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# Change in Taste-altering Non-volatile Components of Blood and Common Orange Fruit during Cold Storage

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**Abstract**

Cold storage may cause changes in the volatile and non-volatile components of orange fruit, in association with the decrement of the characteristic fruit flavour and sensory acceptability. The aim of this work was to evaluate the changes of some non-volatile taste-altering components (total and individual sugars, acids, anthocyanins, putrescine and limonin) that may affect the organoleptic perception of cold-stored orange fruit. Three blood orange varieties ('Tarocco TDV', 'Tarocco Gallo', and 'Moro') and a common variety ('Washington navel') were stored at 6 ± 1 °C and 90-95 % Relative Humidity (RH) for 60 d. Chemical and sensory assessments were performed during fruit storage at 15 d intervals. During storage, no dramatic change of the physicochemical parameters was recorded and the ascorbic acid content remained almost unchanged in all varieties. As expected, total anthocyanins significantly increased during storage. Limonin significantly decreased in all varieties. A consistent and significant increase in putrescine occurred during storage in the fruit of the pigmented varieties, not recorded in the common orange variety. Putrescine behaviour showed direct correlation with the accumulation of off-flavour in cold-stored 'Moro' and 'T. TDV' fruit, showing a clear influence of its relative concentration on the sensory perception of fruit. Finally, principal component analysis showed that the complete quality profile of the four investigated varieties represented clear differentiation without overlapping clusters. Our results suggest that the arise of a negative sensory perception in cold stored blood orange fruit might be linked to their accumulation of putrescine.

**Keywords:** Orange; cold storage; flavour; putrescine; sensory acceptability.

## 1. Introduction

In Italy, the annual citrus fruit production is estimated at approximately 3,000,000 tons with approximately 1,700,000 tons of oranges produced every year. Of the oranges, approximately 70 % is represented by red (blood) orange varieties (*Citrus sinensis* L. Osbeck, 'Moro', 'Tarocco', and 'Sanguinello') (FAOSTAT, 2017). A significant amount of this production is destined to foreign markets. Thus, maintenance of fruit quality during postharvest commercialization is of great relevance. Postharvest storage at low temperature is necessary to extend the commercial shelf-life of citrus fruit. The majority of blood orange genotypes reach maturity in the winter and spring seasons. Thus, postharvest management of these varieties extend the presence of blood oranges in the market until the summer months. During postharvest storage, soluble sugars, organic acids, and the solid/acid ratio are important parameters influencing quality of the fruit and they can be affected by both internal and external factors. Although oranges are considered to be non-climacteric fruit, storage treatment may be responsible for the occurrence of respiration phenomena of the fruit (Grierson & Ben-Yehoshua, 1986). Senescence is the most important internal factor that determines storage lifetime and final quality of the fruit. Moreover, depending on several external factors, such as temperature, humidity and climatic condition, the development of negative taste affecting compounds with a low threshold concentration for human perception can occur during storage of fruit and their derived products. As widely documented in literature, the development of undesirable volatile compounds in citrus products during storage has been associated to the sensory perception of negative flavours, which irreparably damage the final organoleptic quality of the product (Tatum, Nagy & Berry, 1975; Naim *et al.,* 1994). The organoleptic quality and the consumer related acceptance of cold-stored citrus fruit is a matter of great interest as reported in many recent works focused on the evaluation of the impact of new post-harvest treatments (i.e. irradiation, coating formulations, vapour treatments with natural compounds, etc…) on overall fruit quality, including sensory assessments (Ramakrishnan *et al.,* 2019; Baswal *et al.,* 2020; Habibi et al., 2020). Haider et al. (2020) recently demonstrated that postharvest salicylic acid (SA) application can be used safely to minimize the decay % and to maintain

the highest level of bioactive compounds in 'Kinnow' mandarin fruit for three months under cold storage. Recently the effects of postharvest treatments with γ-aminobutyric acid (GABA), methyl jasmonate (MeJA) or methyl salicylate (MeSA) on antioxidant systems and sensory quality of blood oranges during cold storage have been evaluated (Habibi et al., 2020) showing that vapour treatments of MeSA and MeJA had positive effect for maintaining bioactive compounds and nutritional and sensory quality of blood orange fruit during long-term cold storage throughout a delay of the fruit senescence process. The authors found that during prolonged storage edible quality of blood orange fruit gradually decreased with the control samples showing the lowest sensory acceptability and they assumed that the main reason for decreasing of edible quality may be probably linked to the production of volatile compounds of the fermentative metabolism. Anyway, no discussion or analytical evidence on which components or class of compounds could be responsible of the onset of undesirable taste in cold stored fruit is provided. Some authors (Shirra *et al.,* 2004) found that a trained sensory panel of judges attributed lower taste and flavour scores to blood orange fruit (cv. 'Tarocco', 'Moro', 'Sanguinello' and 'Doppio Sanguigno') passed through cold quarantine at 1 °C for 16 d, subsequent storage at 8 °C for 3 weeks and an additional week of simulated marketing period at 20 °C, even though their ethanol and acetaldehyde contents were lower than those of the control. Thus, it may be concluded that some components, other than volatiles, accumulate in fruit samples during cold storage and negatively contribute to their organoleptic characteristics. Recently, Vera-Guzmán, et al. (2019) demonstrated, at the transcriptional level, that the modulation of the antioxidant enzymatic systems such as manganese superoxide dismutase (MnSOD), ascorbate peroxidase (APX1), catalase (CAT1) and glutathione reductase (GR2) is involved in the reduction of peel physiological alterations affecting external quality of grapefruit fruit (*Citrus paradisi*) during cold storage. Moreover, a protein analysis of 'Moro' blood orange fruit pulp during storage at low temperature revealed a possible role of defence and other stress related response pathways in anthocyanin accumulation (Carmona et al., 2019), thus stimulating other research studies focused on

the accumulation of some stress-related metabolites that could be linked to negative sensory perceptions of cold stored orange fruit.

Free natural and biogenic amines shape the typical and characteristic taste of mature fruit and vegetables, and they are precursors of certain aroma compounds (Askar & Treptow, 1989). Based on their biochemical origin and physiological function, amines are classified as polyamines or biogenic amines (Abreu Gloria, 2005). Natural amines, commonly known as polyamines, are derived from their precursors during de novo biosynthesis, while the formation of biogenic amines is primarily a consequence of enzymatic decarboxylation of specific free amino acids due to microbial enzyme activity. Most fruit and vegetables normally contain low levels of polyamines although it has been demonstrated that spermidine concentration is high in broccoli and cauliflower and that putrescine is high in citrus fruit with levels ranging from ~ 1382 ± 502 μmol $kg^{-1}$ and ~ 1556 ± 128 μmol $kg^{-1}$, for mandarin and orange respectively (Eliassen, *et al.,* 2002*).* The presence of polyamines at relatively high concentrations in vegetables has also been associated with spoilage due to prolonged storage time (Yen, 1992). In plants, polyamine synthesis is a complex process in which putrescine represents an obligate intermediate, and it serves as a precursor to spermine and spermidine (Moret & Conte, 1996). The biosynthesis pathway for putrescine came from arginine via decarboxylation of ornithine (ornithine decarboxylase, ODC) or through agmatine via decarboxylation of arginine (arginine decarboxylase, ADC), and an additional path is via citrulline (Abreu Gloria, 2005). The presence of putrescine alone does not represent a risk for consumer health even if it interferes with the enzymes that metabolise it. However, the presence of putrescine, if exceeding the detection threshold which has been estimated at 91 nM (Greenman *et al.,* 2004), may adversely affect the sensory quality of orange fruit.

Delayed bitterness in citrus juices is a known phenomenon due to limonin, a bitter compound that is generated in acidic conditions by enzyme hydrolysis of its non-bitter precursor, limonoate A-ring lactone (LARL), after squeezing (Fong, *et al.,* 1992*).* Moreover, the presence of bitter limonoid aglycones in citrus fruit, i.e., limonin and nomilin, has also been widely reported (Li, *et al.,* 2014*).*

Recently, the spatio-temporal distribution of bitter limonoid aglycones in different citrus fruit tissues (flavedo, albedo, segment membrane and juice sacs) has been investigated in different species, such as lemon, pummelo, grapefruit, sweet orange and mandarin (Wang, *et al.,* 2016*).* The authors found that segment membrane accumulates bitter limonoid aglycones more than the other tissues with the lowest levels detected in flavedo. Moreover, limonin is always the most represented regardless of the tissue or the species with sweet orange showing an average concentration almost equal to 4 g $Kg^{-1}$ dry weight. Thus, when cold storage of citrus fruit is concerned, the presence and/or the level of this bitter limonoid aglycone, if exceeding the sensory threshold, must be taken into account as a variable that may affect the final sensory quality of cold-stored fruit.

To the best of our knowledge, the contribution of the onset of undesirable non-volatile components during cold storage of blood orange fruit has not yet been investigated. The purpose of the present study was to investigate the influence of cold storage on the levels of negative non-volatile aroma compounds, such as putrescine and limonin, in three pigmented varieties (cv. 'Tarocco TDV', 'Tarocco Gallo' and 'Moro') and a common variety (cv. 'Washington navel') of orange fruit [*Citrus sinensis* (L.) Osbeck] stored at 6 ± 1 °C and 90-95 % RH for 60 d. Juice quality parameters, individual sugars (glucose, fructose and sucrose), antioxidant components (total anthocyanins and ascorbic acid), putrescine and limonin were determined during fruit storage. In addition, sensory assessments were performed by a trained sensory panel at 15 d intervals. Multivariate statistical analysis, such as principal component analysis (PCA) was used to observe the differences and similarities of the analysed samples according to the storage period.

## 2. Materials and Methods

### 2.1 Chemicals

Ascorbic acid, putrescine dihydrochloride, and limonin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade, and the solvents used for chromatography were HPLC grade (Merck KGaA, Darmstadt, Germany).

**2.2 Plant material and cold storage treatment**

Samples of orange fruit of three pigmented (or blood) varieties ('Tarocco Gallo', 'Tarocco TDV', and 'Moro') and a common variety ('Washington navel') were collected at the Palazzelli (Siracusa, Italy) Experimental Farm of the CREA-Centro di Ricerca Olivicoltura, Frutticoltura e Agrumicoltura; Acireale, Italy). Based on the aim of this study, 'Moro' variety has been chosen as its fruit are notoriously the most pigmented blood oranges. 'Tarocco Gallo' and 'Tarocco TDV' differ one another for their ripening time, being respectively a medium and a early variety, and for their antocyanin content, poor in the first and relevant in the latter. Thus these varieties have been chosen in order to understand if their behaviour respect to the accumulation of taste-altering non-volatile components could differ. 'Washington navel' fruit have been chosen as a control common orange fruit. Samples of 70 fruit were harvested at commercial maturity from 5 trees from the outer part of the canopy and from the four cardinal points. Fruit were treated in a washing tank with a solution containing Imazalil fungicide (2 g $kg^{-1}$) and then stored at 6 ± 1 °C and 90-95 % RH for 60 d. Fruit sampling was performed before storage (T0) and at 15 d intervals for a total storage period of 60 d (T1, T2, T3, and T4). Juice was extracted by a domestic squeezer and used for chemical and sensory analyses for each sampling time.

**2.3 Physicochemical analyses**

Titratable acidity (TA), total soluble solids (TSS), and pH were determined according to conventional methods (Kimball, 1991).

Sugars (sucrose, fructose and glucose) were determined by High Performance Liquid Chromatography (HPLC) (Caggia, *et al.,* 2004*)*. Samples (10 mL) of centrifuged juices (15,000 g for 20 min at 4 °C) were purified through a Sep-Pak C18 cartridge (Waters Corporation, Milford, MA), diluted in water, filtered through a 0.45-μm filter, and injected directly into the column. Separation and identification of sugars were performed using a Waters 600-E HPLC system (Waters

Corporation, Milford, MA) equipped with a Waters 410 refractive index (RI) detector and a Luna-$NH_2$ column (250 x 4.6 mm i.d., 5 µm; Phenomenex, Torrance, CA). The elution was performed with an acetonitrile:water (80:20 v/v) solution at a flow rate of 1.8 mL $min^{-1}$. Sugars were identified by comparing their retention times with those of pure standards and confirmed by co-injection. Quantification of each compound was performed using an external standard calibration curve.

**2.4 Antioxidant components**

Total anthocyanin content (expressed as mg $L^{-1}$ of cyanidin-3-glucoside) was determined spectrophotometrically (Varian UV-Vis Spectrophotometer model Cary 100 Scan) by the pH differential method (Rapisarda, Fanella & Maccarone, 2000).

Ascorbic acid concentration was evaluated by liquid chromatography using a Waters Alliance 2695 HPLC (Waters Corporation, Milford, MA) equipped with a Waters 996 photodiode array detector and Empower software (Rapisarda & Intelisano, 1996). Briefly, 5 mL of centrifuged juice was poured into a flask and brought to a total volume of 50 mL with 3 % metaphosphoric acid solution. An aliquot of the solution was filtered (0.45-µm filter), and then 20 µL was injected into the HPLC. The mobile phase was 0.02 M $H_3PO_4$, and the detector was set at 260 nm.

**2.5 HPLC analysis of putrescine and limonin**

Putrescine in juice samples was derivatized according to the method of Flores & Galston (1982) with some modifications. Putrescine was toluene-extracted and analysed by HPLC. The mobile phase for dansyl chloride-derivatives consisted of water (solvent A) and acetonitrile (solvent B). The elution programme was as follows: held at 65 % of B for 1 min; ramped at 80 % of B (10 min), 90 % of B (12 min), 100 % of B (18 min), and 65 % of B (25 min); and held until the end of the run (35 min) with a flow rate of 0.8 mL $min^{-1}$. Measurements were recorded at an absorbance of 254 nm.

Limonin was determined by liquid chromatography according to Abbasi *et al.* (2005) with some modifications using a Waters Alliance 2695 HPLC (Waters Corporation, Milford, MA) equipped

with a Waters 996 photodiode array detector and Empower software (Rapisarda & Intelisano, 1996). Briefly, 10 mL of centrifuged juice (15,000 g for 20 min at 4 °C) was passed through a Sep-Pak C18 cartridge (Waters Corporation, Milford, MA) and eluted with an acetonitrile:water (50:50 v/v) solution. The solution was filtered (0.45-µm filter), and 20 µL of the solution was analysed using a Hypersil C18 ODS column (250 x 4.6 mm i.d., 5 µm; Phenomenex, Torrance, CA), and the elution was performed with an acetonitrile:water (35:65 v/v) solution at a flow rate of 1.5 mL $min^{-1}$. Limonin was identified by comparing the retention time with that of the pure standard and confirmed by co-injection. Quantification of this compound was performed using an external standard calibration curve and expressed as g 1000 $L^{-1}$.

**2.6 Sensory analysis**

The profile method (UNI EN ISO 13299:2016) was used for the sensory evaluation during storage. The standard provides guidelines on the global process of developing a sensory profile. Sensory profiles can be defined for all products that can be evaluated by sight, smell, taste, tact, or hearing. This method involves the quali-quantitative description of sample sensory attributes made by a trained panel (EN ISO 8586:2014). Twenty panellists (8 males and 12 females aged between 28 and 45 years old) were selected among the staff of CREA-Centro di Ricerca Olivicoltura, Frutticoltura e Agrumicoltura; Acireale, Italy. During the training period of about three months, the judges selected the attributes to describe the aroma, acidity, sweetness, flavour, off-flavour, bitterness of the juice using fresh products as a reference. In the sessions, judges evaluated the intensity of each chosen attribute by assigning a score between 1 (absence of the sensation) and 9 (extremely intense) on a numerical unipolar scale (ISO 4121:2003). Sensory analyses were performed at time 0 the same day of the sampling and every 15 d thereafter for a total of 60 d of refrigerated storage of fruit at 6 ± 1 °C. All sensory analysis tests were conducted in a sensory analysis laboratory in accordance with UNI EN ISO 8589: 2014.

### 2.7 Statistical Analysis

Analyses performed by STATISTICA 6.0 for Windows (Statsoft 2001, Vigonza (PD), Italy) were used to test the significance of each variable ($p<0.01$) during storage, and separation of the means was executed using Tukey's post-hoc test. To determine the relationships between the evaluated parameters, Pearson correlation coefficients (r) were used. Finally, the results were subjected to principal component analysis (PCA) to observe the differences and similarities of the analysed samples according to storage time. PCA, as an unsupervised multivariate analysis, was developed starting from the correlation matrix. The Paleontological statistics software package for education and data analysis (Past 3.0 Hammer, Harper, D.A.T., Ryan, P.D. 2001) was used for these objectives.

## 3. Results

### 3.1 Physicochemical analysis

Fruit organoleptic quality is affected by major biochemical and sensory changes that different components, such as sugars, acids, polyphenols, amino acids and other minor components, can undergo. The changes of quality standard parameters for 'Moro', 'Tarocco TDV', 'Tarocco Gallo', and 'Washington Navel' oranges stored at 6 ± 1 °C for 60 d are shown in Table 1. TA remained constant in the early stages of storage and then decreased in all varieties, significantly only in 'Moro' and 'Washington Navel' varieties. Citric acid has been reported to decrease in stored citrus fruit, and this decline may be due, in part, to the use of organic acids for energy production and alcoholic fermentation (Echeverria & Valich, 1989). Comparable pH values were observed in all the varieties throughout the storage time. The lack of any significant correlation between TA and the pH for all varieties (Supplementary Table 1 a-d) may be due to the buffering capacity of orange juice, which counters small TA variations. The TSS values of the four varieties at T0 ranged from 9.56 °Brix ('T. TDV') to 11.03 °Brix (Moro). During storage, an increase in TSS occurred in all samples between T0 and T4 (Table 1). TSS content is commonly used to evaluate the quality of citrus fruit, and its ratio respect to TA is commonly employed to determine fruit commercial maturity. In ripe citrus fruit,

the main soluble sugars are sucrose, fructose and glucose. Fructose and glucose contents increased significantly in all varieties after 60 d of storage, and their behaviour significantly correlated with TSS levels for all varieties (Supplementary Table 1 a-d). This behaviour may be caused by hydrolytic scission phenomena of various glycosylated components of juice, which can occur in response to the need of free monosaccharides as substrate for the onset of several metabolic processes of fruit during cold storage (Rapisarda, Bellomo & Intelisano, 2001).

This trend was not reflected in the sucrose content, which remained almost constant and did not significantly correlate with fructose, glucose and TSS levels during storage. 'Tarocco Gallo' fruit had a slight but not significant increase in sucrose content after 45 d of storage (Supplementary Table 1 a-d).

**3.2 Antioxidant components**

The ascorbic acid content remained almost constant during cold storage in all varieties, except for the 'Moro' cultivar where a significant decline (-16.70 %) was recorded after 45 d of cold storage (Table 1). Various studies have shown that ascorbic acid loss is slight in citrus fruit during cold storage (Rocha & Morais, 1995; Pannitteri *et al.,* 2017). As previously reported by other authors (Rapisarda, Bellomo & Intelisano, 2001), in the present study the decay of ascorbic acid in the 'Moro' variety could be explained by the possible interaction with anthocyanins, which are present in high concentrations in this genotype (Table 1). As shown in Supplementary Table 1 a, a significant indirect correlation was recorded between ascorbic acid and total anthocyanin levels in 'Moro' orange during storage ($r= -0.900$; $p\leq0.05$). Indeed, direct condensation of ascorbic acid with anthocyanin pigments may be responsible for this phenomenon, which occurred only in the 'Moro' variety, while the lower anthocyanin levels recorded in the two 'Tarocco' varieties could be linked to a negligible degradation of ascorbic acid in these clones. At the end of storage (T4), all the varieties maintained relatively high levels of ascorbic acid throughout storage as ascorbic acid levels were equal to 472.6 ± 5.6, 695.0 ± 21.1, 845.8 ± 7.4 and 625.9 ± 20.0 mg $L^{-1}$ in 'Moro', 'T. TDV', 'T. Gallo' and 'Washington navel'

varieties, respectively. A marked significant increase of total anthocyanins in both 'Tarocco' clones and 'Moro' was observed during storage. It has been reported that anthocyanin accumulation in blood oranges during storage at low temperatures depends on activation of enzymes involved in phenylpropanoid metabolism (Lo Piero *et al.,* 2005; Butelli *et al.,* 2012; Carmona *et al., 2017).* Anthocyanin levels increased by approximately 2-fold (from 14.82 ± 3.28 to 27.56 ± 1.09 mg $L^{-1}$) in 'T. Gallo' and by approximately 5-fold (from 22.34 ± 4.86 to 99.91 ± 1.51 mg $L^{-1}$) in 'T. TDV', whereas 'Moro' pigmentation increased slightly by 24.18 % (from 213.31 ± 4.03 to 264.88 ± 1.83 mg $L^{-1}$). Total anthocyanin levels in all the pigmented varieties showed a significant direct correlation with fructose and glucose content (Supplementary Table 1 a-d), except for glucose in 'Moro', suggesting that fruit presumably accumulate free monosaccharides as substrate for the glycosylation reaction, thereby allowing accumulation of anthocyanin pigments catalyzed by flavonoid-3-O-glucosyltransferase (UFGT) as it is known that expression of this enzyme is strongly induced during low temperature exposure (Carmona *et al.,* 2019; Lo Piero *et al*., 2005).

**3.3 Putrescine and Limonin content**

The results showed that free putrescine increased in all blood varities. Statistically significant differences were found after 15 d of cold storage in 'T. Gallo' and 'T. TDV', while a significant increase was recorded after 60 d of storage in 'Moro' oranges, showing a clear increasing trend during the entire cold storage period (Fig. 1). At the end of cold storage, free putrescine levels reached concentrations exceeding 120 mg $L^{-1}$ in both Tarocco clones (122.95 ± 10.28 mg $L^{-1}$ in 'T. Gallo' and 121.96 ± 6.41 mg $L^{-1}$ in 'T. TDV) and a concentration equal to 78.35 ± 6.21 mg $L^{-1}$ in 'Moro' orange. In contrast, putrescine levels remained constant in the common variety 'W. Navel' during cold storage and did not exceed 60 mg $L^{-1}$ with a concentration equal to 53.36 ± 1.61 mg $L^{-1}$ recorded at the end of the cold storage after 60 d (T4). The amount of free putrescine in a fruit or vegetable commodity can change during production, processing or storage. The increase of free putrescine in response to low temperature stress has been mentioned by different authors. Recently a study on mango fruit

(Marco-Medina & Casas, 2012) showed that the increase in putrescine is induced by low temperature. Moreover, Oufir *et al* (2008) demonstrated that cold stress up-regulates the expression of arginine decarboxylase (DC) and S-adenosylmethionine decarboxylase (SAMDC), which are enzymes involved in putrescine biosynthesis. Furthermore, putrescine accumulates in different products exposed to cold temperatures, such as lemon (Valero *et al., 1998)*, broccoli (Ohta *et al., 1993)*, grapefruit (Mc Donald & Kushad, 1986), pepper (Mc Donald & Kushad, 1986), and zucchini squash (Kramer & Wang, 1989; Serrano *et al., 1998;* Wang & Ji, 1989). According some auhors (Serrano *et al., 1998)*, polyamines, particularly putrescine, accumulate in fruit tissues exposed to cold temperature to prevent chilling injury by protecting membrane lipids from peroxidation. As reported in section 3.2, anthocyanin pigmentation also increased in all pigmented varieties, confirming the cold dependency of anthocyanin accumulation. These results showed a direct correlation between the rise of anthocyanin pigmentation and the significant increase of putrescine during the cold storage period in all pigmented varieties ($r=0.749$, $r=0.822$, and $r=0.826$ in 'Moro', 'T. Gallo' and 'T. TDV', respectively, $p\leq0.05$). Habibi & Ramezanian (2017) also showed that vacuum infiltration of putrescine increases anthocyanin concentrations in blood orange fruit ('Moro' and 'Tarocco') during cold storage. The same authors assessed that the direct and indirect putative mechanism involved in the increase of anthocyanin concentration by putrescine treatment is maintaining energy status by preventing chilling injury, increasing phenylalanine ammonia lyase (PAL) activity, and lowering respiration rate. Indeed, it must be also noticed that, in higher plants, polyamines occur in the free form or conjugated to other molecules, such as flavonoids and cinnamic acids (p-coumaric, ferulic and caffeic acids), resulting in hydroxycinnamic acid amides. These components are implied in several physiological processes of plants involving the response of plant and/or fruit to both biotic and abiotic stresses (Walters, 2003). Thus, the direct correlation of putrescine and anthocyanin accumulation in all investigated pigmented varieties may also be linked to a defence strategy of the fruit aimed at preventing the negative effects due to cold storage, which can be considered as a postharvest abiotic stress. Furthermore, it may be hypothesized that the 'Moro' variety, which

notoriously presents higher levels of anthocyanin and hydroxycinnamic acids (Rapisarda, Bellomo & Intelisano, 2001) can preferably accumulate putrescine in its conjugated form, thus building up an endogenous reserve from which to draw in event of prolonged abiotic stress. This may explain the lower levels of free putrescine recorded in the present study in the 'Moro' variety with respect to the two 'Tarocco' clones whose levels exceeded 120 mg $L^{-1}$ at the final stage of cold storage.

In *Citrus* species, limonin, although present in low concentration, is known to impart bitterness, thereby reducing juice quality (Hasegawa *et al., 1989)*. Limonin concentration changed during storage in all the genotypes showing a significant decreasing trend (Fig. 2). The limonin content showed a significant decline at the early stages of storage, reaching the lowest values after 60 d in 'T. Gallo' and 'T. TDV', after 30 d in 'Moro' and after 45 d in 'W. navel' (Fig. 2). At the end of cold storage, limonin levels reached an average concentration equal to 0.82 ± 0.01 mg $L^{-1}$, 2.84 ± 0.03 mg $L^{-1}$, 0.84 ± 0.05 mg $L^{-1}$ and 0.88 ± 0.03 mg $L^{-1}$ in 'Moro', 'T. Gallo', 'T. TDV' and 'W. navel', respectively. Chaudhary *et al* (2017) evaluated limonin levels in Rio Red grapefruit stored for 12 weeks at 11°C, 5°C or conditioned at 16°C for 7 d before storing at 5°C, and they found that limonin content is maintained in conditioned fruit but significantly declined in both the cold storage treatments, confirming the trend recorded in the present study. Limonin is a limonoid aglycone that has tissue-specific synthesis and accumulation. As cold storage proceeds, this limonoid aglycone is converted into non-bitter limonoid glucosides by limonoid glucosyltransferase. Indeed for all the genotypes, a significant inverse correlation of limonin with monosaccharide levels was recorded (Supplementary Table 1 a-d), showing that limonin content decreases while glucose and fructose levels increase during the cold storage period in response to the need of free monosaccharides as substrate for glycosylation reactions (Carmona *et al., 2019;* Lo Piero *et al.,* 2005).

### 3.4 Sensory analysis

The results of the sensory analysis (Table 3, Supplementary Figure 1) showed that there was no significant decrease in the aroma, flavour and sweetness descriptors in all the tested varieties. Instead,

an increase in off-flavour during the fruit storage period was highlighted by the panel. For the bitterness descriptor, only Moro and W.Navel presented an increase at T4.

The study of the correlation among physicochemical parameters, putrescine levels and sensory descriptors showed a correlation between the content of total anthocyanin and a significant increase of off-flavour during the cold storage period in all pigmented varieties (r=0.607, r=0.630, and r=0.301 in 'Moro', 'T. TDV', and 'T. Gallo', respectively). Furthermore, a direct correlation between the content of putrescine and an increase of off-flavour was observed during the cold storage period in 'Moro' and 'T. TDV' varieties, while the correlation value was not significant in 'T. Gallo' and 'W. Navel' (r=0.614, r=0.566, r=0.296 and r=0.202 in 'Moro', 'T. TDV', 'T. Gallo' and 'W. Navel', respectively).

**3.5 Multivariate analysis**

To obtain a broader understanding and to identify trends towards the grouping of samples, physico-chemical (pH, TA, TSS, and individual sugars,) antioxidant (ascorbic acid and total anthocyanins), aroma-related (putrescine and limonin) and sensory (aroma, flavour, sweetness, acidity, off-flavour, and bitterness) data were analysed using PCA. PCA, unsupervised pattern recognition method, is a way of identifying patterns in data and expressing the data in a way as to emphasize their similarities and differences based on the time of storage.. On the basis of eigenvalues >1, PCA evolved two first principal components explaining approximately 72.78 % of the total variance at harvest. Fig.4a shows the eigenvectors and accumulative contributions of variance for the first two PCs of fruit samples according to the T0 (0 d) storage time. The first principal component (PC1, 46.27 % of the total variance) was positively (loadings >0.7) correlated with the limonin (0.97), sweetness (0.94), glucose (0.76), flavour (0.76) and fructose (0.73) descriptors but negatively (loadings <-0.7) correlated with acidity (-0.96 %) and TA (-0.94) (Fig.4a). The major contribution to the second principal component (PC2), which accounted for 26.51 % of the variance, was positively due to putrescine (0.92), TSS (0.85) and anthocyanin content (0.80). In Fig. 4a, the scores of the sample in the four groups are plotted on the plane defined by the two first principal components. According to PC1, ‘Moro’ and

'Tarocco TDV' were separated from the other varieties, which cannot be distinguished due to a notable overlapping of clusters. PC2 allowed for separation among 'Tarocco TDV', 'Tarocco Gallo' and 'Washington Navel' varieties.

After 15 d of storage, PCA evolved two principal components as follows: PC1 explained 40.63 % of total variability; and PC2 explained 30.06 % of total variability. The analysis of the eigenvectors and accumulative contributions of variance for the first two PCs of fruit samples according to the T1 (15 d) storage time showed the highest values of factor coordinates for PC1 (40.63 %). The highest variable contributions based on correlations were limonin (0.97 %), sweetness (0.85 %) and fructose (0.74 %). PC2 accounted for 25.40 % of the total variance and was associated with TSS (0.91 %), glucose (0.91 %), sucrose (0.75 %) and off-flavour (0.73 %) (data not shown).

At the last stage of storage, PCA evolved two principal components explaining approximately 74.72% of the total variance on the basis of eigenvalues >1 (Fig.4b. According to the eigenvalue criterion, only PCs with eigenvalues greater than one are considered important. The scatter plot for the first principal components assumed a widespread score distribution. The first two PCs (PC1 and PC2) were selected to provide the highest variation of data objects (49.87 % and 24.85 % of the variation) for convenient visualisation and differentiation. As shown in the score plot of PC1 (*x*- axis) and PC2 (*y*-axis), the samples were classified into four separate groups at the end of cold storage (T4) (Fig. 4b). The clear separation among the samples highlighted the differences in certain sensory (flavour, acidity, sweetness and off-flavour), physico-chemical (TA and TSS), antioxidant (ascorbic acid and anthocyanins) and aroma-related (putrescine) parameters. Thus, PCA was successfully utilized to obtain a visualisation of the complete quality profile of the four investigated varieties at the end of the storage, showing clear differentiation of different clusters without overlap.

## 4. Conclusions

The occurrence of off-flavours in cold-stored citrus fruit has been previously related to the accumulation of some volatiles (i.e. polyvinylguaiacol, furaneol, ethanol, and acetaldehyde), but the

contribution of the onset of undesirable non-volatile components during cold storage has not been clearly elucidated until now. The present investigation aimed at evaluating the influence of cold storage on the levels of some negative non-volatile flavour compounds, such as putrescine and limonin, in three blood orange varieties ('Tarocco TDV', 'Tarocco Gallo', and 'Moro') and a common variety ('Washington navel'). The oranges were subjected to a period of cold storage at 6 ± 1 °C and 90-95% RH for 60 d. No dramatic change in physicochemical parameters was recorded, and the ascorbic acid content remained almost unchanged in all varieties. However, total anthocyanins significantly increased during storage, confirming the cold-dependency activation of enzymes involved in phenylpropanoid metabolism. Putrescine behaviour showed direct correlation with the accumulation of off-flavour in cold-stored 'Moro' and 'T. TDV' pigmented fruit, showing a clear influence of its relative concentration on the sensory perception of fruit. Putrescine content increased in all the blood varieties, changing significantly after 15 d of cold storage in 'T. Gallo' and 'T. TDV', and this trend is putatively linked to the prevention of chilling injury. Moreover, a direct correlation between the rise of anthocyanin pigmentation and the significant increase of putrescine was observed during the cold storage period in all pigmented varieties. The 'Moro' variety, which notoriously presents higher levels of anthocyanins and hydroxycinnamic acids, preferably accumulates putrescine in its conjugated amide form, thus building up an endogenous reserve from which to draw from in the event of prolonged abiotic stress. Hence, future investigations will be performed at a molecular level on the putative interaction between polyamine metabolism and the phenylpropanoid pathway in fruit or vegetables that undergo storage periods at cold temperatures. Limonin showed a decreasing trend during cold storage and bitterness was not perceived in any of the fruit at any timepoint, demonstrating that its level could not influence the organoleptic quality of fruit. Taking into consideration all the investigated physicochemical, antioxidant, non-volatile, taste-altering and sensory parameters, PCA confirmed that the complete quality profile of the fruit successfully differentiated the different clusters without overlap.

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| Cultivar | Parameters | | | | | | |
|---|---|---|---|---|---|---|---|
| | TSS (°Brix) | TA (% citric acid) | Glucose (g $L^{-1}$) | Fructose (g $L^{-1}$) | Sucrose (g $L^{-1}$) | Ascorbic Acid (mg $L^{-1}$) | Total Anthocyanins (mg $L^{-1}$) |
| **Moro** | | | | | | | |
| T0 (0 d) | 11.03±0.18 b | 1.41±0.07 b | 2.19±0.05 c | 2.36±0.15 c | 4.63±0.57 | 589.1±12.0 b | 213.31±4.03 c |
| T1 (15 d) | 12.03±0.12 a | 1.48±0.06 ab | 2.69±0.05 a | 2.32±0.10 c | 4.94±0.23 | 657.6±20.5 a | 213.27±1.88 c |
| T2 (30 d) | 11.94±0.15 a | 1.57±0.05 a | 2.50±0.03 b | 2.65±0.02 ab | 4.80±0.14 | 604.6±19.8 b | 234.60±5.99 b |
| T3 (45 d) | 12.31±0.07 a | 1.43±0.07 ab | 2.46±0.02 b | 2.56±0.10 bc | 4.44±0.20 | 490.7±15.1 c | 258.27±5.43 a |
| T4 (60 d) | 12.17±0.24 a | 1.24±0.04 c | 2.64±0.03 a | 2.86±0.02 a | 4.80±0.03 | 472.6±5.60 c | 264.88±1.83 a |
| **Tarocco 'TDV'** | | | | | | | |
| T0 (0 d) | 9.56±0.06 c | 1.30±0.07 ab | 1.21±0.02 c | 1.34±0.03 d | 4.46±0.23 | 674.0±9.60 | 22.34±4.86 d |
| T1 (15 d) | 9.60±0.05 c | 1.36±0.03 a | 1.52±0.17 ab | 1.74±0.02 b | 4.45±0.07 | 667.5±28.3 | 37.58±4.83 c |
| T2 (30 d) | 9.76±0.01 b | 1.33±0.02 ab | 1.47±0.08 b | 1.62±0.04 c | 4.38±0.00 | 663.6±11.9 | 37.95±1.77 c |
| T3 (45 d) | 9.98±0.07 a | 1.28±0.01 ab | 1.65±0.03 ab | 1.79±0.02 ab | 4.57±0.07 | 694.0±19.5 | 84.71±8.88 b |
| T4 (60 d) | 9.98±0.05 a | 1.24±0.01 b | 1.74±0.05 a | 1.86±0.02 a | 4.31±0.13 | 695.0±21.1 | 99.91±1.51 a |
| **Tarocco 'Gallo'** | | | | | | | |
| T0 (0 d) | 10.53±0.16 b | 0.96±0.03 a | 2.24±0.06 b | 2.41±0.06 c | 4.82±0.09 | 855.2±46.8 | 14.82±3.28 b |
| T1 (15 d) | 11.22±0.43 ab | 0.89±0.02 ab | 2.31±0.05 b | 2.43±0.17 c | 4.69±0.35 | 772.3±16.5 | 16.31±1.30 b |
| T2 (30 d) | 11.25±0.35 ab | 0.79±0.02 b | 2.34±0.01 b | 2.59±0.09 bc | 4.67±0.54 | 827.2±33.7 | 24.65±0.24 a |
| T3 (45 d) | 11.39±0.34 ab | 0.85±0.05 ab | 2.56±0.01 a | 2.73±0.02 ab | 4.92±0.04 | 839.1±31.8 | 27.34±1.27 a |
| T4 (60 d) | 11.83±0.26 a | 0.75±0.11 b | 2.63±0.05 a | 2.87±0.04 a | 5.27±0.17 | 845.8±7.40 | 27.56±1.09 a |
| **Washington Navel** | | | | | | | |
| T0 (0 d) | 10.63±0.33 b | 0.87±0.02 a | 2.49±0.11 b | 2.57±0.11 b | 4.21±0.22 c | 622.5±15.6 | - |
| T1 (15 d) | 11.90±0.18 a | 0.87±0.04 ab | 2.55±0.03 b | 2.73±0.08 ab | 4.72±0.22 ab | 653.9±13.0 | - |
| T2 (30 d) | 12.09±0.45 a | 0.84±0.01 abc | 2.55±0.02 b | 2.78±0.03 a | 4.85±0.16 a | 658.3±4.70 | - |
| T3 (45 d) | 12.34±0.08 a | 0.83±0.02 bc | 2.71±0.01 a | 2.89±0.01 a | 4.43±0.11 abc | 620.4±21.1 | - |
| T4 (60 d) | 12.53±0.40 a | 0.82±0.02 c | 2.70±0.03 a | 2.83±0.02 a | 4.36±0.02 bc | 625.9±20.0 | - |

The data are expressed as mean ± Standard Deviation.: $p \leq 0.05$ – small letters; no letters - no significant difference.

**TABLE 1.** Changes in physicochemical parameters and antioxidant components in orange fruit cv. 'Moro', 'Tarocco Gallo', 'Tarocco TDV' and 'Washington navel' during storage time (6 ± 1 °C and 90-95 % RH for 60 d).

| MORO | time | Aroma | Flavour | Sweetness | Acidity | Off-flavour | Bitterness |
|---|---|---|---|---|---|---|---|
| | T0 | 4.4 | 4.2 | 3.1 | 5.4 a | 2.0 B | 2.5 b |
| | T1 | 4.8 | 4.0 | 3.6 | 4.9 ab | 2.6 AB | 3.5 ab |
| | T2 | 5.0 ns | 3.6 ns | 3.1 ns | 5.2 ab | 2.7 AB | 3.8 a |
| | T3 | 4.8 | 3.9 | 3.6 | 3.7 b | 3.0 AB | 3.2 ab |
| | T4 | 3.5 | 3.8 | 3.2 | 4.8 ab | 4.0 A | 3.8 a |
| **T.GALLO** | **time** | **Aroma** | **Flavour** | **Sweetness** | **Acidity** | **Off-flavour** | **Bitterness** |
| | T0 | 4.5 | 5.8 | 6.1 | 2.8 | 1.5 B | 1.8 |
| | T1 | 4.3 | 4.9 | 5.8 | 2.7 | 1.4 B | 1.6 |
| | T2 | 4.4 ns | 5.3 ns | 6.2 ns | 2.5 ns | 1.5 B | 1.4 ns |
| | T3 | 4.4 | 5.6 | 5.9 | 2.3 | 1.7 AB | 1.5 |
| | T4 | 4.4 | 5.6 | 5.9 | 2.9 | 2.7 A | 2.0 |
| **W.NAVEL** | **time** | **Aroma** | **Flavour** | **Sweetness** | **Acidity** | **Off-flavour** | **Bitterness** |
| | T0 | 4.3 | 5.4 | 6.3 AB | 2.3 | 1.4 b | 1.1 B |
| | T1 | 4.8 | 5.3 | 6.3 AB | 2.1 | 1.5 b | 1.8 AB |
| | T2 | 4.0 ns | 5.4 ns | 6.9 A | 2.8 ns | 1.8 ab | 1.7 AB |
| | T3 | 4.5 | 5.0 | 4.5 B | 2.1 | 2.1 ab | 2.5 AB |
| | T4 | 4.7 | 5.3 | 5.3 AB | 2.0 | 2.4 a | 2.6 A |
| **T.TDV** | **time** | **Aroma** | **Flavour** | **Sweetness** | **Acidity** | **Off-flavour** | **Bitterness** |
| | T0 | 4.6 | 4.5 ab | 3.5 | 6.5 A | 1.4 B | 1.9 |
| | T1 | 4.9 | 5.2 a | 4.0 | 4.1 B | 1.5 B | 1.7 |
| | T2 | 4.4 ns | 3.9 ab | 3.3 ns | 4.7 AB | 1.9 AB | 2.5 ns |
| | T3 | 4.2 | 4.3 ab | 3.8 | 4.6 AB | 2.4 AB | 2.6 |
| | T4 | 4.2 | 3.3 b | 3.6 | 4.2 B | 2.9 A | 3.2 |

**TABLE 2** Changes in sensory descriptors in orange fruit cv. 'Moro'. 'Tarocco Gallo'. 'Tarocco TDV' and 'Washington navel' during storage time (6 ± 1 °C and 90-95 % RH for 60 d).

## Figure captions

**Fig. 1.** Changes in Putrescine content of blood oranges cv. 'Moro', 'Tarocco Gallo' and 'Tarocco TDV' and blond orange cv. 'Washington Navel' during storage time.

**Fig. 2.** Changes in Limonin content of blood oranges cv. 'Moro', 'Tarocco Gallo' and 'Tarocco TDV' and blond orange cv. 'Washington Navel' during storage time.

**Fig. 3a.** – Scatter plot of the fruit samples on the first and second principal component scores according to the storage time (T0).

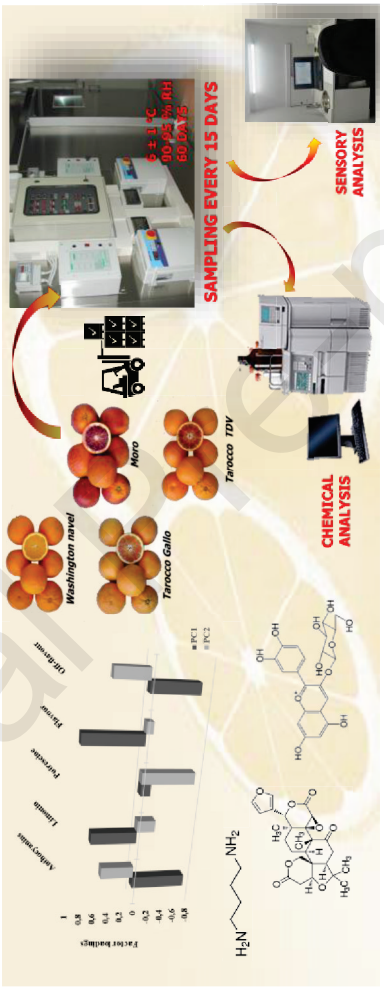
**Fig. 3b.**– Scatter plot of the fruit samples on the first and second principal component scores according to the storage time (T4).

**Fig. 4a** The eigenvectors and accumulative contributions of variance for the first 2 principal components (PCs) of fruit samples according to the storage time (T0).

**Fig. 4b** The eigenvectors and accumulative contributions of variance for the first 2 principal components (PCs) of fruit samples according to the storage time (T4).

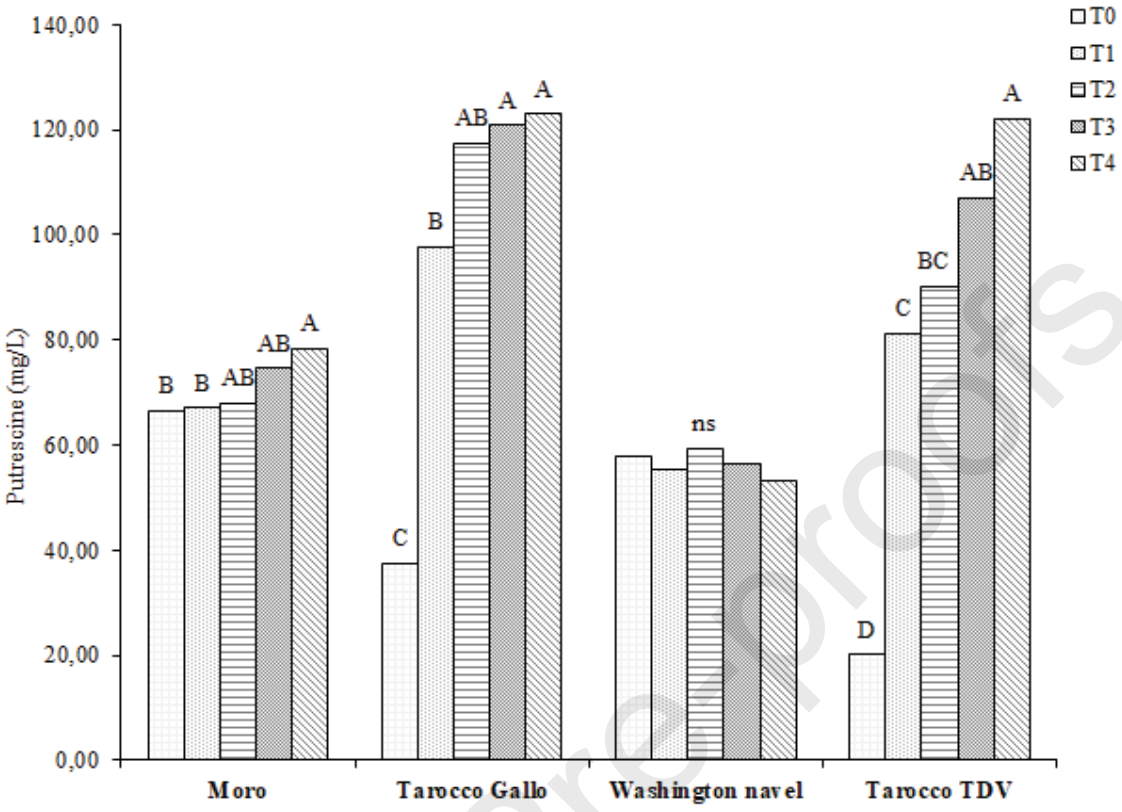
## Supplementary Figure caption

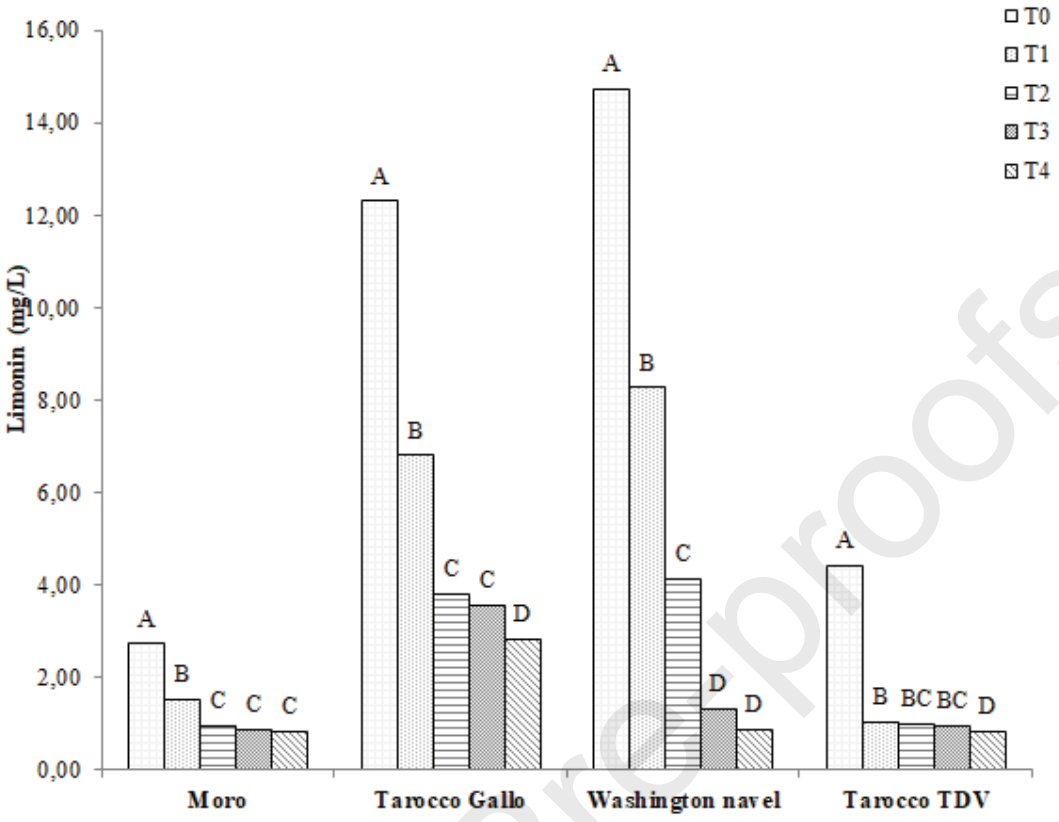
**Supplementary Fig. 1. -** Sensory profiles of 'Tarocco TDV', 'T. Gallo', 'Washington Navel' and 'Moro' oranges during storage (6 ± 1 °C and 90-95 % RH for 60 d).

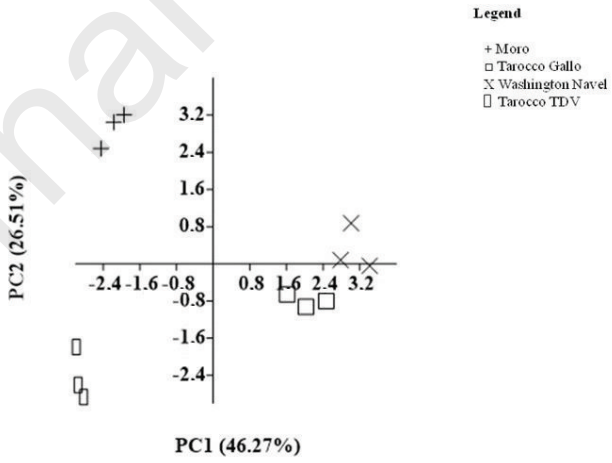


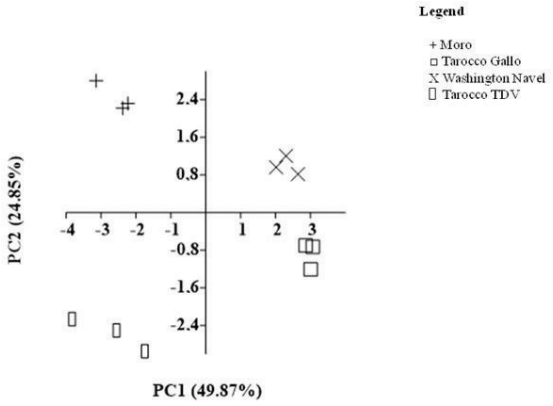
**Highlights**

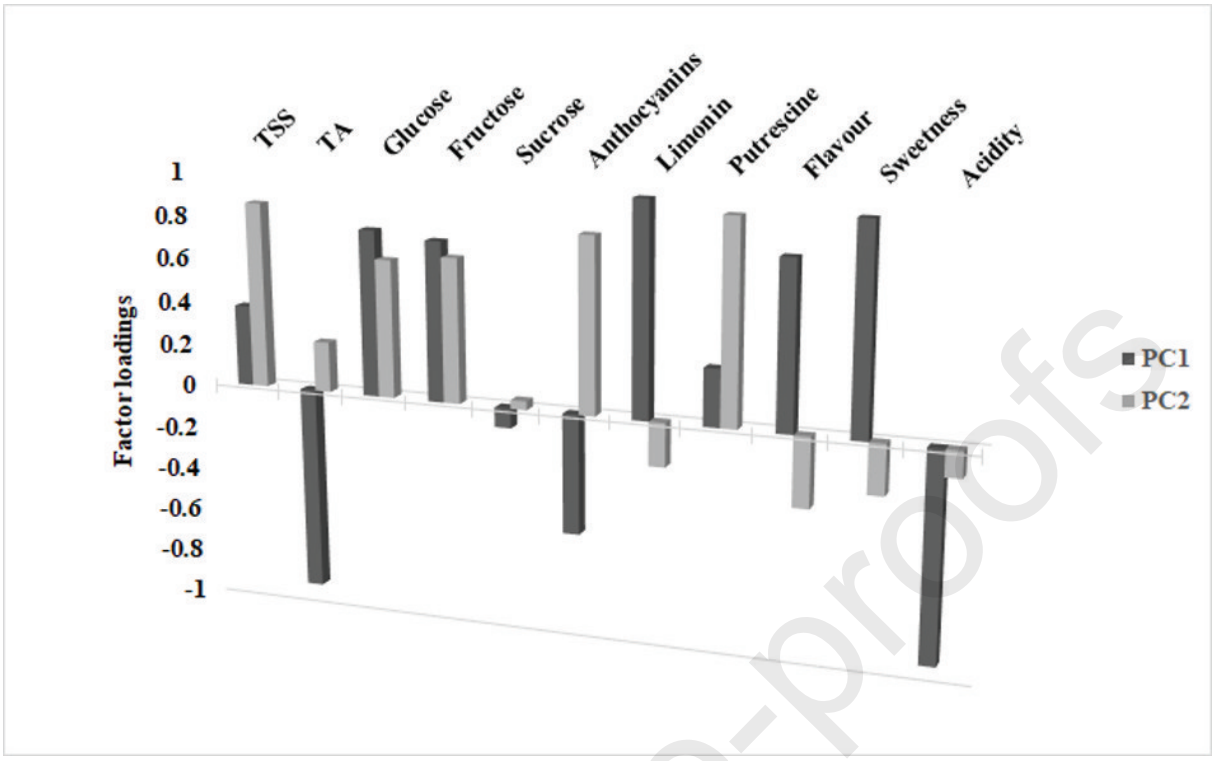
- Physicochemical parameters and ascorbic acid remained unchanged in all varieties;
- Putrescine showed correlation with off-flavour in 'Moro' and 'T. TDV';
- A correlation between anthocyanin pigmentation and putrescine was observed;

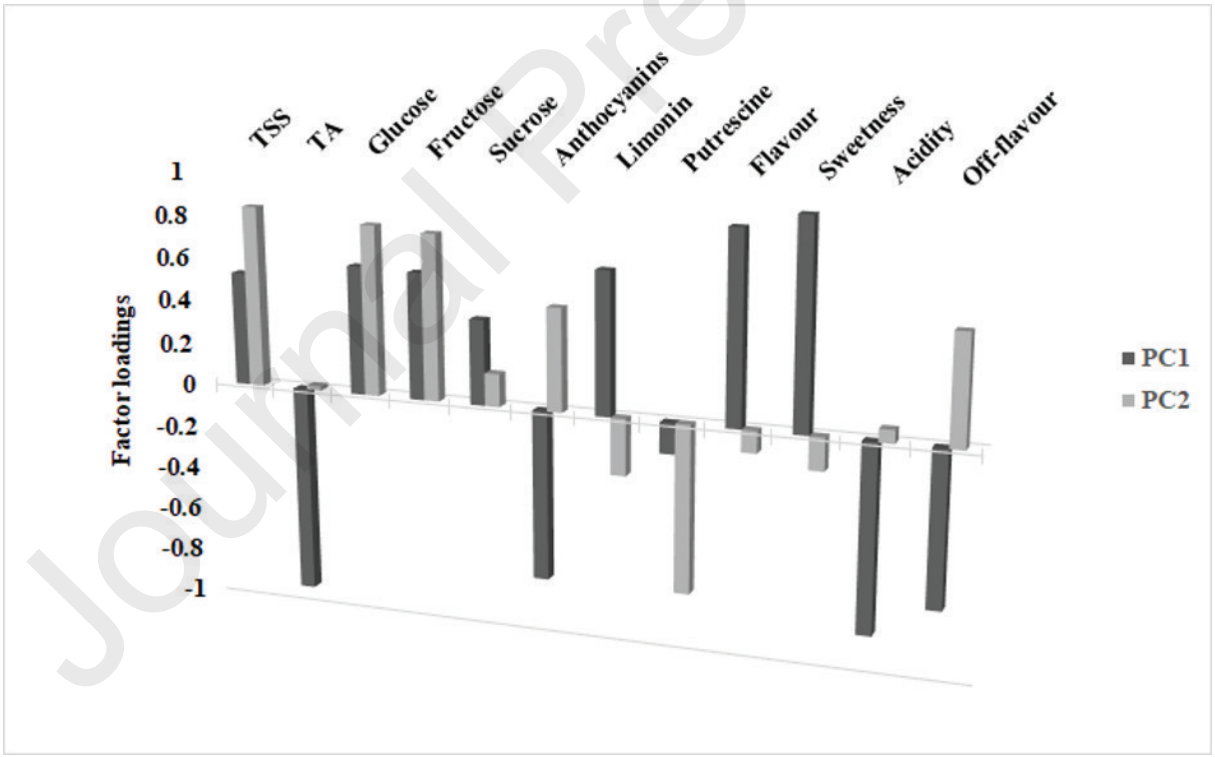








**Simona Fabroni:** Methodology, Formal analysis, Investigation, Data curation, Writing- Original draft preparation, Writing- Reviewing and Editing. **Margherita Amenta**: **Methodology, Validation, Formal analysis, Data curation,** Writing- Original draft preparation. ***Nicolina Timpanaro*: Methodology, Investigation, Data curation,** Writing- Original draft preparation. **Aldo Todaro**: Investigation, Visualization. **Paolo Rapisarda:** Conceptualization; Supervision; Project administration.

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Regards

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