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Metabolite content and antioxidant potential of blood oranges as affected by rootstock

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ABSTRACT

Rootstocks influence several fruit qualitative parameters. The aim of the study was to investigate the role of the rootstock on the accumulation of primary and secondary metabolites in the juices of sweet blood orange cv. Tarocco Sciré at different maturation stages. The tested rootstocks were Carrizo citrange, widely spead in the citrus industry, in comparison with C35 citrange, Bitters and Furr citrandarins recently introduced and on which little informations are available. Three simple sugars and 28 different specialized metabolites were quantified by HPLC/Uv-vis/DAD and HPLC/ESI-MS, the antioxidant potential was assessed by $ABTS^{+}$ and $DPPH^{\bullet}$. Analytical data obtained were processed by robust statistical tools that allowed us to highlight the role of the metabolites during maturation for the nutraceutical value. Bitters and C35 anticipated fruit ripening of nearly one month; Bitters and Furr reached the highest pigmentation one month earlier than the other genotypes.

1. Introduction

Blood or pigmented orange (*Citrus sinensis* L. Osbeck) cultivation is growing randomly in the Mediterranean basin and is well diffused in South Italy; blood oranges represent a good source of health-promoting compounds, such as antioxidants (Grosso et al., 2013). The most important Italian blood orange cultivar is Tarocco; this variety displays a large number of lines with different maturation periods (Caruso et al., 2016) and other peculiar features (Legua et al., 2022). Blood orange fruits are also a reservoir of secondary/specialized metabolites among which anthocyanins, that are responsible for the red/purple colour of blood orange peel and juice (Habibi et al., 2020; Legua et al., 2022). The presence of these compounds depends on different intrinsic and environmental conditions; in particular, temperature plays a crucial role (Modica et al., 2022; Rapisarda et al., 2001). Among anthocyanins, cyanidin 3-*O*-glucoside is the most represented metabolite; other antocyanins (delphinidin, peonidin and petunidin) are also present. On the whole, the high content of hydroxycinnamic acids and flavanones (Cebadera-Miranda et al., 2019; Kelebek et al., 2008; Rapisarda et al., 1998; Rapisarda et al., 2008) confers to pigmented orange juice a superior nutraceutical value (Legua et al., 2022).

During the past years, a large amount of research focused on the set of physiological changes characterizing blood orange fruits during ripening (Lana et al., 2021; Ordóñez-Díaz et al., 2020). The maturation of pigmented fruits is defined by different phenological stages from 81 BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemical Industry), in which the fruit colouration begins, to 89 BBCH, being characterized by an increase in soluble solids content, a decrease in organic acids, and remarkable changes in peel and juice colour. Noticeably anthocyanin accumulation in these two fruit parts is not correlated (Forner-Giner, Ballesta-de los Santos, et al., 2023; Lo Cicero et al., 2018; Lo Piero, 2015).

In citriculture, rootstock is a pivotal agronomic tool aimed to confer vigor to the whole plant, and tolerance to pathogens and to biotic and abiotic stresses; besides, the rootstock may influence several vegetative and productive features (Bennici et al., 2021; Caruso et al., 2020; Caruso et al., 2024; Continella et al., 2018; Incesu et al., 2013; Legua et al., 2014; Morales et al., 2021). Previous investigations assessed that orange cultivars grafted on vigorous rootstocks, such as rough lemon, Rangpur lime, *Citrus macrophylla*, *C. volkameriana*, showed larger peel thickness and lower sugar and organic acid concentrations (Lado et al., 2018). Rootstocks also affect the accumulation of primary (glucose, fructose, sucrose) and secondary (anthocyanins, flavanones, flavones, hydroxycinnamic acids) metabolites in fruits, and consequently the antioxidant

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activity of the juice (Continella et al., 2018; Legua et al., 2022; Modica et al., 2022). Finally rootstock influences ripening period by modulating several biochemical processes related to ripening (Continella et al., 2018; Lana et al., 2021; Morales et al., 2021; Ordóñez-Díaz et al., 2020).

With the aim to evaluate the effect of rootstock genotype on the biochemistry evolution during maturation process, we monitored several key biochemical processes were monitored from November to March (from 184 to 304 days after full bloom, DAFB), on four different rootstock/scion combination. On the collected fruits, quality traits such as colour index, pH and acidity were measured; the contents of selected primary and specialized metabolites involved in fruit quality and palatability were assessed and the antioxidant potential of juices was estimated.

2. Materials and methods

2.1. *Plant material & experimental design*

Fruits of Tarocco Sciré blood orange were harvested from 9 years old trees grafted on four different rootstocks: Carrizo citrange [*C. sinensis* (L.) Osb. cv. Washington navel x *P. trifoliata* (L.) Raf.], C35 citrange (*C. sinensis* cv. Ruby x *P. trifoliata*), Bitters and Furr citrandarins [*C. sunki* Hort. ex Tan. x *P. trifoliata* (L.) Raf.]. Plants were cultivated in an experimental field located in Lentini (37°17′04′’N, 14°53′16′’E, Siracusa, Italy) and were subjected to standard cultural practice. The experimental design was a complete randomized block, with 4 plots of 10 trees and each tree spaced 5 m × 3 m apart. Thirty fruits were collected from 8 trees of each scion/rootstock combination at each sampling. The phenological phases considered were from the breaking of the colour (81 BBCH) until the complete ripeness (89 BBCH). Therefore, fruits were selected every 30 days at mid-month, from November to March for two years (2018–19 and 2019–20) (184, 214, 245, 276 and 304 days after full bloom, DAFB). Fruit juice was extracted with a commercial juice extractor (Kenwood Citrus Juicer JE290, UK), filtered and quickly stored at −80 °C until processed.

2.2. *Citrus colour parameters, total soluble solids content, titratable acidity and pH*

Peel and juice colour was recorded with a Minolta CR-400 chromameter (Minolta Corp., Osaka, Japan). Two reading were performed on the outer equatorial part of the skin of 28 fruits per combination and stages. Results were expressed as L*, a*, b*: L* (brightness or lightness; 0 = black, 100 = white), a* (−a* = greenness, +a* = redness) and b* (−b* = blueness, +b* = yellowness). These values were then used to analyze Hue angle degree [H°* = arctang (b*/a*)], where 0° = red–purple; 90° = yellow, 180° = bluish–green and 270° = blue and chroma [C* = (a*2 + b*2)]1/2, indicate of the colour intensity or saturation. Citrus colour index (CCI = a·1000/L·b), that was widely used in the citrus industry as maturation index, was calculated using coordinates of the Hunter Lab colour space, while a*/b* value was calculated using CIELAB parameters (Caruso et al., 2020; Jiménez-Cuesta et al., 1981). The TSS content was measured using a digital refractometer (Atago Co., Ltd., model PR-32 α, Tokyo, Japan) and expressed as °Brix. Titratable acidity and pH were determined by potentiometric titration (Hach Company, TitraLab AT1000 Series, Loveland, Colorado, USA) of the juice with 0.1 N NaOH beyond pH 8.1 as reported by Consoli et al. (2023); the results expressed as g L^{-1} of citric acid equivalent.

2.3. *Specialized metabolites determination and quantification via HPLC/Uv-Vis /DAD and HPLC/ESI/Orbitrap MS analyses*

All solvents and reagents used in this study were high purity laboratory solvents from VWR (Milan, Italy); HPLC grade water and acetonitrile were also obtained from VWR. High purity standards cyanidin 3-*O*-glucoside, rutin, chlorogenic acid, sinapic acid and p-coumaric acid were purchased from Sigma (VWR chemicals, Milan, Italy), whilst naringin, hesperidin and vitexin were from Extrasynthese (Lyon, France). Small portions (2 mL) of the juices were put in 15 mL plastic sample tubes and 100 μL of formic acid (98 %) were added. Samples were shaked during five minutes, then centrifuged at 4000 rpm for 10 min to separate the solid portion of the juices. 1 mL of the clear supernatants were transferred into 2 mL HPLC amber vials and immediately analysed. Chromatographic analyses were carried out on an Ultimate 3000 UHPLC focussed instrument equipped with a binary high-pressure pump, a Photodiode Array detector, a Thermostatted Column Compartment and an Automated Sample Injector (Thermo Fisher Scientific, Inc., Milan, Italy). Collected data were processed through a Chromeleon Chromatography Information Management System v. 6.80. Chromatographic runs and DAD acquisitions were all performed according to Pannitteri et al. (2017). A series of HPLC/ESI/MS analyses were also performed on a selected number of representative samples to confirm spectrophotometry-based peak assignments. In this case, aliquots (5 mL) of previously centrifuged juices were freeze dried (Lyoquest-85, Telstar Italy, Legnano, Milan, Italy) then re-dissolved in 2 mL HPLC grade water and transferred into 2 mL HPLC amber vials ready to ESI/MS analyses. ESI mass spectra were acquired by a Thermo Scientific Exactive Plu Orbitrap MS (Thermo Fisher Scientific, Inc., Milan, Italy), using a heated electrospray ionization (HESI II) interface. LC/ESI/MS settings and mass spectra acquisition were performed according to our previous works (Modica et al., 2022; Pannitteri et al., 2017) All analyses were carried out in triplicate; results are reported in milligram (mg) of compound per liter (L) of juice.

2.4. *HPLC /ELSD analyses and quantification of simple sugars*

HPLC grade water and acetonitrile were high purity laboratory solvents from VWR (Milan, Italy). Simple sugars (fructose, glucose and sucrose) contents were evaluated using a Varian 9010 instrument (Varian Inc., Palo Alto, CA, USA) equipped with an Alltech 3300 evaporative light scattering detector (ELSD) (BÜCHI Labortechnik, AG, Flawil, CH). Small portions (2 mL) of the juices were transferred in 15 mL plastic centrifuge tubes and centrifuged at 4000 rpm for 10 min. 10 μL of the clear supernatant solutions were injected using a graduated HPLC syringe. A Luna Omega 3 μm SUGAR column (250 × 4.6 mm, 5 μm particle size) from Phenomenex (Torrance, CA, USA) was used for eluting and separating simple sugars with the following conditions: isocratic gradient CH_3CN-H_2O/25:75, 0–30 min; flow rate 1 mL/min. The ELSD was set to a probe temperature of 85 °C, a gain of 1.0, and the nebulizer gas nitrogen adjusted to 2.0 L/min. The retention time was respectively 7.06 min for fructose, 7.66 min for glucose and 10.68 min for sucrose. Analyses were always performed in triplicate.

2.5. *Antioxidant activity (ABTS$^+$ and DPPH$^{\bullet}$)*

Antioxidant activity was determined by ABTS$^+$ and DPPH$^{\bullet}$ methods for each sample. A methanol extract was prepared, using 1 mL of each sample juice sample mixed with 10 mL of MeOH/water (80,20, *v*/v) +1 % HCl, and the mixture was sonicated mat 20 °C for 15 min and left for 24 h at 4 °C. Then, the extract was again sonicated for 15 min, and centrifuged at 10000 ×*g* for 10 min. The radical scavenging activity was evaluated using the DPPH$^{\bullet}$ radical (2,2-diphenyl-1-picrylhydrazyl) method and the ABTS$^+$ [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation method. The decrease in absorbance of all samples was measured in a UV–visible spectrophotometer (NanoDrop 2000, Thermo Scientific) at 515 nm and 730 nm for DPPH$^{\bullet}$ and ABTS$^+$, respectively. A calibration curve was performed with Trolox ((*R*)-(+)-6-hydroxy-2,5,7,8-tetramethyl-croman-2-carboxylic acid) (0 to 20 nmol) from Sigma (Madrid, Spain) and results were expressed as mmol of Trolox equivalent per kg of fresh weight (mM Trolox kg^{-1} FW).

2.6. *Statistical analysis*

Analysis of variance (ANOVA) was carried out using STATISTICA 6.0 (Statsoft Inc., Tulsa, Oklahoma) and used to test the significance of each variable ($P \leq 0.05$). A basic descriptive statistical analysis was followed by an analysis of variance test for mean comparisons. The method used to discriminate among the means (Multiple Range Test) was Fisher's Least Significant Difference (LSD) procedure at a 95.0 % confidence level. The correlation was performed using R software computing package "ggcorrplot" (Kassambara & Kassambara, 2019). Principal component analysis (PCA) was performed using R software computing the "prcomp" function of the package 'tidyverse' (Wickham et al., 2019). The results were represented using the package 'ggplot2' (Wickham et al., 2016).

3. Results

3.1. *Internal and external fruit colour parameters*

The evolution of peel and juice colour in Tarocco Scirè fruits is shown in Table 1 and Fig. S1. As regarding peel colour, at the beginning of the observation period (184 DAFB) citrange C35 registered the highest value (2.1) of Citrus Colour Index (CCI), while Furr the lowest (1.0). The epicarp gradually changed from green to orange/red at 214 DAFB, in which citrange Carrizo had the highest CCI value and C35 the lowest; no statistical differences were observed between Bitters, Furrand the others rootstocks. At the same maturation stage, peel fruit of Tarocco Sciré highly incremented a* values with respect to the previous sampling, meaning that in this period the colour break from green/yellow to orange colour occurred for all combinations. At 245 DAFB, the peel of C35 reached the highest values of a*/b* and CCI, maintaining it until full maturity (304 DAFB). At harvest, Bitters and Carrizo showed the highest CCI and a*/b* values. At the end of the experiment, the a* value ranged from 27.9 of C35, to 31.7 of Carrizo.

With respect to juice colour, a* values were small and negative in all rootstock/scion combinations at both 184 and 214 DAFB, as they represent the balance between red (+) and green (−) colour. Furr had the highest value of a*, while Bitters had the lowest negative parameters because of the green appearance at 184 DAFB (Table 1); nevertheless, at the beginning of observations Bitters manifested the highest a*/b* values, maintaining this trend up to the harvest time (304 DAFB). Juice colour progressively changed from yellow/orange to deep orange/reddish at 245 DAFB, especially in citrandarins which showed the highest value of both a* and a*/b* and the lowest of H°. The two citranges induced a remarkable increase in juice colour at 276 DAFB, as regarding a* value (Table 1), while the two citrandarins reached the highest CCI values (Fig. S1) that maintained up to the harvest (304 DAFB) At full maturity, C35 and Bitters had the highest values of CCI and the lowest of H°, so they showed the deepest reddish juice.

3.2. *Citric acid, pH and simple sugars content of Tarocco Sciré*

The TSS, Citric acid content (g L^{-1}) and pH values of Tarocco Sciré blood orange juice grafted onto different rootstocks are reported in Table 2. At the beginning of the trial, the TSS ranged from 9.7°Brix (Furr) to 10.5°Brix (Bitters). The TSS values increased progressively in all rootstocks tested. At 304 DAFB, C35 and Bitters showed the highest TSS value (12.5 and 11.9, respectivelly). As expected, citric acid contents and pH values in the juices are inversely proportional, the latter increasing while the former decreased, and this was observed for all the studied rootstocks. At 184 DAFB, Furr registered the highest citric acid value (28.3 g L^{-1}), followed by C35 (26.7 g L^{-1}). At 214 DAFB, Carrizo showed a slight decrease in citric acid content, while C35 had the highest decrement (−18.0 %). At 245 DAFB Furr recorded a remarkable diminution in the content of citric acid (−34.2 %) and exhibited the lowest pH (3.2). At 276 DAFB, Bitters reached its minimum value in

Table 1

Changes in peel and juice colour of Tarocco Sciré blood orange grafted onto different rootstocks registered from 184 to 304 days after full bloom (DAFB). Values without letters, within the same row, have no significant differences according to Fisher's LSD procedure at 95 % confidence level (n = 30).

DAFB		L*				a*				b*				C*				H°				a*/b*			
		Bitters	Furr	C35	Carrizo	Bitters	Furr	C35	Carrizo	Bitters	Furr	C35	Carrizo	Bitters	Furr	C35	Carrizo	Bitters	Furr	C35	Carrizo	Bitters	Furr	C35	Carrizo
Peel	184	79.62 b	81.07 ab	82.00 a	82.53 a	10.50 ab	7.51 b	10.66 ab	12.10 a	79.44 a	79.00 a	78.87 b	78.33 b	80.38 a	79.77 b	80.88 a	79.41 b	82.26 b	85.02 a	82.54 b	82.50 b	0.13 b	0.14 ab	0.14 ab	0.15 a
	214	79.16 a	78.52 b	79.56 a	78.82 b	24.94	25.06	24.14	24.96	78.30	79.82	80.79	77.03	82.26	84.31	84.46	82.31	72.25	72.73	73.43	72.51	0.32 b	0.32 b	0.30 b	0.35 a
	245	74.37	76.34	75.05	73.98	30.72	27.90	28.41	29.15	76.44	77.48	76.66	73.92	81.72	83.53	81.97	79.74	68.60	70.40	69.51	68.20	0.41	0.36	0.38	0.40
	276	72.78	72.06	74.48	72.39	32.14 a	32.33 a	29.34 b	31.24 ab	74.53	73.07	75.44	71.99	81.31 a	79.95 ab	81.15 a	77.35 b	66.59 ab	66.12 b	68.62 a	66.53 ab	0.44	0.45	0.40	0.45
	304	72.16	72.67	73.90	72.43	30.57 a	30.90 a	27.93 b	31.65 a	70.95 b	74.49 a	75.32 a	71.82 ab	79.04 b	80.62 a	80.95 a	79.46 b	66.85	67.57	69.25	67.85	0.55 a	0.42 b	0.38 b	0.46 ab
Juice	184	39.59 a	30.04 c	35.87 b	35.90 b	−8.04 c	−3.39 a	−6.12 b	−6.49 b	19.25 a	7.11 c	12.76 b	14.54 b	20.89 a	7.87 c	14.15 b	15.93 b	112.77 b	115.42 a	115.61 a	114.15 ab	−0.42 a	−0.48 b	−0.48 b	−0.45 a
	214	36.10	35.93	35.15	37.71	−5.10	−6.45	−5.42	−6.21	15.46 ab	15.10 ab	14.84 b	17.41 a	16.28 ab	16.43 a	15.81 b	18.48 a	108.26	113.17	110.06	109.59	−0.33 a	−0.43 b	−0.36 ab	−0.37 ab
	245	33.84 ab	31.87 b	33.86 ab	34.76 a	3.78 a	3.79 a	0.65 b	0.52 b	17.14 ab	16.20 b	18.48 a	17.24 ab	17.56 ab	16.75 b	18.80 a	17.52 ab	77.66 b	76.70 b	86.72 ab	87.44 a	0.22 a	0.24 a	0.06 b	0.05 b
	276	29.94 ab	30.00 a	28.43 b	27.88 b	9.01 a	4.53 b	7.76 ab	5.52 b	11.35 ab	12.64 a	11.07 b	10.33 b	14.63 a	13.49 a	13.52 a	11.79 b	51.72 b	70.12 a	53.32 b	61.83 a	0.81 a	0.30 b	0.70 a	0.54 b
	304	28.67	29.97	28.91	28.26	7.87 a	4.07 b	9.01 a	6.64 ab	9.72 b	10.94 a	11.07 a	9.91 ab	12.65 ab	12.04 ab	14.47 a	11.96 b	51.27 b	67.67 a	50.48 b	56.31 ab	0.83 a	0.40 b	0.85 a	0.67 b

Table 2
Total soluble solids (TSS), citric acid and pH values of Tarocco Sciré blood orange juice grafted onto different rootstocks, measured from 184 to 304 days after full bloom (DAFB). Values followed by the same lowercase letter, within the same row, have no significant differences according to Fisher's LSD procedure at 95 % confidence level (n = 30).

DAFB	TSS (°Brix)				Citric acid (g L^{-1})				pH			
	Bitters	Furr	Carrizo	C35	Bitters	Furr	Carrizo	C35	Bitters	Furr	Carrizo	C35
184	10.5 a	9.7 b	10.2 a	10.3a	24.5 b	28.3 a	22.6 b	26.7 ab	0.33	0.40	0.47	0.33
214	11.0 a	10.5 b	10.8 b	11.4 a	23.5 b	27.8 a	21.9 b	21.9 b	0.43	0.40	0.47	0.40
245	11.7 a	11.5 a	10.9 b	11.7 a	16.2	18.3	15.8	19.1	3.77 a	3.18 b	3.80 a	3.73 a
276	11.9	11.5	11.5	11.9	14.6 b	17.9 a	13.9 b	17.0 a	3.80	3.51	3.84	3.60
304	11.9	11.6	11.6	12.5	14.6 b	19.0 a	12.3 b	15.0 b	3.57	3.70	3.88	3.80

citric acid along the observation period (up to 304 DAFB). The same phenomenon occurred in Carrizo and C35 citranges, but only at full maturity (304 DAFB), with C35 showing the greatest decrement (−11.9 %) (Table 2).

As shown in Fig. 1, total sugar (glucose + fructose + sucrose) content in Tarocco juices depended on ripening stage. At the beginning of the observation period (184 DAFB) total sugars ranged from 62.5 (Furr) to 64.8 g L^{-1} (Bitters); a noticeable increase was observed at 214 DAFB for all the rootstocks studied that continued to raise up sugar content until 245 DAFB, with the exception of Carrizo citrange, whose sugar content raised up only at 276 DAFB, nearly one month later. At harvest (304 DAFB), the total sugar content ranged from 105.5 g L^{-1} in fruits of Tarocco Scirè grafted onto Carrizo to 125.9 g L^{-1} in those grafted onto C35, which exhibited an important increment at the last sampling. Fruits on Furr and Bitters citrandarins reached the highest sugar amount already at 245 DAFB and maintained this quantity up to the harvest time.

The effects exerted by the different rootstocks studied is noticeable when considering the individual sugar contents along the entire observation period (Fig. S2). At 184 DAFB, Bitters recorded the lowest values in glucose content (12.7 g L^{-1}), even if no statistical difference was observed among the rootstocks tested. An increase in glucose levels was already observed at 214 DAFB in fruit grafted onto Bitters (+51 %), Furr (+41 %) and C35 (+30 %) but not in Carrizo, whose glucose level peaks only at 276 DAFB (+53 %). At 304 DAFB, C35 had the highest concentration of glucose (31.1 g L^{-1}), and no statistical difference was registered between Bitters and Furr (Fig. S2). Regarding the content of fructose, no statistical difference was appreciable at 184 DAFB for all juices. At 245 DAFB, fruits of Tarocco Sciré grafted onto Furr showed the highest values of fructose (21.4 g L^{-1}), followed by fruits grafted onto Bitters (20.5 g L^{-1}). At harvest (304 DAFB), C35 registered the highest fructose level (Fig. S2). Similarly to glucose and fructose, sucrose concentration was also affected by rootstocks, even no statistical difference was found at 184 DAFB. As previously observed, Bitters and Furr recorded the highest increase in sucrose at 245 DAFB, while in Carrizo occurred a month later (276 DAFB) and in C35 two months later (304 DAFB). At full ripening, the concentration of sucrose in Tarocco juices ranged from 57.8 of Carrizo to 68.6 g L^{-1} of C35 citrange.

3.3. *Analysis of specialized metabolites accumulation during maturation*

3.3.1. *Hydroxycinnamic acids*

Eleven different hydroxycinnamic acid-based metabolites were identified and quantified in the juices of Tarocco Scirè, namely two caffeic acid derivatives (caffeoyl –hexose and chlorogenic acid), four p-coumaric acid derivatives (p-coumaroyl hexose and three p-coumaroylquinic acid isomers), four ferulic acid derivatives (feruloyl hexose, two feruloylquinic acid isomers and ferulic acid), and sinapic acid (Table S1). Molecules belonging to this group were gathered according to the hydroxycinnamic acid nucleus (caffeic, p-coumaric, ferulic and sinapic) and their variations monitored along the observation period, from 184 to 304 DAFB, as showed in Fig. S3. At 184 DAFB, p-coumaroyl acids were the most abundant compounds, followed by ferulic acids. Significant statistical differences were observed in both groups as depending on the rootstock applied; e.g. Tarocco juice from fruits grafted on Bitters showed the highest contents (103.96 mg L^{-1} of p-coumaric acids and 41.40 mg L^{-1} of ferulic acid – based metabolites). At 214 DAFB a notable increase of caffeic acids and ferulic acids was

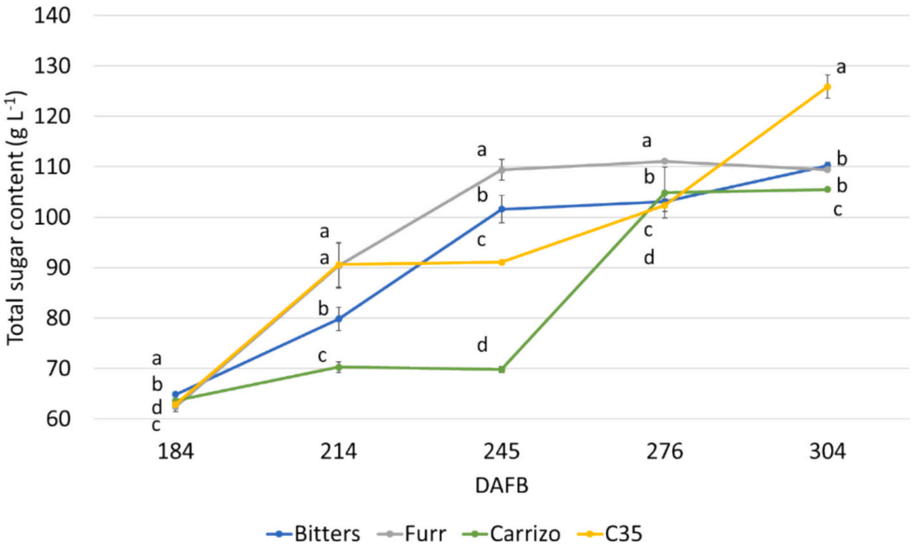

Fig. 1. Total sugar content in the juice of Tarocco Sciré blood orange grafted onto different rootstock from 184 to 304 days after full bloom (DAFB). The values represented are the mean of three replications. Values without letters have no significant differences according to Fisher's LSD procedure at 95 % confidence level (*n* = 30).

observed in Furr genotype until full ripening (304 DAFB). Indeed, a decrement of ferulic acids was observed at 245 DAFB in all rootstocks tested (Fig. S3).

3.3.2. Flavanones, flavones and flavonols

A total of six metabolites belonging to the biochemical subclass of flavanones, flavones and flavonols (Table S1) were identified in the flesh of Tarocco Scirè, namely three flavanones (hesperetin and two narigenin derivatives, narirutin and dydimin), the flavone vitexin, and the two quercetin derivatives rutin and quercitrin (Fig. S4). Hesperidin was registered as the most abundant flavanone, present in remarkable amounts already at the beginning of the observation period (184 DAFB). A significant difference in hesperidin levels was noticed among the rootstocks: in fact, while Carrizo citrange and Furr citrandarin underwent a remarkable decrease in hesperidin at 214 DAFB, C35 citrange and Bitters citrandarin experienced this phenomenon only one month later (245 DAFB). Naringenin-based metabolites were found as the second most abundant compounds in juice of Tarocco Scirè. At 184 DAFB, their amount ranged from 26.87 mg L^{-1} (Furr) to 33.04 mg L^{-1} (Bitters). Similarly to what observed for hesperidin, the amount of naringenin derivatives decreased at 214 DAFB in Tarocco Scirè juices grafted on Carrizo and Furr and nearly only one month later in fruits grafted on Bitters and C35 genotypes. The amounts of the sole flavone detected, vitexin, ranged from 31.17 mg L^{-1} (Carrizo) to 35.35 mg L^{-1} (Furr); for all the roostocks considered in this study, a decrease in vitexin levels was observed at 214 DAFB with no statistical difference. Two flavonols sharing a common aglycone (quercetin) were also identified in the juices from Tarocco Scirè: rutin (quercetin 3–O rutinoside) and quercitrin (quercetin 3- O rhamnoside). At the beginning of the observation period (184 DAFB), the amount of quercetin derivatives ranged from 7.39 mg L^{-1} (Carrizo) to 8.95 mg L-1 (Furr); even if there was no statistical difference, the highest amount was recorded in C35 at 304 DAFB.

3.3.3. Anthocyanins

Eleven different anthocyanins contributed to the juice colour; six cyanidin derivatives, two delphidin, two peonidin and a petunidin were identified and quantified in the flesh (Table S1). Juice pigmentation appeared at the second observation period (214 DAFB) in juices from fruits grafted on all the roostock; only cyanidin-based metabolites were detected, ranging from 0.039 mg L^{-1} (Furr) to 0.386 mg L^{-1} in Bitters (Fig. S5). At 245 DAFB, the whole anthocyanins pool appeared, and a remarkable increase was registered again for cyanidin-related molecules, peaking at a value of 5.18 mg L^{-1} in Bitters. This rootstock also showed the highest values of peonidin (0.93 mg L^{-1}) and petunidin (0.18 mg L^{-1}). An increase in all individual anthocyanins was observed at 276 DAFB. Cyanidins confirmed to be the most abundant pigment family in the juice, as they ranged 4.61 mg L^{-1} (Furr) to 11.62 mg L^{-1}(C35). The second most abundant subclass of compounds were that derived from peonidin, that ranged from 0.93 to 1.99 mg L^{-1} in Furr and

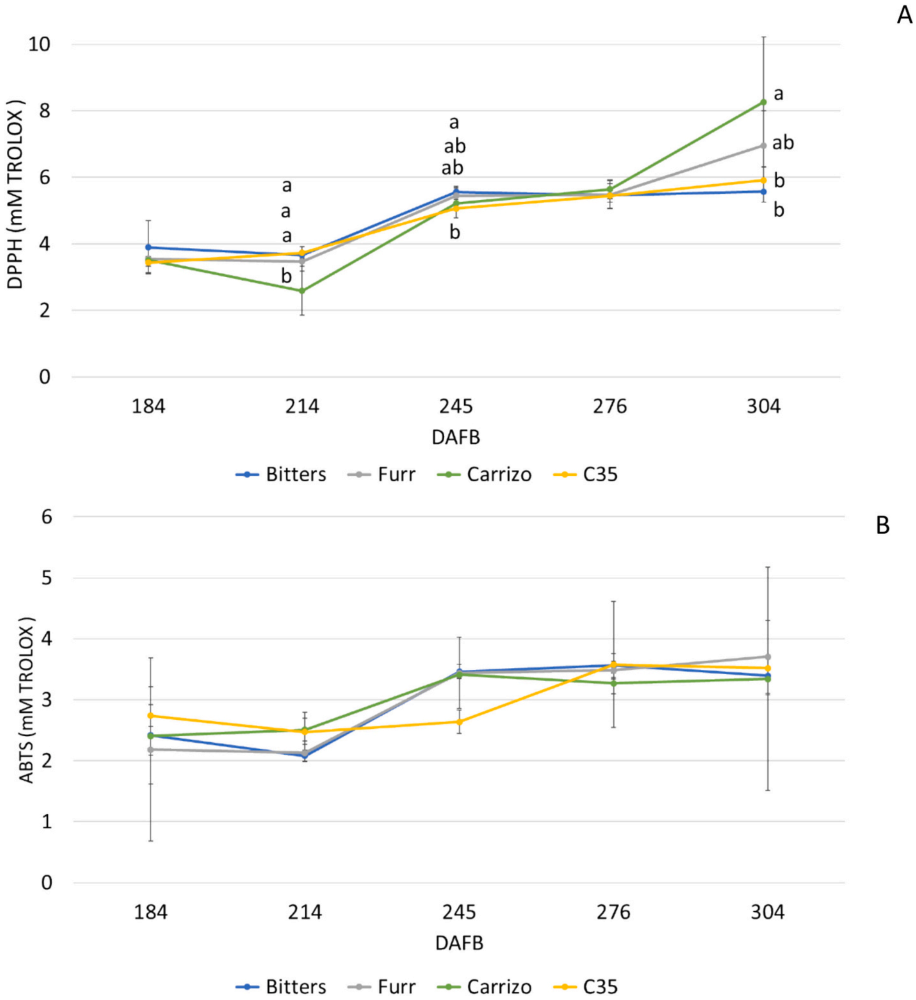

Fig. 2. The antioxidant activity measured by DPPH (A) and ABTS (B) of the juice of Tarocco Sciré blood orange grafted onto different rootstock from 184 to 304 days after full bloom (DAFB). The values represented are the mean of three replications. Values without letters have no significant differences according to Fisher's LSD procedure at 95 % confidence level (n = 30).

C35, respectively (Fig. S5). At full ripening (304 DAFB) juices from fruits grafted on Bitters and C35 showed the most intense colour, corresponding to the highest values registered for all anthocyanins.

3.4. Antioxidant activity evaluation

Regarding the total antioxidant activity, two different tests were used for its determination (Fig. 2 A). About DPPH$^{\bullet}$ assay, significant differences between rootstocks are found only at full maturity (304

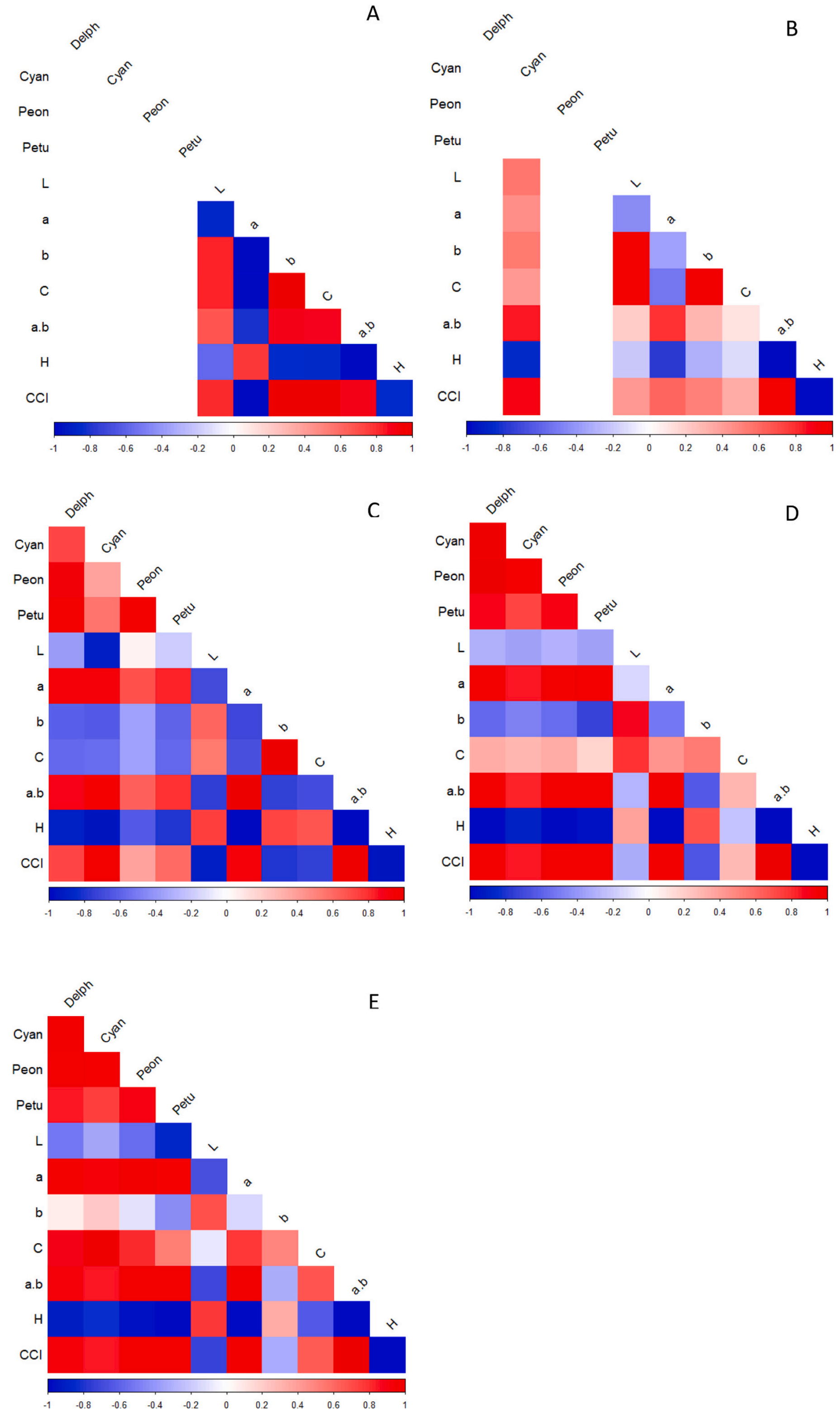

Fig. 3. Correlation of CIELab colour variables (L - Lightness, a - red/green, b - yellow/blue, C -chroma, H - hue angle, CCI - citrus colour index, a.b – a/b value), and concentrations of individual anthocyanins (Delph - Delphinid, Cyan – Cyanidin, Peon – Peonidin, Petu - Petunidin) at 184 (A), 214 (B), 245 (C), 276 (D) and 304 (E) day after full bloom (DAFB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DAFB). Fruits of Tarocco Scirè grafted on Carrizo citrange had the highest value (8.3 mM Trolox), while Bitters showed the lowest value (5.6 mM Trolox). As regards ABTS$^+$ methodology, an increase in antioxidant activity was found from 184 DAFB to 245 DAFB. After this date, the antioxidant activity was constant until complete maturation (304 DAFB). At full ripening ABTS$^+$ values ranged from 3.3 mM Trolox in fruits of Tarocco Sciré grafted on Carrizo citrange, to 3.7 mM Trolox in juice on Bitters genotype (Fig. 2 B), even if no statistical difference was recorded in any sampling date.

3.5. Correlation between colour parameters and individual anthocyanins

The correlation of CIELab colour variables of the juice and the quantification of anthocynins is shown in Fig. 3, from 184 to 304 DAFB. No anthocyanins were found in the first survey (184 DAFB). At 214 DAFB, anthocyanins of cyanidin group were quantified and they were intensely correlated with CCI, a* and a*/b* ratio. The anthocyanin profile was fully expressed one month later (245 DAFB); it was noted that CCI is largely correlated with cyanidin and less with delphinidin, peonidin and petunidin. While a* value and a*/b* ratio were mostly related with delphinidin and cyanidin and less with peonidin and petunidin. A significant negative correlation between L* and b* coordinates, Chroma and H° parameters versus all individual anthocyanins was observed. One month before harvesting (276 DAFB) a similar trend is observed for all the biochemical compounds responsible for juice colour: in detail, a significant positive correlation between CCI, a* value and a*/b* ratio with delphynidin, peonidin and petunidin was noted, while L* and b* were negatively correlated with anthocyanin pigments (Fig. 3). At full ripening, most CIELab variables were positively correlated with individual anthocyanins. Delphinidin, peonidinin and petunidin were related with CCI and a* value. Peonidin and petunidin were also highly correlated with a*/b* ratio and a little less with cyanidins and delphynidin. It was noted that the parameter Chroma was mostly correlated with all the individual anthocynins and especially with the cyanidins, but also with the a* value and the a*/b* ratio (Fig. 3).

3.6. Correlation between individual phenols and antioxidant activity

A correlation was carried out to determine the relationships between the juice polyphenols content and the antioxidant activity measured during the maturation, from 184 to 304 DAFB (Fig. 4). At 184 DAFB, ABTS$^+$ was extremely negatively correlated with hesperidin, vitexin and rutin (−0.73, −80 and − 91.0, respectively), while all other compounds showed a positive correlation with each other (Fig. 4A). At 214 DAFB, only antioxidant activity measured by ABTS$^+$ assay showed negative correlation values (Fig. 4B), and especially with naringerin, rutin and ferulic acid. On the contrary, it was observed that all juice polyphenols were positively correlated with each other at 245 DAFB (Fig. 4C). A variation was observed at 276 DAFB: ABTS$^+$ showed a good correlation with all individual compounds, especially with naringerin (0.91) and sinapic acid (0.93), while DPPH$^\bullet$ exhibited a negative correlation with all individual phenols (Fig. 4D). ABTS$^+$ was positively related with caffeic acids, while a strong negative correlation was observed with ferulic acids at full repining (Fig. 4E). On the contrary, DPPH$^\bullet$ measurements were negatively correlated with all biochemical compounds (at 304 DAFB).

3.7. Principal component analysis (PCA)

We performed a PCA to analyze the relationship between the four rootstocks, metabolite compounds analysed (i.e, glucose, fructose, sucrose, citric acid, pH, TSS, individual metabolites of the anthocyanins, flavanones, flavones, flavonols and hydroxycinnamic acids and derivatives classes) and CCI of peel and juice of each sampling period from 184 to 304 DAFB (Fig. 5).

The cumulative percent variance of the two principal variables was found to be 84.4 % of the cumulative variance at 184 DAFB. The PC1 was positively associated with caffeoyl hexose (0.307), p-coumaroyl quinic acid (0.301), sucrose (0.297), fructose (0.289) and CCI of the juice (0.269). Whereas PC2 was positively correlated with pH (0.330), CCI of the peel (0.320) and ABTS (0.310), while it was negatively related with the content of vitexin (−0.349), hesperidin (−0.334) and rutin (−0.334). A division between rootstocks was markedly observed: in detail, Bitters was positively related with PC1 (0.700), while Furr was negatively correlated with PC1 (−0.452) and PC2 (−0.558). Carrizo showed the highest correlation PC2 (0.644) and it was related with citrus colour index of peel and pH of juice.

At 214 DAFB, the principal components disclosed 79.91 % of the cumulative variance, with PC1 detailing 56.73 % and PC2 23.18 %. Carrizo was positively affected by PC1 (0.734), while C35 was negatively linked with PC1 (−0.356) and positively correlated with PC2 (0.654). Furr was negatively influenced by PC2 (−0.438) as it had the highest accumulation of citric in the juice (Table 1).

At 245 DAFB, PCA explained 88.94 % of data and it was noticed that PC1 was negatively related with glucose (−0.231), fructose (−0.228), sucrose (−0.209) and delphinidin (−0.223). Meanwhile, PC2 was positively affected by citric acid (0.463) and TSS (0.392), and negatively related with CCI of the peel (−0.347) and ABTS (−0.319). C35 citrange showed the highest correlation with PC1 (0.508) and PC2 (0.510), while Bitters (−0.468) was negatively correlated with PC1. Furr was related to PC2 and especially it was affected by citric acid.

At 276 DAFB, the cumulative percentage variance was 78.24 % (PC1 50.14 % and PC2 28.1 %). The anthocyanins cyanidin (0.273), delphinidin (0.269), peonidin (0.268) and TSS (0.270) were positively linked to PC1. PC2 was negatively related with vitexin (−0.355), glucose (−0.349) and citrus colour index of the peel (−0.323). C35 citrange (0.530) and Bitters citrandarin (0.319) were correlated with PC1 and especially with anthocynins accumulation and TSS value, while Furr (−0.358) was negatively influenced by PC2. Indeed, Bitters (−0.482) was negatively correlated with PC2.

At full maturation (A 304 DAFB) the cumulative percentage variance was 84.01 % of data and it was noticed that PC1 (53.25 %) was related with delphinidin (0.258), peonidin (0.241), cyanidin (0.266), while it was negatively affected by citrus colour index of the peel (−0.264). A separation of C35 and Furr from Carrizo and Bitters was noted; C35 (0.698) was more influenced by PC1 for the anthocyanin content in the juice. Furr (−0.449) was negatively influenced by PC1 and it was related with PC2 (0.595), while Bitters (−0.396) was negatively affected by PC2 due to the content of sucrose, hydroxycinnamic acids, citric acid and pH value of the juice.

4. Discussion

Citrus fruits are non-climacteric fruits and the ripening process imply changes that occur in the internal and external part of the fruit. Peel and flesh colour are fundamental attributes in citrus fruit quality which influence consumer perception and acceptance (Cebadera-Miranda et al., 2019). The remarkable variety of fruit coloration within citrus cultivars is directly correlated with the presence of pigments of different biochemical nature, such as chlorophylls, carotenoids, and anthocyanins (Lado et al., 2014). During maturation, sugars and pigments accumulate in the juice of citrus fruits, while the content of organic acids decreases in the pulp. The latters, especially citric acid, plays a pivotal role for fruit flavor and juice (Kelebek & Selli, 2011). Among all bioactive compounds, carbohydrates are the main soluble components in citrus fruit flesh and these include sucrose, glucose and fructose, with a general ratio of 2:1:1. The TSS/TA ratio is an important indicator of commercial and sensory maturity and it is widely used for citrus fruits, and in oranges and mandarins, because it helps to define their ripening stage and commercialization (Lado et al., 2018; Legua et al., 2022). There are several pre-harvest factors that affect ripening; beyond the selected cultivar, a decisive role is played by the rootstock which affects the

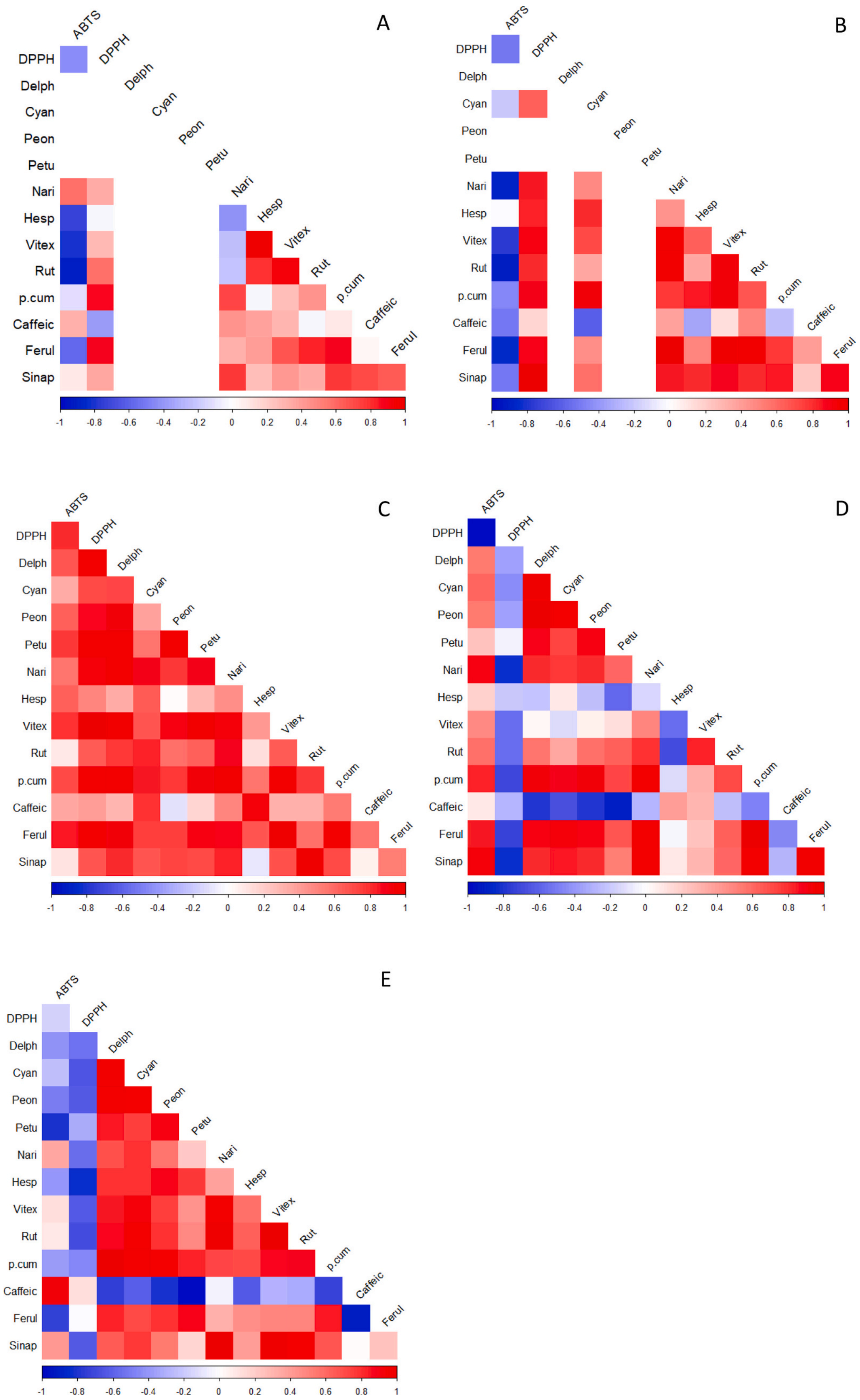

Fig. 4. Correlation of individual metabolite (Delph-delphinidin, Cyan-cyanidin, Peon-peonidin, Petu-petunidin, Nari-narigenin, Hesp-hesperidin, Vitex-vitenin, Rut-rutin, p.cum-pcumaric acid, Caffeic-caffeic acid, Ferul- ferulic acid, Sinap-sinapic acid) and antioxidant activity (DPPH and ABTS) at 184 (A), 214 (B), 245 (C), 276 (D) ad 304 (E) day after full bloom (DAFB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

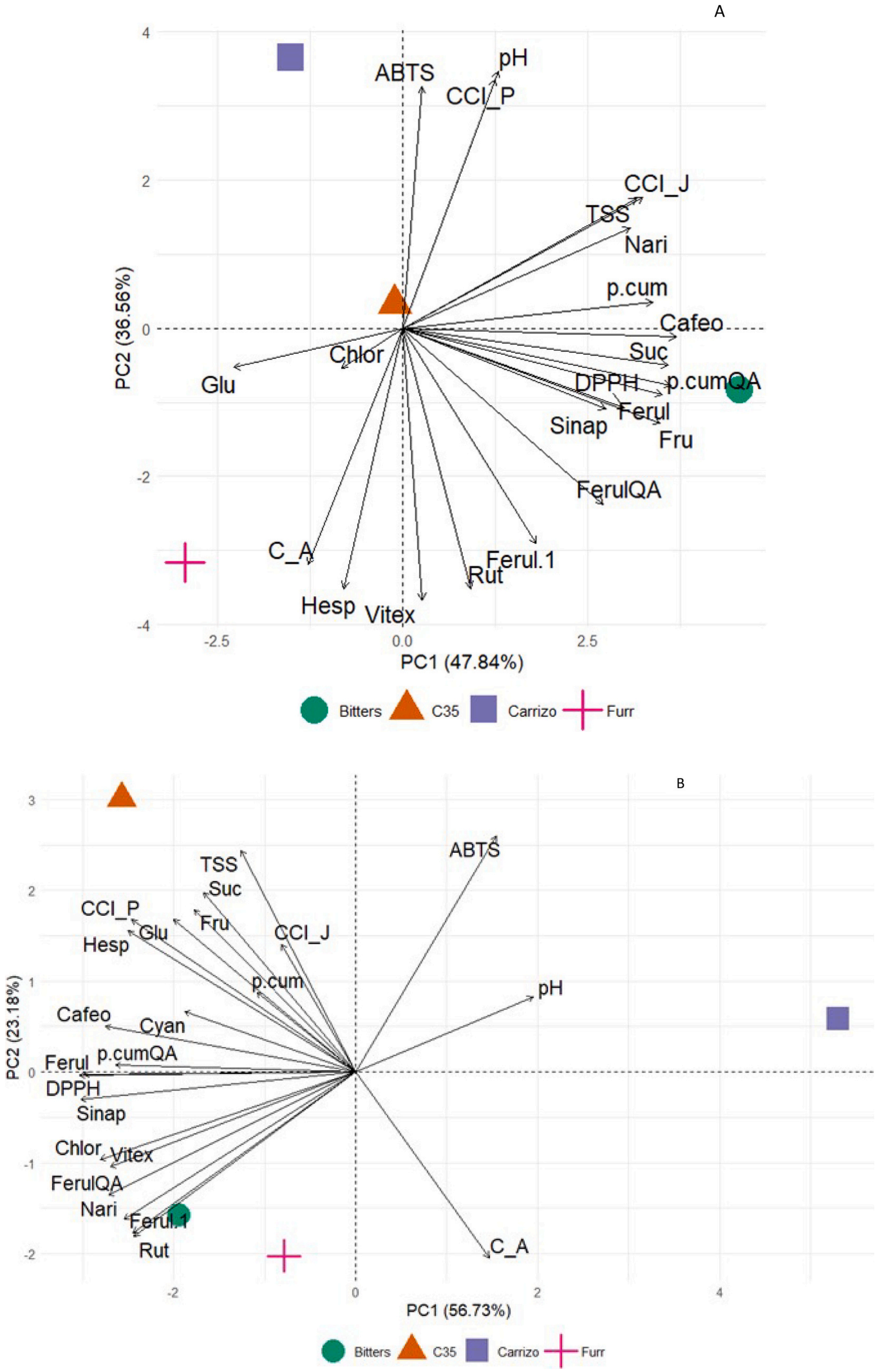

Fig. 5. PCA of glucose (Glu), fructose (Fru), sucrose (Suc), citric acid (C_A), delphinidin (Delph), cyanidin (Cyan), Peonidin (Peon), petunidin (Petu), naringenin (Nari), hesperidin (Hesp), vitexin (Vitex), rutin (Rut), p.cum-pcumaric acid (p-cum), ferulic acid (Ferul), sinapic acid (Sinap), p-coumaroyl quinic acid (p-cumQA), chlorogenic acid (Chlor), feruloyl quinic acid (FerulQA), caffeoyl hexose (Cafeo), feruloyl hexose (Ferul), total soluble solids (TSS), pH, Citrus colour index of the peel (CCI-P) and of the juice (CCI-J) at 184 (A), 214 (B), 245 (C), 276 (D) and 304 (E) day after full bloom (DAFB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

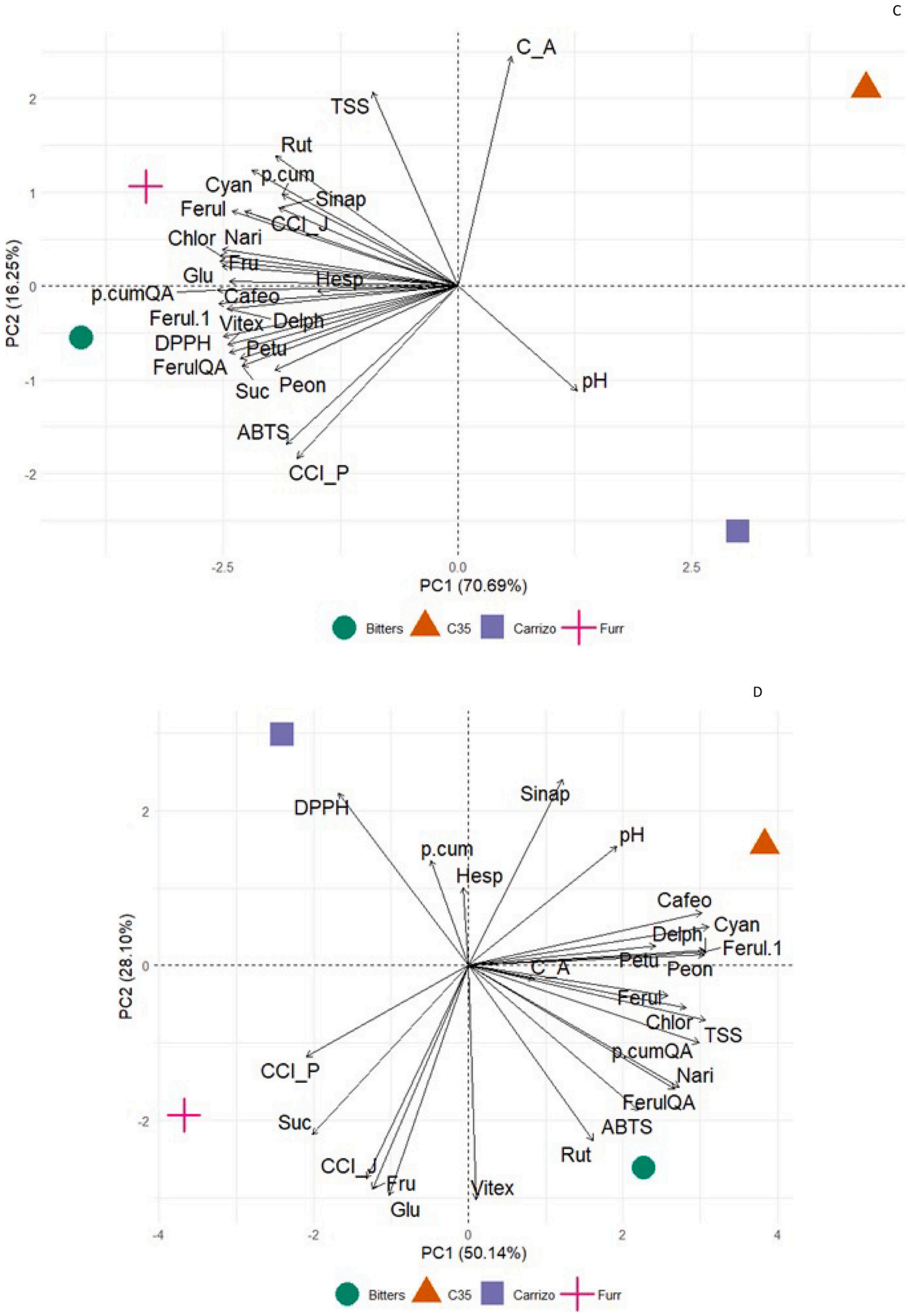

Fig. 5. (*continued*).

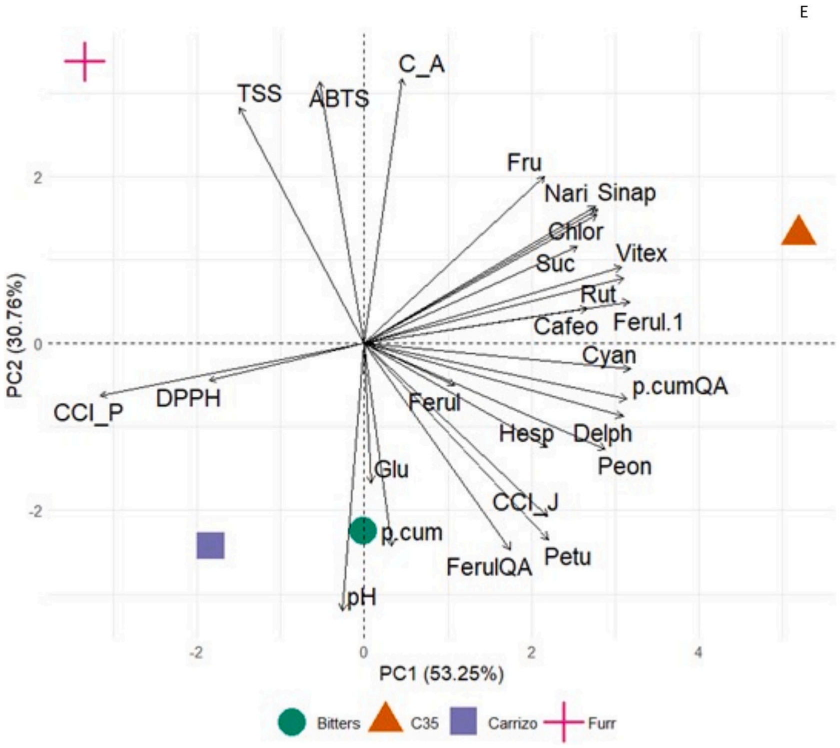

Fig. 5. (*continued*).

variety by influencing the biosynthetic pathway of metabolic processes (Lana et al., 2021; Modica et al., 2022; Modica et al., 2024).

4.1. *Citrus sinensis specialized metabolic pool*

As broadly known, specialized (synonymous with secondary) metabolites are small molecules biosynthesized and accumulated by plants for their own health, defense and communication purposes whose presence is family, genus and species dependent (Siracusa & Ruberto, 2014). The genus Citrus is characterized by the presence of several subclasses of flavonoids, organic acids, hydroxycinnamic acids and anthocyanins, being the latest a peculiarity of the pigmented varieties. A distinctive trait of this genus is also the large abundance of flavanones in its fruits, particularly that of flavanones glycosides; in *C. sinensis*, the derivatives of hesperetin (4'methyl-eriodictyol) and narigenin are the most representative compounds, being the first (hesperidin) largely the more abundant regardless the variety considered. Another flavanone, sakuranetin (4'methyl-naringenin) is also present in orange juices, even if in smaller extents. Notably, all these metabolites are accumulated in *C. sinensis* pulp as 7-*O*- rutinosides. The other subclasses of flavonoids are less represented in oranges, apart from the flavone apigenin, present in the form of various C-glucoside derivatives, such as vitexin and vicenin-2, and the flavonol quercetin as its glucoside and rutinoside derivatives (Gattuso et al., 2007; Multari et al., 2020; Sommella et al., 2017). Hydroxycinnamic acids (caffeic, ferulic and sinapic) and their quinic acid derivatives are largely present in the juices of *C. sinensis* fruits, and in particularly large amounts in pigmented varieties, as stated by Rapisarda et al. (1998).

4.2. *Biochemistry-dependent accumulation of specialized metabolites during maturation of Tarocco Scirè fruits*

As mentioned in the previous paragraph, three classes of specialized metabolites, all possessing phenol/polyphenol structures, were evaluated as markers of the maturation progress along the observation period, consisting in a total of 120 days from 184 to 304 DAFB. The three classes were flavonoids (flavanones, one flavone and flavonols, Fig. S4), hydroxycinnamic acid derivatives (Fig. S3) and anthocyanins (Fig. S5). Flavonoids and hydroxycinnamic acids were detected already at the beginning of the observation period, whilst anthocyanins appeared only one month later, at 214 DAFB. From a biochemical point of view, hydroxycinnamic acids (C6C3 scaffolds) and flavonoids (C6C3C6 scaffolds) are linked by a precursor/final product relationship; in fact, while the cinnamates, holding one aromatic ring only, are biosynthesized in plants via the shikimate pathway alone, flavonoids are regarded as mixed biosynthesis molecules, requiring the intervention of the acetate pathway to gain the second ring (ring B) to complete their structures (Dewick, 2009). The beginning of our observation period, 184 DAFB, is too late to assess if differences in accumulation between the two class of phenolic compounds occur; further studies, starting the collection of samples few weeks after the full bloom, are needed to clarify this aspect. As already mentioned, hesperidin is the most representative flavanone in *C. sinensis*, and its accumulation in fruit tissues raises up along the observation period. The same positive trend was observed for the other two flavanones identified, narirutin and dydimin. The accumulation of vitexin and citrus flavonols (rutin and quercitrin) followed the same tendency, indicating a continuous production (and accumulation) of these metabolites by the plant until ripening. A general increment during maturation was observed also for hydroxycinnamates, with differences ascribable only to the rootstock used. As regarding anthocyanin accumulation in pigmented species, notable differences were observed, from a biochemical point of view, among the different subclasses of flavonoids. The progressive biochemical pathway that leads to the biosynthesis of all these polyphenols is a multistep process, each step being characterized by the action of different enzymes catalyzing specific reactions. Flavanones are located at the very first step of this synthetic route, followed by flavones/isoflavones and flavonols, whilst anthocyanins lie at the top. For these reasons, anthocyanins are accumulated for last along the maturation process in all pigmented species, regardless the temperature for *Citrus* spp. (Rodrigo et al., 2013). In our study, cyanidin-based pigments were the first to appear, followed by the complete pool at 245 DAFB. All anthocyanins kept increasing until full ripening (304 DAFB), showing only rootstock-dependent differences as discussed in detail in the next paragraph.

4.3. Rootstock-dependent accumulation of specialized metabolites during maturation of Tarocco Scirè fruits

In our previous works (Caruso et al., 2020; Continella et al., 2018; Modica et al., 2022) we have demonstrated that, in the specific conditions of our study, rootstock genotype plays a pivotal role in the accumulation of specialized metabolites in pigmented citrus. In this work, we observed that rootstock influences also the maturation process, and this phenomenon is here corroborated by the study of the individual metabolic accumulation along the observation period. In fact, as reported in paragraph 3.3, the accumulation of the several subclasses of phenolic compounds demonstrated to be strongly rootstock-dependent. As example, the amount of flavanones in fruits generally increased from 184 to 304 DAFB; only a slight decline has been observed, at 214 DAFB for Carrizo and Furr and nearly one month later (245 DAFB) for Bitters and C35. This phenomenon, which could appear as a slowdown in the accumulation of these metabolites confirmed similar results in 'Ruby Red' grapefruit. Furthermore, previous researchers noted that naringin accumulated more in young tissues compared to mature tissues. In other citrus species, during fruit maturation both hesperidin and naringin were observed to temporarily decrease in the same stage in the flesh, whereas in the while they increased in the peel. In another study on Satsuma mandarin a decrease in flavanones during fruit ripening was reported (Chaudhary et al., 2016).

These results can be plausibly explained by the utilization of these compounds in biochemical processes finally leading to the biosynthesis of anthocyanins (see further in this paragraph) (Multari et al., 2020). On the contrary, no differences were detected in the accumulation of vitexin, rutin and quercitrin as a function of rootstock, as the same decrement was observed at 214 DAFB for vitexin and 245 DAFB for rutin and quercitrin, regardless the rootstock used. As regarding hydroxycinnamic acids, the rootstock Bitters seemed to quantitatively prompt the accumulation of these metabolites even if no rootstock-driven differences in the whole accumulation trend were observed. It was noted only the remarkable exception of the caffeic acid-based molecules, that registered amounts almost one order of magnitude higher than the others starting from 214 DAFB when grafted on Furr, and the para-coumaric acid derivatives which followed a trend similar to flavanones at 214 DAFB when grafted on Bitters. Remarkable quantitative and rootstock-dependent differences were observed in the amounts of anthocyanins detected; in fact, fruits grafted on Bitters and C35 showed the highest contents of these pigments. Notably, based on our data the rootstock used influenced also pigment accumulation trend, and this is particularly evident for cyanidin-based anthocyanins as shown in fig. S5. On the contrary, pigmentation started at 214 DAFB for all the rootstock tested, corroborating the biochemistry- dependent nature of anthocyanin biosynthesis.

4.4. Rootstock-dependent evolution of qualitative features during maturation of Tarocco Scirè fruits

The main characteristic of blood orange is pigmentation, caused by the presence of high levels of anthocyanins. The accumulation of these compounds in the peel and pulp depends on several of factors, including cultivar, rootstock, maturity stage, growing region and environment. Peel and juice colour, as reported by previous authors, are not correlated; therefore, a deep red peel colour does not necessarily correspond to a pigmented flesh colour and viceversa. In this study, pigmentation was mostly affected by rootstocks and harvest time, confirming previous results (Cebadera-Miranda et al., 2019; Morales et al., 2021). In fact, it was notable that C35 had the highest value of peel CCI at 184 DAFB, but all rootstocks enhanced this parameter, recording Bitters and Carrizo with the highest peel CCI and a*/b* values at harvest time (304 DAFB), and with the exception of Furr that exhibited the maximum CCI at 245, maintaining it up to harvest. Juice colour changed progressively from yellow/orange to deep orange/reddish at 245 DAFB, especially in fruits grafted on Bitters and Furr, in line to what reported by other authors who have stated that citrandarin genotypes achieved the highest CCI first (Domingues et al., 2021). At 276 DAFB, Bitters and C35 had the highest values of a* and CCI, also showing the lowest values of H°, confirming their influence on the pigmentation of the juice even one month earlier with respect to Furr and Carrizo.

Sugars play a pivotal role in fruit quality as one of the molecular signals regulating maturity, but also contribute in modulating physiological functions, metabolism, genetic expression, providing energy and substrates for plant growth, development and senescence. Organic acids also have a primary role in regulating plant growth and development as metabolically active solutes in cellular osmoregulation, as key components in response to nutritional deficiencies, accumulation of metal ions and plant-microorganism interaction. Citric acid is the main organic acid present in ripe citrus fruits, while sucrose, fructose and glucose are the primary sugars (Kelebek & Selli, 2011). During maturation, the accumulation of total soluble solids (especially sugars) and the decrease in the content of citric acid in blood oranges are the main responsible for the sensory properties of the juice. Certainly, it well-recognized the contribution of the sugar/acid ratio in determination of the fruit sweetness and their profile and concentration was associated to cultivar, harvest time, environment conditions and agronomic tecquique including rootstocks (Continella et al., 2018; Legua et al., 2014; Ordóñez-Díaz et al., 2020). The effect of the rootstocks genotype on the individual content of sugars has been investigated in different studies on citrus juice (Legua et al., 2014; Morales et al., 2021). In the present study, as verified by other researchers, sucrose was the main sugar in the juice of Tarocco Sciré, followed by glucose and fructose (Legua et al., 2022; Morales et al., 2021). As declared, the sweetness of orange juice is related to the levels of the individual sugars and the ratio sucrose: glucose:fructose is approximately 2:1:1 (Legua et al., 2022). Indeed, it was noted that the content of glucose slightly exceeds the concentration of fructose, even if it was reported that orange juice contains glucose and fructose in almost equal quantities (Kelebek & Selli, 2011). In the present study, the profile of individual and the total sugars content were affected by rootstocks, confirming previous result in blood orange (Continella et al., 2018; Forner-Giner et al., 2023; Incesu et al., 2013). It was noticed that Furr, Bitters and C35 had the highest increase of total sugar content in the juice at 214 DAFB, while Carrizo increased this parameter one month later, in accordance with the results obtained by Lana et al. (2021). The same trend was observed for total soluble solids, confirming previous result (Lana et al., 2021). Furthermore, blood oranges are generally more acid than blonde ones at harvest (Legua et al., 2022). Citric acid content in the juice decreased during the maturation in the fruits of Tarocco Sciré grafted with all rootstocks. At 304 DAFB, the highest citric acid was observed in Furr, confirming what previously reported by other researchers (Continella et al., 2018). Contrarily, in other studies it was detected that Carrizo and C35 citrange had the highest values of citric acid in fully ripen juice of Tarocco Rosso (Morales et al., 2021). Therefore, among the rootstocks evaluated, the two citrandarins were effective in causing early ripening of Tarocco Sciré fruits. These results are in agreement with Domingues et al. (2021).

Colorimetric parameters such as the a*/b* ratio or the CCI (Citrus Colour Index, 1000*a/L*b), are widely used as an objective and reliable parameter commonly applied to evaluate colour changes in citrus peel during maturation (Lado et al., 2014). Among the main objectives of this work, there was the investigation on the evolution of the juice colour in relation to individual anthocyanin accumulation in Tarocco Sciré cultivar, and in particular to highlight any differences in the colour of the anthocyanins in relation to the different ripening period (Fig. 3). At 214 DAFB, cyanidin group anthocyanins were quantified and consequently a positive correlation with CCI, a* and a*/b* ratio was observed, as reported in other species (Gonçalves et al., 2007; Han et al., 2008).

Indeed, it was noted that a* value and a*/b* ratio were mostly related with delphinidin and cyanidin and less with peonidin and petunidin at 245 DAFB. In agreement with these researchers, the correlation between CIELab parameters describing the deep red coloration and anthocyanins is explained by a higher concentration of these pigments in the juice, namely delphinidin, peonidin and petunidin that contributes to darker colour tones). Among the colour parameters investigated, during the whole maturation ant at harvest time (304 DAFB) CCI and a*/b* were the most discriminant parameters.

All polyphenols participate in different functions in the fruit and are in fact associated with colour, sensory characteristics (flavor, astringency, hardness), nutritional characteristics and antioxidant activity (Legua et al., 2022). Changes in antioxidant activity in blood orange were tested from 184 DAFB to 304 DAFB via radical cation $ABTS^+$ scavenging capacity and $DPPH^\bullet$ free radical scavenging capacity. Contrary to what has been reported for other citrus varieties, the maximum $ABTS^+$ and $DPPH^\bullet$ level were recorded at full ripeness (Hou et al., 2021; Zhang et al., 2022). By contrast, the antioxidant activity of Tarocco Sciré juice was influenced by the rootstock used, as reported by other authors (Ordóñez-Díaz et al., 2020). The correlation between antioxidant activities ($ABTS^+$ and $DPPH^\bullet$ assays), and polyphenolic compounds was created using a correlation map that adopts the Pearson correlation coefficient. As shown in Fig. 4, at 184 DAFB $ABTS^+$ measurement was high negatively correlated with hesperidin, vitexin and rutin (−0.73, −0.80 and − 0.91 respectively), while $DPPH^\bullet$ assay was extremely high correlated with ferulic acids, p-coumaric acids and sinapic acids conferming previous studies (Hou et al., 2021). It has been noted that ferulic acids, p-coumaric acids and sinapic acids are closely related to the antioxidant capacity expressed with $DPPH^\bullet$ during the early stages of maturation (from 184 DAFB to 245 DAFB), probably because they are antioxidant compounds that play an important role during the ripening of oranges (Hou et al., 2021). It was distinguished a significant correlations between $ABTS^+$, $DPPH^\bullet$ and all individual polyphenols, according wiyh Palma et al. (2015): it was also noticed that $DPPH^\bullet$ was greatly related with delphinidin (0.98), petunidin (0.97), naringenin (0.93), vitexin (0.99), p-coumaroyl QA (0.98), chlorogenic acid (0.94), feruloyl QA (0.97), caffeoyl hexose (0.95) and feruloyl hexose (0.97). It was observed that $ABTS^+$ had a good correlation with all individual compounds, particularly with naringerin (0.91) and sinapic acid (0.93), while $DPPH^\bullet$ maintained a negative correlation with all individual compounds from 276 DAFB to 304 DAFB. At full maturity $ABTS^+$ value had a large relationship with caffeic acid (0.91), while it showed the highest negative correlation with petunidin (−0.82). It was also observed that all anthocyanins were related to each other, had a positive relation with p-coumaric acids and they showed a negative association with caffeic acids.

The principal component analysis was performed to discriminate scion/rootstocks combination at different maturity stages. The data consisted of glucose, fructose, sucrose, citric acid, pH, TSS, individual metabolites of anthocyanins, flavanones, flavones, flavonols and hydroxycinnamic acids and derivatives, citrus colour index of peel and of juice of Tarocco Sciré (Fig. 5). The PCA effectively separated the rootstocks into different growth stages. At 184 DAFB, the cumulative percent variance of two principal variables was found to be 84.4 % of the cumulative variance. It was noticed a separation of Bitters from Furr, Carrizo and C35 citrange, due to the positive correlation with CCI of the juice, hydroxycinnamic acids and PC1. Instead, Carrizo was correlated with citrus colour index of peel and pH of juice. At 214 DAFB, the principal components disclosed 79.91 % of the cumulative variance; Carrizo was related with PC1 due to the pH value and the lowest content of citric acid (Table 2). At 245 DAFB, it was distinguished that Bitters was negatively related with PC1 for the highest pigmentation of the peel and the juice. Indeed, Furr was noticeably related to PC2 for the highest content of citric acid in the juice, even if no statistical difference was observed between the rootstocks tested (Table 2). At 276 DAFB, the cumulative percentage variance was 78.24 %. It was observed that C35 citrange and Bitters citrandarin were mostly related with the individual anthocyanins; Furr was influenced by the citrus colour index of the peel; Bitters was negatively correlated with PC2 due to the highest pigmentation and the vitexin accumulated in the juice (Fig. S4). At 304 DAFB, a separation of C35 and Furr from Carrizo and Bitters was recorded; C35 was affected by the individual anthocynins, especially for the accumulation of cyanidin-based and peonidin, although no statisticaldifference was noted for the latter (Fig. S5). Furr was negatively related with PC1 and positively correlated with PC2 for the highest content of citric acid in the juice (Table 1) and the lowest CCI of the peel (Fig. S1). Bitters was was negatively related with PC2, especially for accumulation of hydroxycinnamic acids and the lowest pH value, even if no statistical difference was noted between the rootstocks tested (Table 2).

In summary, the PCA highlighted a clear separation of C35 citrange and Bitters citrandarin from Furr citrandarin and Carrizo citrange in the different growth stages. These imply that multiple pathways may occur regarding fruit ripening and individual metabolites biosynthesis regulated by citrus roostock and an understanding of these mechanisms can optimize the production of high-quality citrus (Forner-Giner et al., 2023; Lana et al., 2021; Liu et al., 2015).

5. Conclusions

The results highlighted that rootstock genotype played a pivotal role by influencing both the timing and trend of accumulation.

- In particular: Bitters and Furr citrandarins anticipated the ripening of the fruits by approximately one month with respect to Carrizo citrange in terms of sugars accumulation and acids decrease, while C35 increased sugars one month later reaching the highest amount;
- Bitters and Furr citrandarins reached the highest pigmentation one month earlier than Carrizo and C35 citrange, for which an increase in anthocyanin content was recorded till the last sampling period;
- all rootstocks affected the accumulation extent of individual polyphenols and the corresponding antioxidant activity during maturation.

Overall, it was noticed that rootstocks C35 citrange and Bitters citrandarin were the most promising rootstocks to be used for pigmented oranges in terms of qualitative traits determined in fruits under the tested conditions.

CRediT authorship contribution statement

Giulia Modica: Writing – original draft, Investigation, Formal analysis. **Luana Pulvirenti:** Writing – original draft, Investigation, Formal analysis. **Tonia Strano:** Investigation. **Stefano La Malfa:** Writing – review & editing, Supervision. **Alessandra Gentile:** Writing – review & editing, Supervision, Funding acquisition. **Carmelo Drago:** Writing – review & editing, Supervision, Methodology. **Alberto Continella:** Writing – review & editing, Project administration, Methodology, Supervision. **Laura Siracusa:** Writing – review & editing, Supervision, Methodology.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.143634.

Data availability

Data will be made available on request.

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