMR and phenolic compounds by HPLC-ESI-DAD-MS^{n}. Overall, varieties grafted onto *Citrus macrophylla* showed higher metabolite values in the peel compared to those grafted onto *Citrus reshni*. These findings are relevant to the agri-food industry as they allow for the selection of optimal rootstock/variety combinations to improve peel quality and meet market demands.

1. Introduction

Citrus fruits, including oranges, are crops of great importance and productivity worldwide (Andrade et al., 2023; Forner-Giner et al., 2023a). According to FAOSTAT data, global production of these fruits reached 158 million tons in 2020, representing a significant increase of 7.5 % compared to 2017 (FAOSTAT, 2022). Among *Citrus* species, oranges (*Citrus sinensis* [L.] Osbec.) hold a prominent position as one of the most relevant varieties globally (Forner-Giner et al., 2023a). In fact, worldwide orange production reaches approximately 79 million tons (Andrade et al., 2023). Oranges can be classified into two main groups: white oranges, cultivated in numerous *Citrus*-producing countries, and blood oranges, primarily grown in specific regions due to particular climatic requirements (Forner-Giner et al., 2023a). Blood oranges, known for their distinctive reddish pigment, are highly valued for their unique flavor and visual appeal. Additionally, they present high concentrations of bioactive compounds, such as phenolic compounds, and especially anthocyanins, which are beneficial to human health due to their strong antioxidant activity (Habibi et al., 2024). The synthesis of red pigments conferred by anthocyanins depends on various factors, including varieties, rootstocks, and environmental factors such as the contrast between daytime and nighttime temperatures (Habibi et al., 2020).

Although some studies have investigated the accumulation of anthocyanins in blood orange varieties, a significant influence of rootstocks on the concentrations of phenolic compounds in the juices of blood organge varieties has been observed (Continella et al., 2018). Furthermore, the effects of rootstocks and varieties have also been observed in aspects such as yield and quality of blood oranges (Morales et al., 2021a). However, the number of studies that have thoroughly

Abbreviations: ANOVA, analysis of variance; ^{1}H NMR, ^{1}H-Nuclear Magnetic Resonance Spectroscopy; HPLC-ESI-DAD-MS^{n}, HPLC-Diode Array Detection-Electrospray Ionization-Mass Spectrometry; PCA, Principal Component Analysis; PC, Principal Component; RT, retention time.

* Corresponding author.
E-mail addresses: forner_margin@gva.es (M.Á. Forner-Giner), manuel.ballestas@umh.es, mballestad@uoc.edu, manuelballestadelossantos@gmail.com (M. Ballesta-de los Santos), pablo.melgarejo@umh.es (P. Melgarejo), juanjose.martinez@umh.es (J.J. Martínez-Nicolás), roberto.gomezp@umh.es (R. Gómez-Pérez), acontine@unict.it (A. Continella), p.legua@umh.es (P. Legua).

https://doi.org/10.1016/j.scienta.2025.114042
Received 5 February 2025; Received in revised form 18 February 2025; Accepted 25 February 2025
0304-4238/© 2025 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

evaluated the effect of rootstocks and varieties on the bioactive compounds of these orange varieties is still limited, and in some cases, the results obtained are contradictory (Forner-Giner et al., 2023b). Along these lines, in many *Citrus*-producing countries, one of the main challenges faced by the *Citrus* industry is the search for new *Citrus* rootstocks that enhance crop performance in terms of quantity, fruit weight and size, cumulative production per tree, and especially, nutritional quality (Legua et al., 2014). Therefore, further studies are needed to generate knowledge about the influence of rootstocks on the bioactive compounds and overall parameters of fruit quality, with the aim of obtaining rootstock/variety combinations that increase the nutritional value of the fruit (Continella et al., 2018).

On the other hand, it is important to highlight that the processing of blood oranges, especially for juice extraction, generates large amounts of co-products, mainly peels (Wang et al., 2008). Globally, it is estimated that around 15 million tons of *Citrus* co-products are generated annually (Leporini et al., 2020), emphasizing the need for efficient utilization and valorization of these resources that can be used in various food applications. In fact, these *Citrus* peel co-products contain significant amounts of bioactive and nutritional compounds that are not properly utilized (Andrade et al., 2019). In this regard, several studies have demonstrated the antioxidant capacity and beneficial properties of *Citrus* peel extracts in extending the shelf life and improving the nutritional and organoleptic quality of various foods (Tayengwa et al., 2020). These findings support the utilization of *Citrus* co-products in the agri-food industry, promoting circular economy and effective waste management in the fruit industry (Smeriglio et al., 2019). Considering the potential of *Citrus* peel co-products to enhance organoleptic properties and prolong the shelf life of different food products, it is essential to investigate the nutritional composition and bioactive compounds of blood orange peel (Andrade et al., 2023). However, despite its relevance, there is again a scarcity of available information on the nutrient composition and bioactive compounds present in blood orange peel for potential applications in different food products (Smeriglio et al., 2019).

To address these knowledge gaps, this study aimed to characterize eight rootstock/variety combinations of blood oranges to determine how rootstocks, varieties, and their interaction influence fruit morphology, peel color, and metabolite composition. We hypothesized that rootstock selection significantly affects the accumulation of primary and secondary metabolites in blood orange peel, with *Citrus macrophylla* expected to enhance metabolite concentrations compared to *Citrus reshni*. This effect may be attributed to differences in nutrient uptake efficiency, hormonal signaling, and stress tolerance conferred by each rootstock, which could modify the biosynthesis and accumulation of sugars, organic acids, and phenolic compounds. By analyzing key morphological parameters, peel color, and metabolic composition in four “Tarocco” varieties grafted onto these two rootstocks, we sought to identify the most favorable combinations for improving fruit quality and potential industrial applications. The results of this study may contribute to optimizing rootstock selection strategies in *Citrus* cultivation, enhancing the nutritional and commercial value of blood oranges.

2. Materials and methods

2.1. Plant material and sample preparation

In this study, a comprehensive evaluation of the morphological characteristics, color, and the content of primary and secondary metabolites in the peel of the blood orange varieties ’Tarocco Ippolito’, ’Tarocco Lempso’, ’Tarocco Tapi’, and ’Tarocco Fondaconuovo’ grafted onto *Citrus macrophylla* and *Citrus reshni* rootstocks (seedlings from Viveros Calipant S.L., Murcia, Spain) was conducted. These varieties were cultivated in an experimental farm located in Orihuela, Spain (38.06733781,-0.98229272). During the harvest period, the farm had an electrical conductivity (EC) of 0.47 dS m^{-1} (20 °C), a pH of 7.56, a temperature of 17 °C, and a relative humidity (RH) of 49 %. To carry out the study, a total of 200 fruits were collected from 24 trees that were 3 years old. Additionally, to ensure empirical rigor, the 200 fruits were randomly collected from various trees distributed across homogeneous plots at each cultivation site. Moreover, any fruits exhibiting visible damage or anomalies were systematically excluded according to the recommendations of Forner-Giner et al. (2023a). Uniform management practices were implemented throughout the experiment to minimize external influences on the evaluated parameters. The sampling was performed in February 2023, as the commercial consumption period for blood oranges in Spain extends from January to March. Only fruits that reached commercial maturity were manually selected according to strict standards and immediately transported to the laboratory for further processing.

In the laboratory, a thorough cleaning of the blood oranges’ surface was carried out using distilled water to remove any potential dirt residues. Subsequently, the color of the peel was measured in 25 fruits, with two measurements taken for each fruit ($n = 50$), and the weight and size of an additional 25 fruits were determined. Following that, a destructive analysis of the fruits was conducted by cutting them in half to determine the number of carpels, peel thickness, and peel weight ($n = 25$). Next, the juice from each blood orange variety was extracted using a manual commercial juicer (Citromatric Deluxe, MPZ-22, Braum, Madrid, Spain). Finally, the remaining peel, consisting of albedo and flavedo, was cut into small pieces and divided into six replicates ($n = 6$), with approximately 180 g of peel per replicate. These peel samples were subjected to freeze-drying using a lyophilizer (LyoMicron, Coolvacuum, Barcelona, Spain) for subsequent metabolomic analysis.

2.2. Fruit morphological characterization

In this study, analyses of the fruit morphological parameters were conducted using the method described by Forner-Giner et al. (2023a), with specific modifications for this research. To determine the average weight of the fruit and rind in the blood orange varieties, a precision digital scale (model BL-600; Sartorius, Germany) was used. The number of carpels was visually counted by observing the interior of the fruit. To determine the peel thickness and measure the fruit size, including the equatorial diameter and length, a digital electronic sliding caliper (model CD-15 DC; Mitutoyo, Japan) was employed. This instrument allowed for precise and accurate measurements of the morphological parameters of the studied fruits. These measurements were crucial in obtaining quantitative and detailed data on the shape and physical characteristics of the analyzed blood oranges.

2.3. Peel color determination

To assess color parameters, a Minolta C-300 chroma meter (Minolta Corp., Osaka, Japan) coupled with a DP-301 data processor (Minolta Corp.) was used. Measurements were taken at two equidistant points on the rind of each fruit, following the guidelines of the International Commission on Illumination (CIE) and expressed in L^*, a^*, and b^* values. All color determinations were performed under standardized conditions using D65 light (6500 K) to simulate natural daylight. Furthermore, the Minolta CR-400 chroma meter was calibrated daily with a certified white standard to ensure measurement accuracy. Based on the recorded values, the hue angle (H°) was calculated using the equation $H^\circ = \arctan(b^*/a^*)$, while chroma ($C^*$) was determined using the formula $C^* = (a^{*2} + b^{*2})^{1/2}$. Previous studies (McGuire, 1992) have identified these two parameters as the most representative and easily interpretable color variables. Additionally, the color index (CI) was calculated using the equation $CI = 1000\, a^*/L^* b^*$ (Jimenez-Cuesta et al., 1981). These measurements provided a precise characterization of the chromatic properties of the analyzed fruits, offering essential information for evaluating and comparing their visual appearance.

2.4. Primary metabolites analysis by ^{1}H-Nuclear magnetic resonance spectroscopy (^{1}H NMR)

The primary metabolites in the peel of blood oranges, including amino acids, organic acids, and sugars, were analyzed. The method employed was based on the procedure described by van der Sar et al. (2013), with slight modifications specified by Martínez-Nicolás et al. (2023). A 50 mg lyophilized sample was taken for analysis and mixed with a MeOH/H_2O solution (1:1) in 2 mL Eppendorf tubes. The mixture was sonicated (2.7 L Ultrasonic cleaner, Toctech) for 30 minutes at 4 °C. Subsequently, it was centrifuged at 10,000 rpm for 12 minutes at 4 °C. The resulting supernatant was evaporated overnight using a Speed-Vacuum at a maximum temperature of 27 °C until the complete evaporation of the liquid phase. The obtained soluble solid was then reconstituted in 800 µL of 100 mM potassium phosphate buffer (100 mM KH_2PO_4, pH = 6) (dissolved in 100 % D_2O) containing 0.58 mM dissolved TPS. The sample was vortexed for 2 minutes and filtered through 0.45 µm filters (Millipore, Burlington, MA, USA). A volume of 650 µL was transferred to a ^{1}H NMR tube for further analysis. All ^{1}H NMR spectra were recorded at 298 K on a Bruker AVIII HD 500 ^{1}H NMR spectrometer (500.16 MHz for ^{1}H) equipped with a 5 mm CryoProbe Prodigy Broadband Observe cryogenic probe (Biospin; Bruker, Bremen, Germany). Results are reported as g Kg^{-1} DW of blood orange peel.

2.5. Secondary metabolites analysis by HPLC-Diode array detection-electrospray ionization-mass spectrometry (HPLC-ESI-DAD-MS^n)

The analysis of specific phenolic compounds in the peel of various blood orange varieties was carried out using a modified version of the method described by Legua et al. (2016), with slight adjustments outlined below. To initiate the analysis, 50 mg of lyophilized peel was meticulously combined with 5 mL of methanol (MeOH). The resulting mixture was subjected to vigorous vortexing for a duration of 5 minutes, followed by a 12-minute sonication step at 4 °C using an Ultrasonic cleaner (Toctech) with a capacity of 2.7 L. After the sonication, the heterogeneous mixture obtained underwent centrifugation at a speed of 5,000 rpm for 5 minutes. To ensure the purity of the sample, the supernatant was meticulously filtered through a 0.45 µm filter (Millipore, Burlington, MA, USA) before being introduced into the chromatograph system for analysis. The mobile phase employed for the chromatographic separation consisted of a combination of acetonitrile and a water-formic acid solution in a ratio of 5:95 (v/v), while maintaining a constant flow rate of 0.85 mL/min. A precisely measured injection volume of 15 µL was introduced into the chromatograph for each analysis. The chromatographic analyses were performed using a highly sophisticated Agilent series 1100 HPLC-ESI-DAD-MS^n Ion Trap system (Agilent, Waldbronn, Germany). This cutting-edge HPLC system, equipped with a DAD detector series 1100, was seamlessly coupled with a mass spectrometer featuring an ion trap and the electrospray ionization (ESI) interface. This instrumental setup facilitated the comprehensive identification and quantification of the individual phenolic compounds in the blood orange peel. The quantification of the identified phenolic compounds was reported as mg Kg^{-1} DW of blood orange peel.

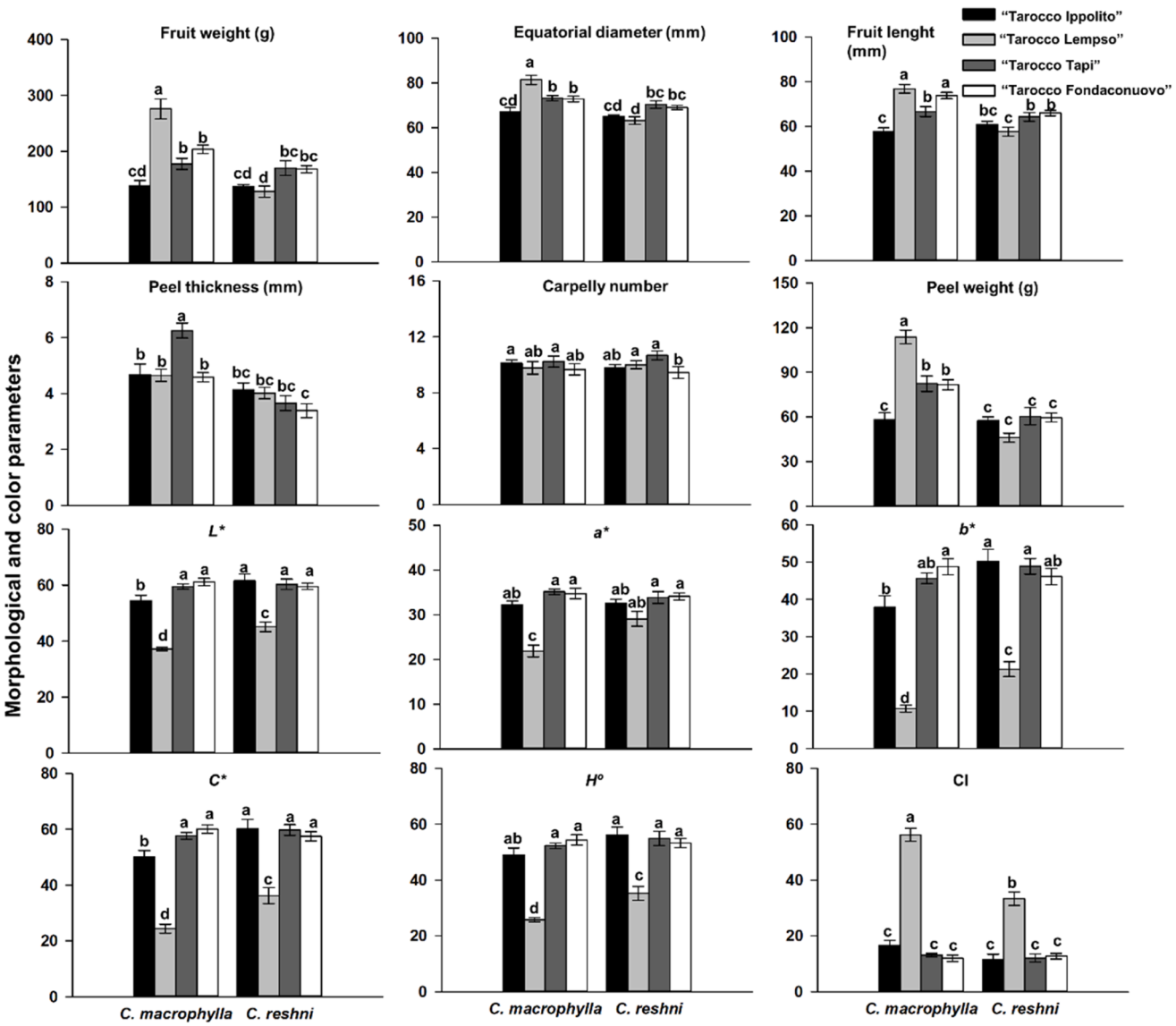
2.6. Statistical analysis

Primary and secondary metabolite data were collected from six replicates ($n = 6$), while morphological parameters were measured using 25 replicates ($n = 25$), and color parameters were assessed with 50 replicates ($n = 50$). Following the recommendations of (Forner-Giner et al., 2023a,b), a sample size of six replicates was selected, as this number is sufficient to capture the inherent variability of metabolomic profiles. Similarly, according to the guidelines of (Forner-Giner et al., 2023a,b), preliminary analyses indicated that 25 replicates for morphological parameters provide a statistical power, allowing for the detection of moderate differences with high reliability. Finally, in accordance with the recommendations of (Legua et al., 2021), it was determined that 50 replicates for color measurements are suitable for identifying subtle variations in pigmentation, thereby ensuring the accuracy of the obtained results. Before performing the ANOVA, assumptions of normality and homogeneity of variances were checked using the Shapiro-Wilk and Levene's tests, respectively, within SPSS 28.0. A variance analysis (ANOVA) (Fraiman and Fraiman, 2018; Jodar-Abellan et al., 2024) was then performed, and significant differences between group means were determined using Tukey's HSD test with a significance level of $p < 0.05$. Additionally, effect sizes (e.g., partial eta squared) were calculated to quantify the magnitude of the observed differences. Furthermore, a Principal Component Analysis (PCA) (Hotelling, 1933) was conducted using Stat Graphics Centurion v. 18.1.12. For PCA, data were first standardized to remove scale effects, and the Pearson correlation matrix was used as input. Components were extracted based on eigenvalues greater than 1, and a Varimax rotation was applied to facilitate interpretation. Data adequacy for PCA was confirmed using the Kaiser-Meyer-Olkin (KMO) measure and Bartlett's test of sphericity.

3. Results and discussion

3.1. Morphological parameters

The morphological parameters of *Citrus* fruits play an essential role in the agri-food industry Martínez-Nicolás et al., (2023). Their accurate measurement and control allow for the selection of high-quality fruits, optimization of sorting and packaging processes, and meeting market demands and consumer preferences (Boz et al., 2020). Understanding the importance of these morphological parameters is crucial for *Citrus* fruit producers, distributors, and processors as it contributes to improving the quality and competitiveness of products in the agri-food industry (Curzi et al., 2015). In this study, we investigated the effects of rootstocks and varieties on the morphological characteristics of blood oranges. Eight different combinations of rootstocks and blood orange varieties were evaluated, and measurements were taken for fruit weight, equatorial diameter, fruit length, peel thickness, number of carpels, and peel weight (Fig. 1). The results revealed that both rootstocks and varieties had a significant impact on the morphological characteristics of blood oranges. Significant differences ($p < 0.05$) were observed among rootstock and variety combinations in terms of fruit weight, equatorial diameter, fruit length, peel thickness, and peel weight. However, not significant differences ($p > 0.05$) were found in the number of carpels among the different rootstock and variety combinations evaluated. For example, regarding fruit weight, "Tarocco Lemso" grafted onto *Citrus macrophylla* obtained the highest value, while "Tarocco Lemso" grafted onto *Citrus reshni* recorded the lowest value. For equatorial diameter and fruit length, the highest values were found in "Tarocco Lemso" grafted onto *Citrus Macrophylla*. The peel thickness had the highest value in "Tarocco Tapi" grafted onto *Citrus Macrophylla*, while "Tarocco Fondaconuovo" grafted onto *Citrus reshni* showed the lowest peel thickness. These parameters are important as they provide information about the overall quality of *Citrus* fruits (Forner-Giner et al., 2023b). Fruit weight and equatorial diameter are indicators of their size, which is an important factor for consumers and for marketing (Dono et al., 2016). A heavier and larger fruit is often perceived as more attractive and of better quality. Peel thickness is an important parameter for fruit resistance and protection (Zhang et al., 2019). A thicker peel can better protect the pulp from possible physical damage, such as impacts or injuries during handling and transportation. Additionally, peel thickness can influence the shelf life of the fruit (Fernández-Muñoz et al., 2022), as a thicker peel can help prolong its freshness and resistance to deterioration. Overall, this study provides further evidence that both rootstocks and varieties play a crucial role in influencing the morphological characteristics of blood oranges. Similar observations have been made in previous studies conducted by Forner-Giner et al. (2023a,b) on blood



p-value	Fruit weight	Equatorial diameter	Fruit length	Peel thickness	Carpelly number	Peel Weight	L*	a*	b*	C*	H°	CI
A: Rootstock	0,000 *	0,000 *	0,000 *	0,000 *	0,664	0,000 *	0,000 *	0,013 *	0,000 *	0,000 *	0,000 *	0,000 *
B: Variety	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *
A*B	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *

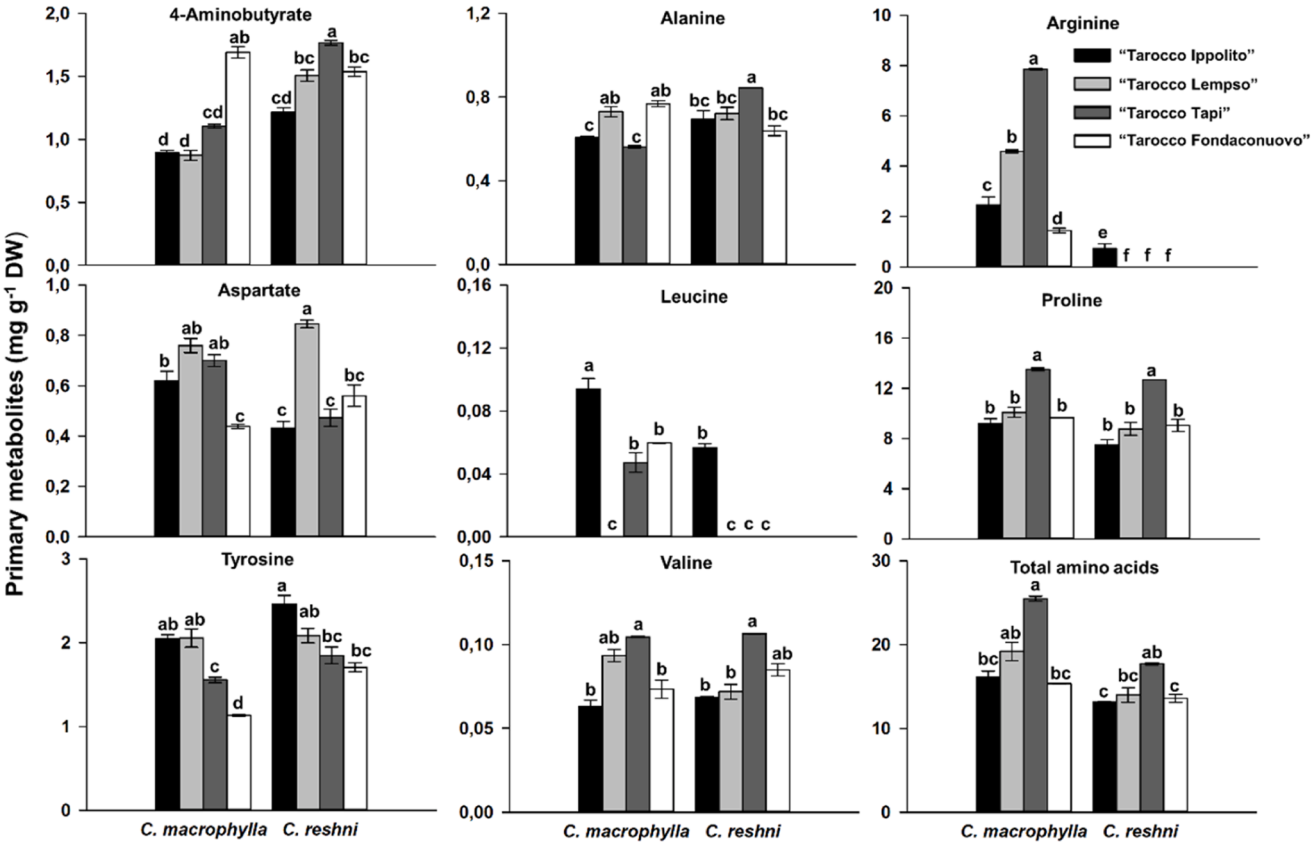
Fig. 1. Morphological and color parameters of four blood orange cultivars ("Tarocco Ippolito", "Tarocco Lempso", "Tarocco Tapi" and "Tarocco Fondaconuovo") grafted onto *Citrus macrophylla* and *Citrus reshni*. Values are the mean ± SE ($n = 25$ in morphological parameters or $n = 50$ in color parameters). Values followed by the same letter, within the same graph, were not significantly different ($p < 0.05$), according to HSD Tukey's least significant difference test. In the *p*-value table, the main effects of rootstock (A), variety (B), and their interaction (A*B) are presented for all measured morphological and color parameters.

oranges, Legua et al., (2018) on lemons or Maciá-Vázquez et al., (2024) on mandarins. Thus, our findings suggest that the appropriate selection of rootstock-variety combinations can influence fruit weight, size, peel thickness, and other important morphological characteristics. These findings can be used by *Citrus* producers and growers to improve the quality and yield of fruits and meet market demands and consumer preferences.

3.2. Peel color

Color parameters in the peel of *Citrus* fruits play a key role in evaluating quality, ripeness, and consumer acceptance (Kayesh et al., 2013). Their objective measurement and quantification allow for precise characterization of the visual properties of the fruits, as well as differentiation between varieties and maturity stages (Ranganath, 2022). These parameters are highly relevant in the agri-food industry as they influence consumer perception, product selection, and food production

(Kayesh et al., 2013; Maciá-Vázquez et al., 2024). Therefore, it is essential for *Citrus* producers, distributors, and manufacturers to carefully consider color parameters to ensure quality and competitiveness in the agri-food market. In this study, the effects of rootstocks and varieties on the main color parameters of blood orange peel were investigated (Fig. 1). The analyzed parameters included brightness (L*), red-green component (*a**), yellow-blue component (*b**), chroma (*C**), hue angle (*H*°), and chroma intensity (CI). The results obtained in this study obtained by Minolta C-300 chroma meter clearly confirm the influence of both rootstocks and varieties on the color parameters of blood orange peel, coinciding with what was observed by (Forner-Giner et al., 2023a, b) in blood oranges. The results showed that rootstocks had a significant effect on the color parameters of blood orange peel. This indicates that the appropriate choice of rootstock can contribute to obtaining fruits with brighter, more intense, and visually appealing colors. On the other hand, the variety also showed a significant influence on the color parameters of the peel. In fact, significant differences ($p < 0.05$) were observed in L*, *a**, *b**, *C**, *H*°, and CI among the different evaluated varieties. This means that the selection of specific varieties can allow for obtaining fruits with different colors, adapted to market trends. The interaction between rootstocks and varieties was also revealed as an important factor as observed in previous studies on blood orange peel (Morales et al., 2021a,b). The results suggest that the choice of rootstock and blood orange variety can have a combined impact on the color of the fruit peel. This implies that the effect of each factor cannot be considered separately, but rather the specific combination of rootstock and variety can influence the appearance and color of the fruit peel differently than if they were considered individually. The color results extracted from this study can have several practical applications in the agri-food industry. Firstly, the results can be used by *Citrus* producers to select the most suitable rootstocks and varieties based on production goals and market preferences. On the other hand, these findings can also be used in *Citrus* breeding programs. Identifying rootstocks and varieties that enhance the color parameters of the peel can serve as a basis for developing new varieties with superior color characteristics. This would contribute to strengthening the industry's competitiveness and meeting consumer demands for the quality of *Citrus* products.



p-value	4-Aminobutyrate	Alanine	Arginine	Aspartate	Leucine	Proline	Tyrosine	Valine	Total amino acids
A: Rootstock	0,000 *	0,037 *	0,000 *	0,571	0,000 *	0,018 *	0,002 *	0,869	0,000 *
B: Variety	0,000 *	0,238	0,000 *	0,114	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *
A*B	0,003 *	0,000 *	0,000 *	0,412	0,072	0,784	0,193	0,080	0,013 *

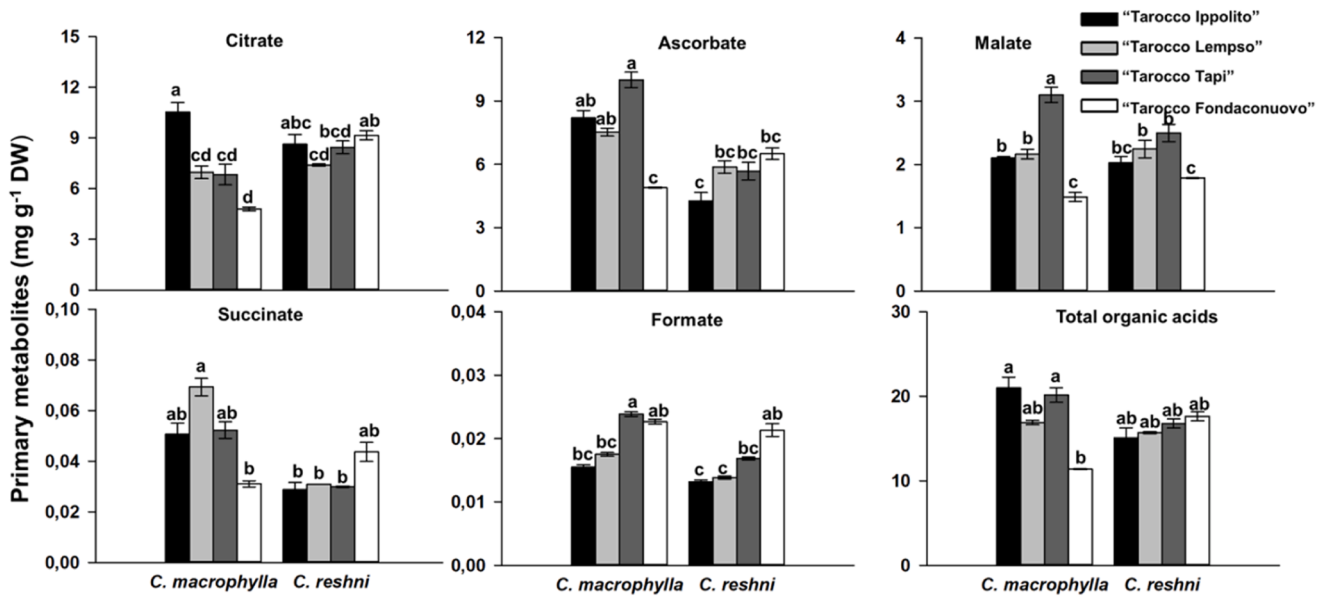
Fig. 2. Content of individual and total amino acids in the peel of four blood orange cultivars ("Tarocco Ippolito", "Tarocco Lempso", "Tarocco Tapi" and "Tarocco Fondaconuovo") grafted onto *Citrus macrophylla* and *Citrus reshni*. Values are the mean ± SE ($n = 6$). Values followed by the same letter, within the same graph, were not significantly different ($p < 0.05$), according to HSD Tukey's least significant difference test. In the *p*-value table, the main effects of rootstock (A), variety (B), and their interaction (A*B) are displayed for each amino acid parameter.

3.3. Primary metabolites content in peel

The amino acids present in the *Citrus* peel play an important role in the agri-food industry by contributing to the flavor, aroma, sensory quality, functional properties, and nutritional value of food products and co-products (Zhang et al., 2024). Their presence and appropriate proportions are crucial for obtaining high-quality products that meet consumer preferences (Boz et al., 2020). Additionally, they have significant industrial applications as nutritional supplements in functional foods and dietary supplements (Prandi et al., 2019). In this study, amino acid profiles were evaluated in different combinations of rootstocks and varieties, and the concentrations of 4-aminobutyrate, alanine, arginine, aspartate, leucine, proline, tyrosine, and valine were identified and quantified using ^{1}H-Nuclear Magnetic Resonance Spectroscopy (Fig. 2). Regarding the effect of rootstocks, significant differences ($p < 0.05$) were found in the levels of 4-aminobutyrate, alanine, arginine, leucine, proline, tyrosine, and total amino acid content. However, not significant differences ($p > 0.05$) were observed in the levels of aspartate and valine. These results suggest that the rootstock may have an effect on the accumulation of certain amino acids in blood oranges, as observed in previous studies on white orange peel (Liu et al., 2023). Several mechanisms could be involved in the influence of rootstocks on amino acid accumulation in the peel of blood oranges. Firstly, different rootstocks may influence different root characteristics, such as nutrient absorption efficiency (Sarkhosh et al., 2021), which could influence the availability of amino acids for synthesis and accumulation in the peel tissues. Additionally, rootstock has been observed to affect gene expression (López-Hinojosa et al., 2021) and the activity of key enzymes involved in amino acid metabolism (McGee et al., 2022). These factors can influence amino acid synthesis and metabolism, and the choice of rootstock affects not only nutrient availability but also the regulation of key metabolic pathways. An appropriate rootstock can enhance nutrient uptake efficiency, modulate enzyme activity related to amino acid metabolism, and optimize the plant's response to stress factors, enabling better adaptation and higher accumulation of specific metabolites. Regarding the blood orange varieties, significant differences ($p < 0.05$) were observed in the contents of 4-aminobutyrate, arginine, leucine, proline, tyrosine, valine, and total amino acids. This confirms that the selected genotype can have a substantial impact on the amino acid profiles of the peel, as described in previous studies on white orange (Liu et al., 2023). Furthermore, the metabolic pathways related to the biosynthesis and transport of amino acids can also vary according to the genotype (Decouard et al., 2022). These genetic factors may interact with environmental conditions, such as nutrient availability, temperature, or light, to influence the accumulation of amino acids in the orange peel. These results highlight the importance of carefully selecting blood orange varieties to obtain specific amino acid profiles in the peel. Furthermore, the analysis of the significant interaction between rootstock and variety revealed significant differences ($p < 0.05$) in the profiles of several amino acids, including 4-aminobutyrate, alanine, arginine, and total amino acid content. This confirms that the combination of rootstock and variety influences the accumulation of amino acids in the peel of blood oranges. These findings may have important implications for the selection of optimal combinations of rootstocks and varieties to improve the quality and nutritional value of extracts obtained from blood orange peel.

Organic acids play an essential role in the quality and nutritional properties of these *Citrus* fruits (Forner-Giner et al., 2023a; Maciá-Vázquez et al., 2024). Their presence in adequate proportions contributes to the freshness, acidity, and complexity of flavor in products and co-products such as peel extracts (Coban, 2020), which can be incorporated into various food matrices, improving food preservation or producing enhanced flavors or aromas (Delgado and Fleuri, 2016). Additionally, organic acids possess antioxidant and health-promoting properties (Zeng et al., 2022), making them valuable components from a nutritional standpoint. Understanding the importance of organic acids in the peel of blood oranges allows producers and manufacturers to maximize the utilization of these compounds in the agri-food industry, developing high-quality co-products that provide them with a competitive advantage. In this study, the levels of organic acids in the peel of blood oranges were identified and quantified using ^{1}H-Nuclear Magnetic Resonance Spectroscopy in different combinations of rootstocks and varieties (Fig. 3). The results obtained revealed significant differences ($p < 0.05$) in the content of organic acids among the different combinations of rootstocks and varieties evaluated. Indeed, it was observed that the rootstock had a significant effect ($p < 0.05$) on the levels of citrate, malate, succinate, and formate. For example, blood oranges grafted onto *Citrus macrophylla* showed higher levels of ascorbic acid or succinic acid compared to those grafted onto *Citrus reshni*. This could be explained by the fact that the rootstock can influence the efficiency of nutrient absorption (Sarkhosh et al., 2021). Different rootstocks can have varying root characteristics, such as nutrient uptake capacity or carbohydrate translocation efficiency towards the fruit (Liu et al., 2023). These differences can affect the availability of substrates for the synthesis of organic acids and, consequently, their accumulation in the peel of blood oranges. Additionally, the rootstock can also influence the enzymatic activity related to the synthesis and degradation of organic acids in the fruit (Asayesh et al., 2023). These enzymes could be differentially regulated depending on the rootstock used, which can influence the rate of synthesis and accumulation of organic acids in the peel of blood oranges. In this way, the rootstock could modulate the plant's physiological response to these stress factors and, therefore, influence the accumulation of organic acids. This modulation can significantly impact the biosynthesis and accumulation of organic acids, as the rootstock determines the plant's capacity to cope with and adapt to these factors, ultimately affecting the fruit's composition. Regarding the variety, significant differences ($p < 0.05$) were also found in the content of citrate, formate, and total organic acids. For example, the "Tarocco Tapi" variety grafted onto *Citrus macrophylla* exhibited the highest content of ascorbic acid, while the "Tarocco Fondaconuovo" variety grafted onto *Citrus macrophylla* showed the lowest content of this acid. Similarly, the "Tarocco Ippolito" variety grafted onto *Citrus macrophylla* showed the highest content of citric acid, while the "Tarocco Fondaconuovo" variety grafted onto *Citrus macrophylla* had the lowest content of this acid. These results indicate that the selection of specific varieties can influence the profiles of organic acids in the peel of *Citrus* fruits, as previously suggested by (Forner-Giner et al., 2023a) in blood oranges. Gene expression profiles and metabolic pathways related to the production and transport of organic acids can vary according to the variety (Mignard et al., 2022), which could be affect the synthesis and metabolism of organic acids. On the other hand, the interaction between the rootstock and the variety also showed significant effects on the content of citrate, ascorbic acid, malate, succinate, and total organic acids. This suggests that the specific combination of rootstock and variety can impact the composition of organic acids in the peel of blood oranges. These findings provide valuable information for the agri-food industry, offering opportunities to improve the quality and diversity of derived *Citrus* products, in this case, the peel, in the market. The results of this study can be used by food producers and manufacturers to select the most suitable combinations of rootstocks and varieties that meet quality standards and satisfy market preferences.

The sugar profile in blood oranges plays a fundamental role in the sensory quality and nutritional value of these *Citrus* fruits (Legua et al., 2021; Forner-Giner 2023a; Maciá-Vázquez et al., 2024). Its proper understanding and application in the agri-food industry enable the development of products with distinctive flavor profiles (Delgado and Fleuri, 2016). In this study, the sugar contents of different combinations of rootstocks and blood orange varieties were identified and quantified using ^{1}H-Nuclear Magnetic Resonance Spectroscopy (Fig. 4). Regarding the rootstock effect, it was found to have a significant impact ($p < 0.05$) on the contents of fructose, glucose, and total sugar. For example, blood oranges grafted onto *Citrus macrophylla* showed higher levels of fructose,



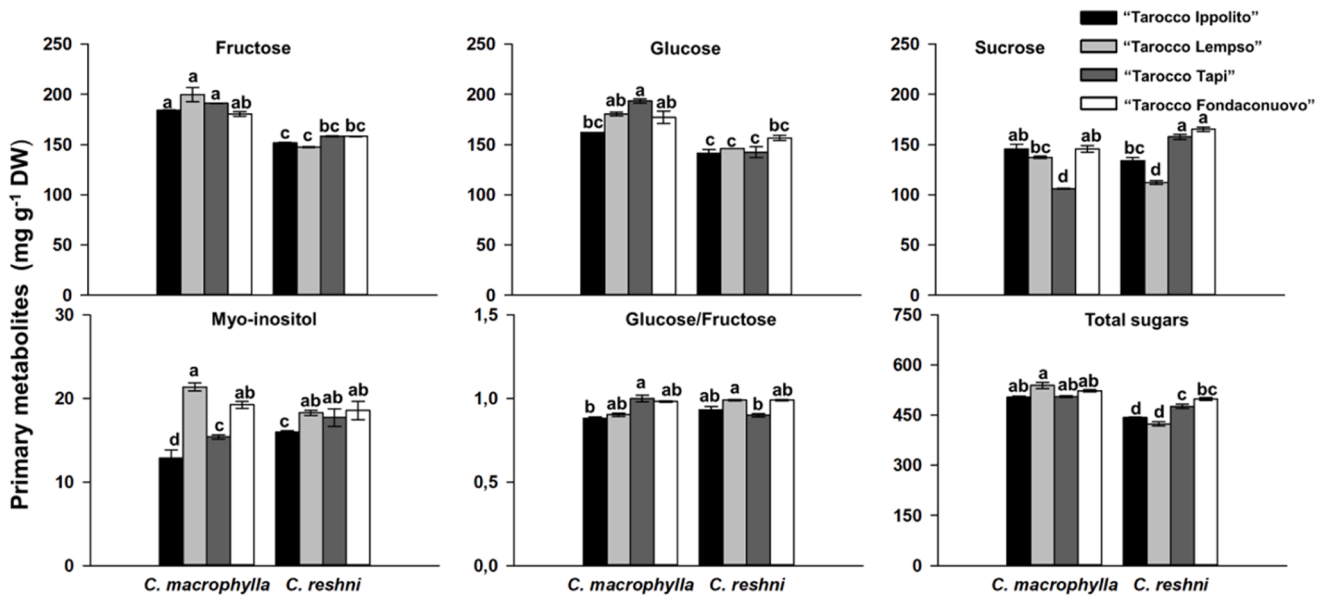
p-value	Ascorbate	Citrate	Malate	Succinate	Formate	Total organic acids
A: Rootstock	0,111	0,000 *	0,000 *	0,000 *	0,000 *	0,308
B: Variety	0,053 *	0,008 *	0,161	0,161	0,000 *	0,046 *
A*B	0,031 *	0,000 *	0,003 *	0,003 *	0,086	0,003 *

Fig. 3. Content of individual and total organics acids in the peel of four blood orange cultivars ("Tarocco Ippolito", "Tarocco Lempso", "Tarocco Tapi" and "Tarocco Fondaconuovo") grafted onto *Citrus macrophylla* and *Citrus reshni*. Values are the mean ± SE ($n = 6$). Values followed by the same letter, within the same graph, were not significantly different ($p < 0.05$), according to HSD Tukey's least significant difference test. In the *p*-value table, the main effects of rootstock (A), variety (B), and their interaction (A*B) are shown for each organic acid parameter.

glucose, and total sugar compared to those grafted onto *Citrus reshni*. However, not significant differences ($p > 0.05$) were found in the sucrose and myo-inositol content, suggesting that these sugars may be less influenced by the rootstock compared to fructose and glucose. This could be due to the rootstock's effect on sugar accumulation in the fruit peel through various physiological mechanisms such as nutrient uptake capacity (Sarkhosh et al., 2021) and metabolites translocation efficiency towards the fruit (Habibi et al., 2022) or influence on enzyme activity related to carbohydrate metabolism (Vittal et al., 2023). Regarding the variety effect, significant differences ($p < 0.05$) were observed in the contents of glucose, sucrose, myo-inositol, or total sugar. For example, "Tarocco Tapi" variety grafted onto *Citrus macrophylla* showed the highest glucose content, while "Tarocco Ippolito" variety grafted onto *Citrus reshni* showed the lowest glucose content. In terms of sucrose, "Tarocco Fondaconuovo" variety grafted onto *Citrus reshni* presented the highest content, while "Tarocco Tapi" variety grafted onto *Citrus macrophylla* showed the lowest content. These differences may be related to the genetic and metabolic characteristics of each variety, which affect sugar synthesis and accumulation in the fruit (Mignard et al., 2022). On the other hand, the observed differences in total sugar content were related to variations in the individual contents of fructose, glucose, and sucrose discussed earlier. Furthermore, the interaction between rootstock and variety also showed a significant interaction ($p < 0.05$) in the contents of fructose, glucose, myo-inositol, glucose/fructose ratio, or total sugar content. This indicates that the specific combination of rootstock and variety can significantly influence the sugar profiles of oranges. Our findings are consistent with Liu et al., (2023), who observed that the specific combination of rootstock and variety can influence the sugar profiles of *Citrus* orange peel. These differences can have important implications for sensory quality, nutritional value, and their potential application as peel extracts in the agri-food industry. The findings of this study can be used by producers and food manufacturers to select the most suitable combinations that allow obtaining co-products, in this case, blood orange peel, with desired sugar profiles that can be incorporated into food matrices, imparting qualities such as sweetness to the final product, thereby meeting market demands.

3.4. Secondary metabolites content in peel

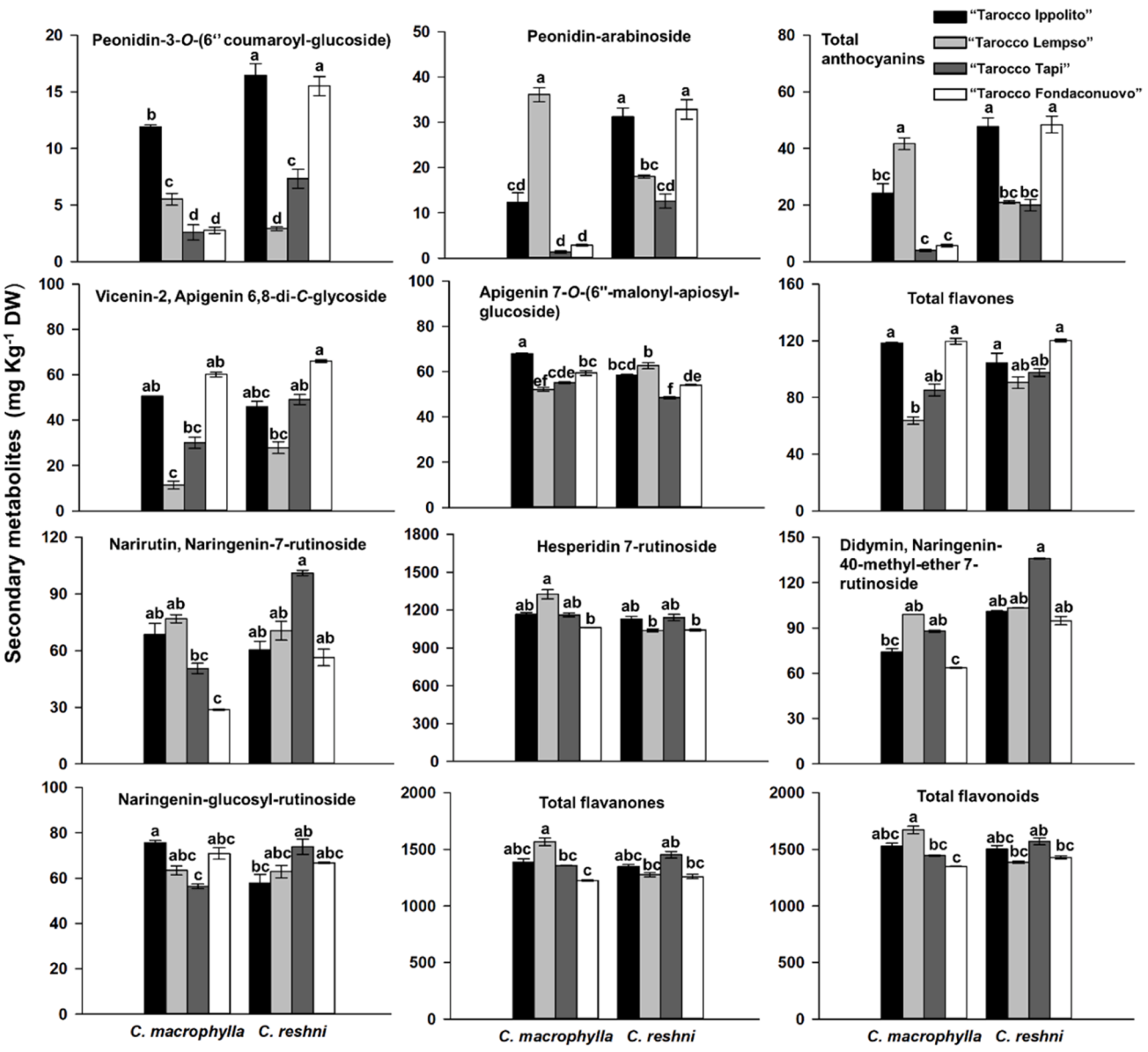
Phenolic compounds, such as flavonoids including anthocyanins, flavones, or flavanones, are present in the peels of fruits (Zhang et al., 2024) play a vital role in both the agri-food industry (Gil-Martín et al., 2022) and the promotion of human health (Sun and Shahrajabian, 2023). The appropriate selection of rootstocks can influence the accumulation of these compounds in blood oranges (Legua et al., 2021; Forner-Giner et al., 2023b; Xiong et al., 2024), offering opportunities to improve and diversify the nutritional and functional profiles of blood oranges. These findings have the potential to develop value-added products in the agri-food industry (Delgado and Fleuri, 2016). For example, the high levels of flavonoids, as suggested in Forner-Giner et al. (2023b), can be harnessed to formulate natural food preservatives (Puttongsiri et al., 2025), while the elevated concentration of anthocyanins documented in Forner-Giner et al. (2023a) supports their use in the development of supplements with potent anti-inflammatory or anti-ulcer disease properties (Prayoga et al., 2025). In this study, the contents of individual secondary metabolites identified and quantified by HPLC-ESI-DAD-MS^n in the peel of the studied varieties are presented in Fig. 5. Two individual anthocyanins (peonidin-3-*O*-(6'-coumaroyl-glucoside)): Rt = 9.5 min, $[M - H]^-$ at m/z 610, 300 and UV/vis = 520 nm and peonidin-arabinoside: Rt = 12.6 min, [M



p-value	Fructose	Glucose	myo-inostol	Sucrose	Glucose/Fructose	Total sugars
A: Rootstock	0,000 *	0,000 *	0,654	0,118	0,000 *	0,000 *
B: Variety	0,452	0,018 *	0,003 *	0,005 *	0,001 *	0,456
A*B	0,044 *	0,021 *	0,105	0,000 *	0,000 *	0,045 *

Fig. 4. Content of individual and total sugars in the peel of four blood orange cultivars ("Tarocco Ippolito", "Tarocco Lempso", "Tarocco Tapi" and "Tarocco Fondaconuovo") grafted onto *Citrus macrophylla* and *Citrus reshni*. Values are the mean ± SE ($n = 6$). Values followed by the same letter, within the same graph, were not significantly different ($p < 0.05$), according to HSD Tukey's least significant difference test. In the *p*-value table, the main effects of rootstock (A), variety (B), and their interaction (A*B) are represented for each sugar parameter.

$-H]^-$ at m/z 448, 300 and UV/vis = 520 nm), two flavones (vicenin-2, apigenin 6,8-di-*C*-glycoside: Rt = 10.8 min, $[M-H]^-$ at m/z 593, 473, 383, 353 and UV/vis = 290 nm and apigenin 7-*O*-(6″-malonyl-apiosyl-glucoside): Rt = 14.7 min, $[M-H]^-$ at m/z 649 and UV/vis = 290 nm), and four flavanones (naringenin-glucosyl-rutinoside: Rt = 14.4 min, $[M-H]^-$ at m/z 741, 579, 433, 271 and UV/vis = 290 nm, narirutin, naringenin-7-rutinoside: Rt = 15.7 min, $[M-H]^-$ at m/z 579, 271 and UV/vis = 290 nm, hesperidin 7-rutinoside: Rt = 17.5 min, $[M-H]^-$ at m/z 609, 301 and UV/vis = 290 nm, and didymin, naringenin-40-methyl-ether 7-rutinoside: Rt = 22.2 min, $[M-H]^-$ at m/z 593, 285 and UV/vis = 290 nm) were identified and quantified. Regarding the effect of rootstocks, significant differences ($p < 0.05$) were observed in the contents of peonidin-3-*O*-(6″-coumaroyl-glucoside), peonidin-arabinoside, total anthocyanins, apigenin 7-*O*-(6″-malonyl-apiosyl-glucoside), hesperidin 7-rutinoside, and didymin, naringenin-40-methyl-ether 7-rutinoside. This suggests that the choice of rootstocks influences the accumulation of phenolic compounds in the peel of blood oranges because rootstocks affect nutrient absorption, enzymatic activity, and the regulation of metabolic pathways related to the synthesis and accumulation of phenols. These variations can determine the quantity and type of phenolic compounds present in the fruit. Following this line, previous studies have demonstrated that different rootstocks can modulate the synthesis and accumulation of these compounds in blood orange (Forner-Giner et al., 2023b). However, not significant differences ($p > 0.05$) were found in the content of vicenin-2, apigenin 6,8-di-*C*-glycoside, narirutin, naringenin-7-rutinoside, naringenin-glucosyl-rutinoside, or total flavones, flavanones, and flavonoids. One of the physiological mechanisms that could explain the differential effect of rootstocks on the accumulation of phenolic compounds is nutrient uptake capacity (Sarkhosh et al., 2021). Rootstocks can influence nutrient absorption and transport through various mechanisms. Specifically, they can alter root architecture, such as the root surface area and root hair density, which affects the plant's ability to take up water and essential nutrients from the soil (Mesquita et al., 2016). Rootstocks may also affect the activity of nutrient transporters that move ions like nitrogen into the plant (Caruso et al., 2025), all of which are critical for the synthesis of phenolic compounds. Furthermore, rootstocks can impact the flow of carbohydrates and other metabolites from the root to the scion (Santos et al., 2024), potentially providing or limiting the availability of specific substrates needed for phenolic biosynthesis, such as sugars or phenolic precursors like amino acids. These physiological changes in the rootstock, in turn, affect the synthesis and accumulation of phenolic compounds in the grafted plant, influencing fruit quality and composition. Regarding variety, significant differences ($p < 0.05$) were observed in the contents of all evaluated compounds, except for naringenin-glucosyl-rutinoside. These variations indicate that different varieties have distinct profiles of these compounds, which can be attributed to their genetic and metabolic characteristics, as observed in other blood orange varieties (Legua et al., 2021; Forner-Giner et al., 2023a,b). Furthermore, the interaction between rootstocks and variety showed significant effects ($p < 0.05$) on the contents of all evaluated compounds. This suggests that the specific combination of rootstocks and variety plays a crucial role in determining the concentrations of these compounds in blood orange peels. Liu et al., (2023) and Forner-Giner et al. (2023b) suggested that the rootstock-variety interaction may play an important role in the accumulation of phenolic compounds. Different blood orange varieties can have different genetic and metabolic profiles, which can influence the plant's capacity to synthesize and accumulate phenolic compounds (López-Hinojosa et al., 2021; Legua



p-value	Peonidin-3-*O*-(6'' coumaroyl-glucoside)	Peonidin-arabinoside	Total anthocyanyns	Vicenin-2, Apigenin 6,8-di-*C*-glycoside	Apigenin 7-*O*-(6''-malonyl-apiosyl-glucoside)	Total flavones	Narirutin, Naringenin-7-rutinoside	Hesperidin-7-rutinoside	Didymin, Naringenin-40-methyl-ether 7-rutinoside	Naringenin-glucosyl-rutinoside	Total flavonones	Total flavonoids
A: Rootstock	0,000 *	0,000 *	0,000 *	0,109	0,002 *	0,287	0,079	0,002 *	0,000 *	0,611	0,092	0,342
B: Variety	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,050 *	0,009 *	0,000 *	0,397	0,001 *	0,008 *
A*B	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *	0,000 *

Fig. 5. Content of individual and total secondary metabolites in the peel of four blood orange cultivars ("Tarocco Ippolito", "Tarocco Lempso", "Tarocco Tapi" and "Tarocco Fondaconuovo") grafted onto *Citrus macrophylla* and *Citrus reshni*. Values are the mean ± SE ($n = 6$). Values followed by the same letter, within the same graph, were not significantly different ($p < 0.05$), according to HSD Tukey's least significant difference test. In the *p*-value table, the main effects of rootstock (A), variety (B), and their interaction (A*B) are provided for each secondary metabolite parameter.

et al., 2021). The interaction between rootstocks and variety can modulate the expression of genes related to phenolic compound biosynthesis (Zhang et al., 2022), which in turn affects the accumulation of these compounds in blood orange peels. These results may be relevant to the agri-food industry as it allows for the selection of specific rootstocks that promote the synthesis of bioactive compounds in blood orange peels, enhancing their nutritional profiles. By choosing the appropriate rootstocks, farmers and producers can optimize the quality and added value of their crops, making them more competitive from a commercial perspective. On the other hand, this information is also valuable for plant breeders and genetic improvement programs as they can focus on developing blood orange varieties with specific profiles of health-beneficial compounds. Finally, the natural co-products industry and dietary supplement industry can utilize this data to develop extracts and formulations from blood orange peels, harnessing their content of bioactive compounds to create value-added products and promote health.

3.5. Principal component analysis (PCA)

The principal component analysis (PCA) revealed significant differences in the rootstock of the studied in terms of morphological parameters, peel color, amino acid content, organic acids, sugars, and secondary metabolites (Fig. 6), which is consistent with previous findings by Legua et al. (2021), Forner-Giner et al. (2023a), and Forner-Giner et al. (2023b) in various blood orange varieties. The first two principal components captured a large proportion of the total variation in each dataset, demonstrating their ability to represent the distinctive characteristics of the varieties (Forner-Giner et al., 2023a). Additionally, all figures depicted the Pearson correlation between the varieties, a

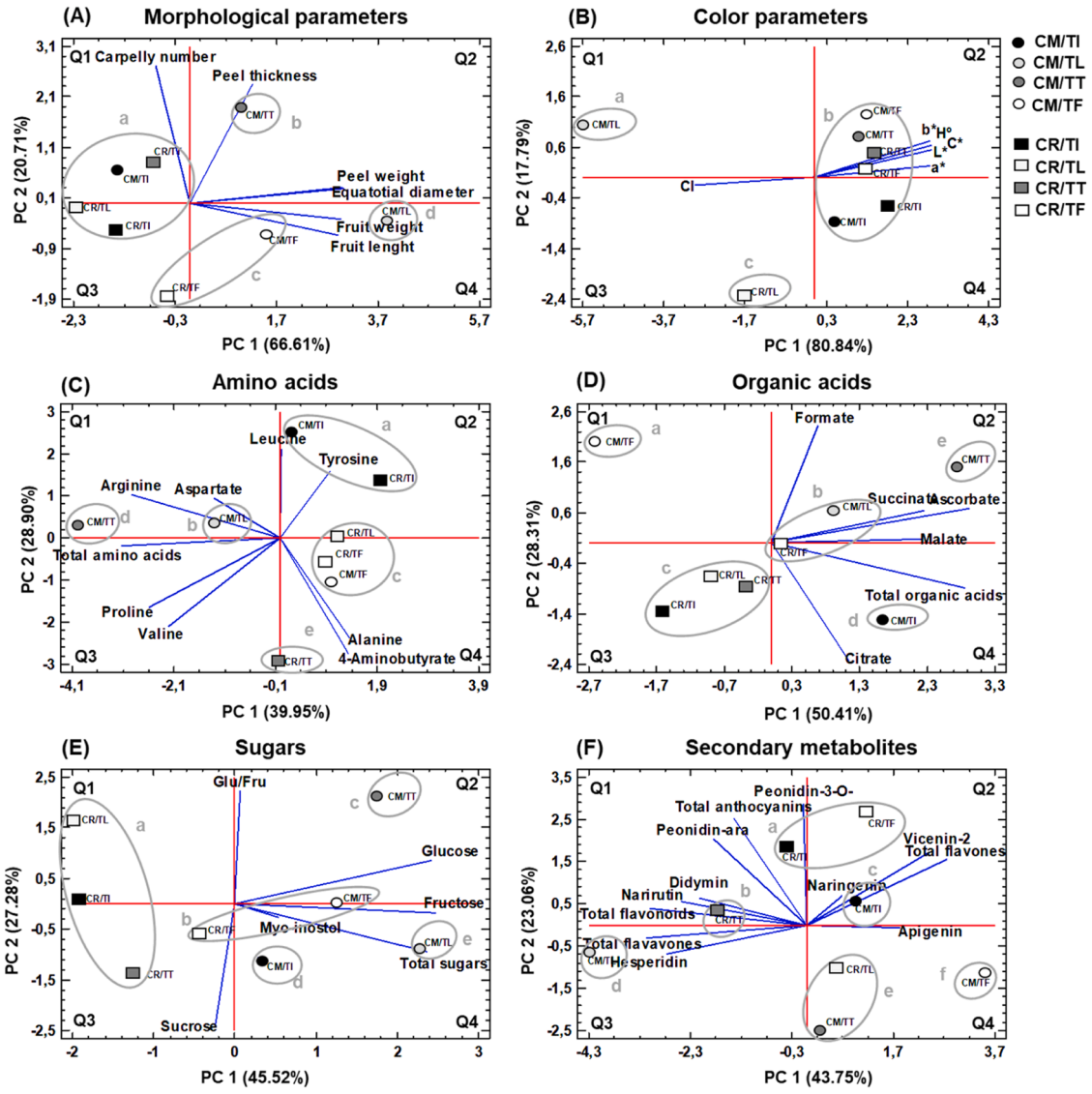


Fig. 6. PCA was conducted to analyze the morphological (A) and color (B) parameters, content of individual and total amino acids (C), content of individual and total organic acids (D), content of individual and total sugars (E), and content of individual and total secondary metabolites (F) in the peel of four blood orange cultivars ("Tarocco Ippolito," "Tarocco Lempso," "Tarocco Tapi," and "Tarocco Fondaconuovo") grafted onto *Citrus macrophylla* and *Citrus reshni*. For PCA, the data were standardized to eliminate scale effects, using the Pearson correlation matrix as the basis. PC with eigenvalues greater than 1 were selected, a criterion indicating that each component retains more variance than a standardized original variable. Subsequently, a Varimax rotation was applied to facilitate the interpretation of the components. Graft-rootstock genotypes were grouped based on identified statistical correlations, revealing distinct groupings in each case. Note: CM/TI: "Tarocco Ippolito" grafted onto *Citrus macrophylla*; CM/TL: "Tarocco Lempso" grafted onto *Citrus macrophylla*; CM/TT: "Tarocco Tapi" grafted onto *Citrus macrophylla*; CM/TF: "Tarocco Fondaconuovo" grafted onto *Citrus macrophylla*; CR/TI: "Tarocco Ippolito" grafted onto *Citrus reshni*; CR/TL: "Tarocco Lempso" grafted onto *Citrus reshni*; CR/TT: "Tarocco Tapi" grafted onto *Citrus reshni*; CR/TF: "Tarocco Fondaconuovo" grafted onto *Citrus reshni*.

valuable statistical tool for establishing relationships between the attributes that define the sample characteristics (Legua et al., 2021; Forner-Giner et al., 2023a). Non-significant results in the correlations, for example between certain amino acids and organic acids, may indicate that additional factors, such as genetic variations, specific environmental conditions, or complex interactions among metabolites, may be modulating the biochemical composition of the blood oranges (Forner-Giner et al., 2023a,b). These unidentified variables could be key to better understanding the observed variability among the studied varieties and warrant further investigation in future studies. The Pearson correlation among the varieties revealed clusters and more specific relationships between the attributes, providing valuable insights into the distinctive features of the analyzed samples. These findings have relevant implications for the classification and characterization of blood orange varieties, as well as for genetic selection and improvement, and they may influence the quality and potential uses of these fruits in the agri-food industry (Puttongsiri et al., 2025; Prayoga et al., 2025).

Regarding morphological parameters (Fig. 6A), the first principal component (PC1) explained 66.61 % of the variability, showing significant positive correlations with peel weight, fruit weight, or equatorial diameter. These results suggest that these variables have a direct and consistent influence on the observed morphological variability among blood orange varieties. In fact, the high component weights in PC1 (0.49 for peel weight, 0.49 for fruit weight, and 0.49 for equatorial diameter) support the importance of these parameters in the morphological differentiation of the varieties, implying a significant relationship with the main physical characteristics of the fruits. On the other hand, the second principal component (PC2), which explained 20.71 % of the variability, showed positive correlations with peel thickness (0.64) and carpel number (0.74), and a negative correlation with fruit length (-0.17), indicating that these characteristics are also important for variety differentiation. Although peel thickness and carpel number have considerable weight in PC2, fruit length shows a negative weight in this component, suggesting that it does not contribute significantly to morphological variability in this case. These results indicate that peel thickness and carpel number are also important morphological characteristics that distinguish the varieties.

Regarding peel color parameters (Fig. 6B), the first principal component (PC1) explained 80.84 % of the variability and showed significant positive correlations with all color variables except chromaticity index (CI), which showed a negative correlation. These results indicate that color variables are strongly related to each other and significantly contribute to the observed variability in blood orange peel pigmentation. Additionally, the high component weights in PC1 for variables such as L* (0.38), a* (0.38), b* (0.37), C* (0.38), and Hº (0.39) reinforce the idea that these characteristics play a key role in the overall variability of peel coloration. The second principal component (PC2) explained 17.79 % of the variability and showed positive correlations with b* (0.20), C* (0.18), and Hº (0.15), suggesting that these characteristics may influence the intensity and hue of the peel color. However, the CI showed a significant negative correlation with PC1 (-0.39) and a much weaker correlation with PC2 (-0.04), suggesting that the chromaticity index may not be as relevant in the differentiation of blood orange varieties compared to other color variables. In this sense, the results confirm that the variability in peel pigmentation is mainly explained by hue, saturation, and luminance variables (L*, a*, b*, C*, and Hº), with a relatively minor impact from CI and fruit weight.

A PCA was also performed to examine the variability in amino acid contents among different varieties (Fig. 6C). The PCA revealed that the first principal component (PC1) explained 39.95 % of the total variability, showing a positive correlation with 4-aminobutyrate (0.22) and alanine (0.23), while presenting negative correlations with arginine (-0.49), aspartate (-0.22), proline (-0.43), and total amino acids (-0.52). These results indicate that 4-aminobutyrate and alanine are the amino acids that most contribute to the observed variation in amino acid contents among the varieties. The second principal component (PC2) explained 28.90 % of the variability, showing positive correlations with leucine and negative correlations with 4-aminobutyrate (-0.51), alanine (-0.44), suggesting that these molecules also play an important role in the differences among the varieties. Together, the first two components explained 68.85 % of the total variability in the data. PC1 is the main contributor to overall variability, especially due to correlations with 4-aminobutyrate, alanine, and arginine, which are key in differentiating the studied varieties. On the other hand, PC2 highlights the importance of leucine and its interaction with other amino acids, suggesting a distinct rootstock in the samples. The negative correlations observed between certain amino acids, such as arginine, proline, and valine, may be related to competition for shared metabolic pathways or protein synthesis regulation (Decouard et al., 2022). This phenomenon suggests that limitations in metabolic resources could influence the relationships between different amino acids. Leucine, with its positive correlation in PC2, may also be an important factor in the differences among varieties, highlighting its relevance in the variability of amino acid contents.

The PCA performed on organic acids (Fig. 6D) shows that the first principal component (PC1) explains 50.41 % of the variability and has a strong positive correlation with ascorbic acid (0.55), malate (0.42), succinate (0.42), and total organic acids (0.53). PC1 also shows a weaker positive correlation with citrate (0.21) and formate (0.13), suggesting that these compounds contribute significantly to the variation in organic acid contents among blood orange varieties. On the other hand, the second principal component (PC2), which explains 28.31 % of the variability, shows a strong negative correlation with citrate (-0.66) and a positive correlation with formate (0.66), indicating that these acids may be key in differentiating the varieties. These correlations suggest that citrate and formate may be involved in competitive or differently regulated metabolic processes between varieties (Mignard et al., 2022), reflecting their possible antagonism or competition in metabolic pathways, while ascorbic acid and succinate are associated with distinct variability in rootstocks.

The PCA performed on sugar contents (Fig. 6E) reveals that the first principal component (PC1) explains 45.52 % of the variability and has positive correlations with fructose (0.58), glucose (0.57), and total sugars (0.56), but a negative correlation with sucrose (-0.06). This indicates that fructose and glucose are key components contributing to the variation in sugar contents among blood orange varieties. PC2 explains 27.29 % of the variability and shows positive correlations with the glucose/fructose ratio (0.63), but a negative correlation with sucrose (-0.68), suggesting that both the glucose/fructose ratio and sucrose could be distinguishing characteristics in terms of sugar contents. These results suggest that the negative correlation with sucrose may reflect a specific metabolic rootstock in which sucrose concentration is inversely related to other sugars, possibly indicating a regulatory mechanism in the studied varieties.

Finally, a PCA was conducted to examine the variability in secondary metabolite contents among different varieties (Fig. 6F). The PCA revealed that the first principal component (PC1) explained 43.75 % of the variability and was positively correlated with various metabolites such as vicenin-2, Apigenin 6,8-di-*C*-glycoside (0.31), apigenin 7-*O*-(6''-malonyl-apiosyl-glycoside) (0.24), total flavones (0.35), and total flavonoids (0.40), while it was negatively correlated with hesperidin 7-rutinoside (-0.36), narirutin, naringenin-7-rutinoside (-0.32), and total flavonones (-0.41). This indicates that these secondary metabolites are important determinants of the variation in contents among blood orange varieties. PC2 explained 23.06 % of the variability and showed positive correlations with metabolites such as, for example, peonidin-3-*O*-(6''-coumaroyl-glucoside) (0.56), peonidin-arabinoside (0.40), and total anthocyanins (0.49), but negative correlations with hesperidin 7-rutinoside (-0.13) and total flavonones (-0.06). These results suggest that these specific metabolites may contribute to differences in secondary metabolite profiles among the varieties. The inverse correlation with certain flavonoids, such as hesperidin, may reflect differences in biosynthetic mechanisms affecting the accumulation of these compounds

(López-Hinojosa et al., 2021; Legua et al., 2021), which in turn could influence the organoleptic and nutritional properties of blood oranges

4. Conclusion

The study investigated the effects of different rootstocks and varieties of blood oranges on morphological characteristics, peel color, and primary and secondary metabolite content of peel. The results revealed that the proper selection of rootstocks and varieties can enhance fruit quality in terms of weight, size or peel thickness. Moreover, both rootstocks and varieties influence significantly peel color, enabling the production of fruits with brighter and more intense colors. Specifically, "Tarocco Lemso" grafted onto *Citrus macrophylla*, showed the highest fruit weight, peel weight, and caliber. It was also found that amino acid, organic acid, sugar, and phenolic compound profiles in blood orange peels are significantly influenced by rootstocks and varieties. Significant differences were observed in the content of most studied metabolites, indicating that the interaction between rootstock and variety can impact the accumulation of these compounds. Specifically, "Tarocco Tapi" grafted onto *Citrus macrophylla* showed the highest concentrations of amino acids. On the other hand, grafts of "Tarocco Lemso" onto *Citrus macrophylla* exhibited high levels of sugars and phenolic compound. In the case of "Tarocco Ippolito" onto *Citrus macrophylla*, high concentrations of total organic acids were found. In general, the varieties grafted onto *Citrus macrophylla* showed higher values of metabolites in blood orange peels compared to those grafted onto *Citrus reshni*. Overall, these findings have important implications for the agri-food industry, genetic improvement programs, and the natural extract industry, as they can be used to improve the quality and nutritional value of incorporating foods and develop high-value-added products.

CRediT authorship contribution statement

María Ángeles Forner-Giner: Investigation, Data curation. **Manuel Ballesta-de los Santos:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pablo Melgarejo:** Visualization, Formal analysis, Data curation. **Juan José Martínez-Nicolás:** Writing – original draft, Visualization, Formal analysis. **Roberto Gómez-Pérez:** Investigation, Formal analysis. **Alberto Continella:** Supervision, Software. **Pilar Legua:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors are grateful to Metabolomics Platform of CEBAS–CSIC for the excellent technical assistance.

Appendices

Not applicable.

Data availability

All data are available via email request to the corresponding author.

Funding

This research was funded by: Generalitat Valenciana, Spain (reference: PID2021-124145OR-C21).

Supplementary information

Not applicable.

Sample availability

Samples of the compounds of blood oranges are available from the authors.

Data availability

Data will be made available on request.

References

Andrade, M.A., Barbosa, C.H., Shah, M.A., Ahmad, N., Vilarinho, F., Khwaldia, K., Silva, A.S., Ramos, F., 2023. Citrus by-products: Valuable source of bioactive compounds for food applications. Antioxidants 12, 38. https://doi.org/10.3390/antiox12010038.

Andrade, M.A., Lima, V., Sanches-Silva, A., Vilarinho, F., Castilho, M.C., Khwaldia, K., Ramos, F., 2019. Pomegranate and grape by-products and their active compounds: Are they a valuable source for food applications? Trends Food Sci. Technol. 86, 68–84. https://doi.org/10.3390/foods10020272.

Asayesh, Z.M., Arzani, K., Mokhtassi-Bidgoli, A., Abdollahi, H., 2023. Enzymatic and non-enzymatic response of grafted and ungrafted young European pear (Pyrus communis L.) trees to drought stress. Sci. Hortic. 310, 111745. https://doi.org/10.1016/j.scienta.2022.111745.

Boz, Z., Korhonen, V., Koelsch-Sand, C., 2020. Consumer Considerations for the implementation of sustainable packaging: A review. Sustainability 12, 2192. https://doi.org/10.3390/su12062192.

Caruso, P., Russo, M.P., Allegra, M., Torrisi, B., Fascella, G., Las Casas, G., Licciardello, C., Caruso, M., Ferlito, F., 2025. Impact of different amounts of biochar as growth media on macronutrient transport systems of carrizo citrange rootstocks and related expression analysis. Agriculture 15 (1), 113. https://doi.org/10.3390/agriculture15010113.

Coban, H.B., 2020. Organic acids as antimicrobial food agents: Applications and microbial productions. Bioprocess. Biosyst. Eng. 43, 569–591. https://doi.org/10.1007/s00449-019-02256-w.

Continella, A., Pannitteri, C., La Malfa, S., Legua, P., Distefano, G., Nicolosi, E., Gentile, A., 2018. Influence of different rootstocks on yield precocity and fruit quality of 'Tarocco Scirè' pigmented sweet orange. Sci. Hortic. 230, 62–67. https://doi.org/10.1016/j.scienta.2017.11.006.

Curzi, D., Raimondi, V., Olper, A., 2015. Quality upgrading, competition and trade policy: Evidence from the agri-food sector. Eur. Rev. Agric. Econ. 42 (2), 239–267. https://doi.org/10.1093/erae/jbu021.

Decouard, B., Bailly, M., Rigault, M., Marmagne, A., Arkoun, M., Soulay, F., Caïus, J., Paysant-Le Roux, C., Louahlia, S., Jacquard, C., Esmaeel, Q., Chardon, F., Masclaux-Daubresse, C., Dellagi, A., 2022. Genotypic variation of nitrogen use efficiency and amino acid metabolism in Barley. Front. Plant Sci. 12, 807798. https://doi.org/10.3389/fpls.2021.807798.

Delgado, C.H.O., Fleuri, L.F., 2016. Orange and mango by-products: Agro-industrial waste as source of bioactive compounds and botanical versus commercial description—a review. Food Rev. Int. 32 (1), 1–14. https://doi.org/10.1080/87559129.2015.1041183.

Donno, D., Cerutti, A.K., Mellano, M.G., Prgomet, Z., Beccaro, G.L., 2016. Serviceberry, a berry fruit with growing interest of industry: Physicochemical and quali-quantitative health-related compound characterisation. J. Funct. Foods. 26, 157–166. https://doi.org/10.1016/j.jff.2016.07.014.

Fernández-Muñoz, R., Heredia, A., Domínguez, E., 2022. The role of cuticle in fruit shelf-life. Curr. Opin. Biotechnol. 78, 102802. https://doi.org/10.1016/j.copbio.2022.102802.

Food and Agriculture Organization of the United Nations (FAO) FAOSTAT. Available online: https://www.fao.org/faostat/en/#data/QCL (accessed on 1 April (2022)).

Forner-Giner, M.Á., Ballesta-de los Santos, M., Melgarejo, P., Martínez-Nicolás, J.J., Melián-Navarro, A., Ruíz-Canales, A., Continella, A., Legua, P., 2023a. Fruit quality and primary and secondary metabolites content in eight varieties of blood oranges. Agronomy 13, 1037. https://doi.org/10.3390/agronomy13041037.

Forner-Giner, M.Á., Ballesta-de los Santos, M., Melgarejo, P., Martínez-Nicolás, J.J., Núñez-Gómez, D., Continella, A., Legua, P., 2023b. Influence of different rootstocks on fruit quality and primary and secondary metabolites content of blood oranges cultivars. Molecules. 28, 4176. https://doi.org/10.3390/molecules28104176.

Fraiman, D., Fraiman, R., 2018. An ANOVA approach for statistical comparisons of brain networks. Sci. Rep. 8, 4746. https://doi.org/10.1038/s41598-018-23152-5.

Gil-Martín, E., Forbes-Hernández, T., Romero, A., Cianciosi, D., Giampieri, F., Battino, M., 2022. Influence of the extraction method on the recovery of bioactive phenolic compounds from food industry by-products. Food Chem. 378, 131918. https://doi.org/10.1016/j.foodchem.2021.131918.

Habibi, F., Liu, T., Folta, K., Sarkhosh, A., 2022. Physiological, biochemical, and molecular aspects of grafting in fruit trees. Hortic. Res. 9, 32. https://doi.org/10.1093/hr/uhac032.

Habibi, F., Ramezanian, A., Guillén, F., Castillo, S., Serrano, M., Valero, D., 2020. Changes in bioactive compounds, antioxidant activity, and nutritional quality of blood orange cultivars at different storage temperatures. Antioxidants 9, 1016. https://doi.org/10.3390/antiox9101016.

Habibi, F., Shahid, M.A., Spicer, R.L., Voiniciuc, C., Kim, J., Gmitter Jr., F.G., Brecht, J. K., Sarkhosh, A., 2024. Postharvest storage temperature strategies affect anthocyanin levels, total phenolic content, antioxidant activity, chemical attributes of juice, and physical qualities of blood orange fruit. Food Chem. Adv. 4, 100722. https://doi.org/10.1016/j.focha.2024.100722.

Hotelling, H., 1933. Analysis of a complex of statistical variables into principal components. J. Educ. Psychol. 24 (6), 417. https://doi.org/10.1037/h0071325.

Jimenez-Cuesta, M., Cuquerella, J., Martínez-Javega, J.M., 1981. Determination of a colour index for citrus fruit degreening. In: *Proceedings of the International Society of Citriculture Citriculture, IV Congress, Tokyo, Japan. 2*, pp. 750–753.

Jodar-Abellan, A., Albaladejo-García, J.A., Aznar-Crespo, P., Ballesta de los Santos, M., Asadollah, S.B.H.S., Derdour, A., Martínez-Nicolás, J.J., Melgarejo, P., Prats, D., Serrano-Bernardo, F., 2024. Exploring expert perceptions towards emerging pollutants and their impacts in reused wastewater and agriculture. Agric. Water. Manage 304 (109098). https://doi.org/10.1016/j.agwat.2024.109098.

Kayesh, E., Shangguan, L., Korir, N.K., Sun, X., Bilkish, N., Zhang, Y., Han, J., Song, C., Cheng, Z.M., Fang, J., 2013. Fruit skin color and the role of anthocyanin. Acta Physiol Plant 35, 2879–2890. https://doi.org/10.1007/s11738-013-1332-8.

Legua, P., Forner, J., Hernández, F., Forner-Giner, M., 2014. Total phenolics, organic acids, sugars and antioxidant activity of mandarin (Citrus clementina Hort. ex Tan.): Variation from rootstock. Sci. Hortic 174, 60–64. https://doi.org/10.1016/j.scienta.2014.05.004.

Legua, P., Forner-Giner, M.Á., Nuncio-Jáuregui, N., Hernández, F., 2016. Polyphenolic compounds, anthocyanins and antioxidant activity of nineteen pomegranate fruits: A rich source of bioactive compounds. J. Funct. Foods. 23, 628–636. https://doi.org/10.1016/j.jff.2016.01.043.

Legua, P., Martinez-Cuenca, M.R., Bellver, R., Forner-Giner, M.A., 2018. Rootstock's and scion's impact on lemon quality in southeast Spain. Int. Agrophys. 32 (3), 325–333. https://doi.org/10.1515/intag-2017-0018.

Legua, P., Modica, G., Porras, I., Conesa, A., Continella, A., 2021. Bioactive compounds, antioxidant activity and fruit quality evaluation of eleven blood orange cultivars. J. Sci. Food Agric. 102, 2960–2971. https://doi.org/10.1002/jsfa.11636.

Leporini, M., Loizzo, M.R., Sicari, V., Pellicanò, T.M., Reitano, A., Dugay, A., Deguin, B., Tundis, R., 2020. Citrus x Clementina Hort. Juice enriched with its by-products (Peels and Leaves): Chemical composition, In vitro bioactivity, and impact of processing. Antioxidants 9, 298. https://doi.org/10.3390/antiox9040298.

Liu, X., Frederick, G., Gmitter, Jr, Jude, W.G., Wang, Y., 2023. Effects of rootstocks on the flavor quality of huanglongbing-affected sweet orange juices using targeted flavoromics strategy. RSC. Adv. 13, 5590–5599. https://doi.org/10.1039/D2RA08182B.

López-Hinojosa, M., de María, N., Guevara, M.A., Vélez, M.D., Cabezas, J.A., Díaz, L.M., Mancha, J.A., Pizarro, A., Manjarrez, L.F., Collada, C., Díaz-Sala, C., Cervera Goy, M. T., 2021. Rootstock effects on scion gene expression in maritime pine. Sci. Rep. 11, 11582. https://doi.org/10.1038/s41598-021-90672-y.

Maciá-Vázquez, A.A., Martínez-Nicolás, J.J., Núñez-Gómez, D., Melgarejo, P., Legua, P., 2024. Influence of rootstock on yield, morphological, biochemical and sensory characteristics of 'Afourer' variety mandarins. Sci. Hortic. 325, 112644. https://doi.org/10.1016/j.scienta.2023.112644.

Martínez-Nicolás, J.J., Hernández, F., Núñez-Gómez, D., García-Sánchez, F., Martínez-Font, R., Legua, P., Melgarejo, P., 2023. Metabolomic approach to study the 'Purple Queen' Pomegranate cultivar response to alternative culture Media and phenological stages. Foods. 12, 352. https://doi.org/10.3390/foods12020352.

McGee, T., Schaffer, B., Shahid, M.A., Chaparro, J.X., Sarkhosh, A., 2022. Carbon and nitrogen metabolism in peach trees on different Prunus rootstocks in response to flooding. Plant Soil. 475, 427–441. https://doi.org/10.1007/s11104-022-05377-6.

McGuire, R.G., 1992. Reporting of objective color measurements. HortScience 27, 1254–1255. https://doi.org/10.21273/HORTSCI.27.12.1254.

Mesquita, G.L., Zambrosi, F.C.B., Tanaka, F.A.O., Boaretto, R.M., Quaggio, J.A., Ribeiro, R.V., 2016. Anatomical and physiological responses of *Citrus* trees to varying boron availability are dependent on rootstock. Front. Plant Sci. 7, 224. https://doi.org/10.3389/fpls.2016.00224.

Mignard, P., Beguería, S., Giménez, R., Font i Forcada, C., Reig, G., Moreno, M.Á., 2022. Effect of genetics and climate on apple sugars and organic acids profiles. Agronomy 12, 827. https://doi.org/10.3390/agronomy12040827.

Morales, J., Bermejo, A., Navarro, P., Quiñones, A., Salvador, A., 2021a. Effect of rootstock on citrus fruit quality: a review. Food Rev. Int. 1–19. https://doi.org/10.1080/87559129.2021.1978093.

Morales, J., Bermejo, A., Navarro, P., Forner-Giner, M., Salvador, A., 2021b. Rootstock effect on fruit quality, anthocyanins, sugars, hydroxycinnamic acids and flavanones content during the harvest of blood oranges 'Moro' and 'Tarocco Rosso' grown in Spain. Food Chem. 342, 128305. https://doi.org/10.1016/j.foodchem.2020.128305.

Prandi, B., Faccini, A., Lambertini, F., Bencivenni, M., Jorba, M., Droogenbroek, B.V., Bruggeman, G., Schöber, J., Petrusan, J., Elst, K., Sforza, S., 2019. Food wastes from agrifood industry as possible sources of proteins: a detailed molecular view on the composition of the nitrogen fraction, amino acid profile and racemisation degree of 39 food waste streams. Food Chem. 286, 567–575. https://doi.org/10.1016/j.foodchem.2019.01.166.

Prayoga, D.K., Aulifa, D.L., Budiman, A., Levita, J., Jiranusornkul, S., 2025. Cyanidin and Cyanidin-3-Glucoside alleviate peptic ulcer disease: insights from in vitro, and in vivo studies. Drug Des. Devel. Ther. 19, 841–856. https://doi.org/10.2147/DDDT.S500645.

Puttongsiri, T., Manichart, N., Teerarak, M., Sikhao, P., Somala, N., Tongsri, P., Pilasombut, K., 2025. Siamese neem tree as a natural preservative: chemical profile, antioxidant properties, and antibacterial efficacy against foodborne pathogens and spoilage bacteria. J. Agric. Food Res. 19, 101559. https://doi.org/10.1016/j.jafr.2024.101559.

Ranganath, K.G., 2022. Pigments that colour our fruits: An overview. Erwerbs-Obstbau 64, 535–547. https://doi.org/10.1007/s10341-022-00698-3.

Sarkhosh, A., Shahkoomahally, S., Asis, C., McConchie, C., 2021. Influence of rootstocks on scion leaf mineral content in mango tree (Mangifera indica L.). Hortic. Environ. Biotechnol. 62, 725–735. https://doi.org/10.1007/s13580-021-00355-w.

Santos, A. da S., Gomes, F.A.L., Silva, L. dos S., Paiva Neto, V.B. de, Silva, M.T.L., Silva, A.C.R. da, Cavalcante, Í.H.L., 2024. Rootstock affects phytotechnical attributes, gas exchange, and carbohydrate accumulation in mango scion. Folia Hortic. 36(3), 337–350. doi:10.2478/fhort-2024-0019.

Smeriglio, A., Cornara, L., Denaro, M., Barreca, D., Burlando, B., Xiao, J., Trombetta, D., 2019. Antioxidant and cytoprotective activities of an ancient Mediterranean citrus (Citrus Lumia Risso) albedo extract: Microscopic observations and polyphenol characterization. Food Chem. 279, 347–355. https://doi.org/10.1016/j.foodchem.2018.11.138.

Sun, W., Shahrajabian, M.H., 2023. Therapeutic potential of phenolic compounds in medicinal plants—natural health products for Human health. Molecules. 28, 1845. https://doi.org/10.3390/molecules28041845.

Tayengwa, T., Chikwanha, O.C., Gouws, P., Dugan, M.E.R., Mutsvangwa, T., Mapiye, C., 2020. Dietary citrus pulp and grape pomace as potential natural preservatives for extending beef shelf life. Meat. Sci. 162, 108029. https://doi.org/10.1016/j.meatsci.2019.108029.

van der Sar, S., Kim, H.K., Meissner, A., Verpoorte, R., Choi, Y.H., 2013. Nuclear magnetic resonance spectroscopy for plant metabolite profiling. The Handbook of Plant Metabolomics. John Wiley & Sons, Ltd., pp. 57–76. ISBN 978-3-527-66988-2.

Vittal, H., Sharma, N., Dubey, A.K., Shivran, M., Singh, S.K., 2023. Rootstock-mediated carbohydrate metabolism, nutrient contents, and physiological modifications in regular and alternate mango (Mangifera indica L.) scion varieties. PLoS. One 18 (5), e0284910. https://doi.org/10.1371/journal.pone.0284910.

Wang, Y.C., Chuang, Y.C., Hsu, H.W., 2008. The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. Food Chem. 106, 277–284. https://doi.org/10.1016/j.foodchem.2007.05.086.

Xiong, B., Li, Q., Yao, J., Wang, C., Chen, H., Ma, Q., Deng, T., Liao, L., Wang, X., Zhang, M., Sun, G., He, S., Zhang, X., Wang, Z., 2024. Combined metabolomic and transcriptomic analysis reveals variation in phenolic acids and regulatory networks in the peel of sweet orange 'Newhall' (C. sinensis) after grafting onto two different rootstocks. Sci. Hortic. 323, 112461. https://doi.org/10.1016/j.scienta.2023.112461.

Zeng, X., Li, H., Jiang, W., Li, Q., Xi, Y., Wang, X., Li, J., 2022. Phytochemical compositions, health-promoting properties and food applications of crabapples: a review. Food Chem. 386, 132789. https://doi.org/10.1016/j.foodchem.2022.132789.

Zhang, W., Fu, X., Zhang, Y., Chen, X., Feng, T., Xiong, C., Nie, Q., 2024. Metabolome comparison of Sichuan dried orange peels (Chenpi) aged for different years. Horticulturae 10 (4), 421. https://doi.org/10.3390/horticulturae10040421.

Zhang, F., Zhong, H., Zhou, X., Pan, M., Xu, J., Liu, M., Wang, M., Liu, G., Xu, T., Wang, Y., Wu, X., Xu, Y., 2022. Grafting with rootstocks promotes phenolic compound accumulation in grape berry skin during development based on integrative multi-omics analysis. Hortic. Res. 9, uhac055. https://doi.org/10.1093/hr/uhac055.

Zhang, W., Yin, S., Yu, T.X., Xu, J., 2019. Crushing resistance and energy absorption of pomelo peel inspired hierarchical honeycomb. Int. J. Impact. Eng. 125, 163–172. https://doi.org/10.1016/j.ijimpeng.2018.11.014.