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Evaluation of Morphological, Qualitative, and Metabolomic Traits during Fruit Ripening in Pomegranate (*Punica granatum* L.)

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Abstract: Pomegranate is characterized by several local accessions and cultivars widespread across different countries, each with different bio-agronomic features. Physiological and biochemical processes occur during fruit ripening, leading to changes in size, color, and flavor, improving the fruit's acceptability for the consumer. The aim of this study was to evaluate the changes in physico-chemical and nutritional traits of three Italian germplasm genotypes ('Santa Lucia', 'Di Benedetto', and 'Arborea') to determine the differences for these parameters both within the same cultivar during four ripening stages, and between individual cultivars in the same ripening stage. Morphological traits and fruit pigmentation showed variation during the ripening process, with higher values at the final stage. The highest fruit weight was detected in 'Di Benedetto' (392.19 g), while 'Arborea' displayed high juice content. Qualitative traits, such as soluble solids, increased until stage III, while titratable acidity values decreased during the ripening stage in 'Di Benedetto' and 'Arborea'. In all three accessions, a reduction in the total polyphenols up to the III ripening stage was observed. Metabolomic analyses using ^{1}H-NMR (proton nuclear magnetic resonance) showed a variation in citric acid and sugar content according to the ripening stage and accession considered. This study found high variability in nutraceutical traits among the analyzed pomegranates. The three pomegranate genotypes showed significant differences in qualitative and metabolomic characteristics. Principal component analysis revealed the main traits that contribute to the positive and negative correlations with PC1 and PC2, highlighting the great variability in the investigated pomegranate genotypes.

Keywords: germplasm; physico-chemical traits; anthocyanins; NMR; polyphenols

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1. Introduction

The pomegranate tree (*Punica granatum* L.) has been known since ancient times. This species, belonging to the family Lythraceae, subclass Rosidae, is native to central Asia, but it is highly adaptive to a wide range of climates and soil conditions, and now it is mainly cultivated in India, Iran, China, Turkey, United States, Spain, South Africa, Peru, Chile, and Argentina [1]. The consumption and market demand for pomegranate fruits and juice worldwide are on the rise, probably also as a consequence of increasing evidence demonstrating that pomegranate is a "super-food", given its nutritional and functional traits. Pomegranate fruits are rich in bioactive compounds. Some of these have nutritional

value, while others are endowed with biological activities that ultimately reduce the risk of developing numerous chronic diseases, thus exerting an important protective action on the health of consumers. In particular, the importance of antioxidants contained in fruits is associated with the beneficial effects that they have on the human body against chronic-degenerative diseases induced by oxidative stress and age [2–4]. The pomegranate is a luxury fruit that sells well in the higher market segment, also as ready-to-eat arils [5]. It is also widely used in the food and beverage industry as a flavoring and coloring agent, or jellified. In many countries, pomegranate seeds are also used to garnish salads and desserts [6]. Furthermore, pomegranate extract is widely used as a dye in cosmetic products, and in skincare creams as a promoter of proliferation and procollagen synthesis [7].

The pomegranate is a non-climacteric fruit and should be harvested when fully ripe, generally 5–6 months after the appearance of blossoms. Fruit growth pattern shows a simple sigmoidal curve from fruit set to maturity. Coordinated physiological, biochemical, and structural processes occur during pomegranate fruit ripening, with changes in size, color, and flavor that make the fruit acceptable for consumption [8]. The major classes of phytochemicals identified in pomegranate fruits are hydrolyzable tannins and related compounds, such as punicalagins, punicalin, gallic acid, and ellagic acid and its glycosidic derivatives. Concerning the anthocyanins, delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, and pelargonidin 3-glucoside have been reported [3]. The beneficial effects against cancer, cardiovascular disease, and other diseases of these and other phytochemicals contained in the edible part of the pomegranate are confirmed by numerous studies [9–11]. Furthermore, pomegranates are also a good source of antioxidants, such as vitamin A, C, and E; minerals, such as potassium, calcium, magnesium, iron, and zinc [12]; and organic acids, such as malic and citric acids [13].

As for other species [14,15], the identification of the optimal moment of harvesting allows one to allocate pomegranate fruits for direct consumption or for transformation. The ripening stage of the pomegranate fruit is commonly assessed by monitoring the exocarp color, and juice color and acidity [16]; skin color change is due to anthocyanin synthesis during the ripening process. The total anthocyanin content increases significantly during fruit ripening [8]. These water-soluble vacuolar pigments are members of the phenolic compounds that contribute to the red, blue, or purple colors of many fruits, including pomegranate skin and seed, and they are well known for their antioxidant activity. However, to obtain a fruit that is tasty and beneficial for the consumers. in addition to the optimal ripening stage, it is also important to consider the quality attributes, such as sugar and nutraceutical content [17,18]. The differences in sugar and organic acid content results in sweet, sour–sweet, or sour genotypes [19].

In Italy, many pomegranate orchards are located in several regions of the south, such as Campania, Apulia, Calabria, and Sicily. Since Italian production is unable to meet the demand, the fruit is also imported from countries such as Turkey, Iran, and Spain. To date, there are only a few studies that characterize the local Italian genotypes [20–23]. Thus, this study aimed to carry out a morpho–pomological and chemical characterization of three Italian germplasm genotypes ('Santa Lucia', 'Di Benedetto', and 'Arborea'). These accessions were compared to detect changes in physico–chemical and nutritional traits during four stages of fruit ripening, with the aim of determining whether some of the accessions may be more acceptable than others to consumers.

2. Materials and Methods

2.1. Experimental Design

Three pomegranate germplasm genotypes (G), 'Santa Lucia', 'Di Benedetto', and 'Arborea', grown in an experimental orchard located in Eboli, Salerno (Center for Applied Research in Agriculture—CRAA—40°33′29″ N; 14°58′28″ E at 15 m s.l.m.) were selected for this study. Thirty pomegranate fruits were randomly harvested from five pomegranate trees at four different stages of ripening: immature, unripe, semi-ripe, and ripe fruit. The

ripening stage was evaluated considering the color of the fruit skin. The immature stage was characterized by green skin color and white seeds, the unripe stage by a light red epicarp with light-pink seeds, the semi-ripe stage by a red epicarp and light red seeds, and the mature stage included ripe pomegranates of intense red skin color and mature red seeds. The four samplings were carried out from 3 September to 3 November (Table 1). The plants were trained to free vase systems, and spaced 4.0 m between the rows and 3.5 m within rows, with a planting density of 714 trees ha^{-1}. The experiment was carried out on medium-textured soil with an adequate content of macro- and microelements. Irrigation was provided using a drip system that was equipped with two self-compensating drippers for each plant, delivering 8 L $\times$ h^{-1}. The orchard received standard horticultural care, and the treatments against the main parasites were established in accordance with the regulations governing integrated production.

Table 1. Description of four different stages of coloring of pomegranate fruits.

Stages of Ripening (S)	Harvest Date	Fruit Description
I	3 September	Immature fruits with green epicarp
II	23 September	Unripe fruit with a light red epicarp
III	22 October	Semi-ripe fruit with a deeper red epicarp
IV	3 November	Ripe fruit with an intense red epicarp

Fruits were selected based on the absence of disease and lack of general defects, packed in single-layer trays, and immediately transported and processed in the laboratory. All of the 30 fruits were used for the morphological parameter measurements, while the chemical analyses conducted on the pomegranate juice were carried out on five replicates, with each biological replicate consisting of three fruits.

2.2. Morphological Characterization of Pomegranate Accessions

For each genotype (G), the weight of the whole fruits and pomegranate seeds (n = 100) was measured with an electronic digital balance (Precisa Instruments AG, model XB220A, Dietikon, Switzerland); the equatorial (mm) and the polar diameters of the fruit (mm), excluding the calyx, were determined using a digital vernier caliper (Mitutoyo, Kawasaki, Japan). The pomegranate seeds were hand-separated from the epicarp and carpellary membranes, counted, and squeezed using a small press. A juice yield of 300 g of pomegranate seeds was measured and expressed as a percentage. Juice (200 mL) was stored at −20 °C and afterwards used for the evaluation of physico–chemical and nutraceutical traits.

2.3. Physico–Chemical Traits

Titratable acidity (TA) was determined through a titration of the juice, diluted with distilled water to a 1:1 ratio, with NaOH 0.1 N, and expressed as g citric acid 100 mL^{-1}. Total soluble solid (TSS) content, expressed as °Brix, was recorded using a digital refractometer (Atago, model PR-101a, Tokyo, Japan), and the pH was measured with a digital pH meter (Crison Instruments, model GLP 21, Barcelona, Spain).

The color attributes of the epicarp were measured using a Minolta CR-400 Colorimeter (Konica Minolta, Inc., Osaka, Japan) to delineate chromaticity values L^* (lightness), a^* (green to red), and b^* (blue to yellow).

2.4. Bioactive Compounds and Antioxidant Activity

The pomegranate juice was used for the analysis. The total phenolic compound (POL) and the total flavonoid (FLA) contents were determined by the Folin–Ciocalteu and the aluminum chloride colorimetric methods, respectively, as reported by Magri et al. [24]. Total polyphenols and total flavonoids were expressed as mg of gallic acid equivalents (GAE) per 100 mL of juice, and mg of catechin equivalent (CE) per 100 mL of juice, respectively.

The total monomeric anthocyanin content was estimated spectrophotometrically according to Adiletta et al. [5], via the pH differential method with potassium chloride (pH 1.0, 0.025 M) and sodium acetate (pH 4.5, 0.4 M) buffer. The anthocyanin content was expressed as mg of cyanidin-3-glucoside equivalent (CGE) per 100 mL of juice. Antioxidant activity was measured using the 1,1-diphenyl-2-picryl-hydrazil (DPPH) method in accordance with Goffi et al. [25], with some modifications. The reaction mixture contained 75 µL of extract and 6.25×10^{-5} M DPPH solution (Sigma Aldrich, Milan, Italy). The antioxidant activity was expressed as µmol Trolox equivalent (TE) per 100 mL of juice.

2.5. Metabolomic Analysis

Aliquots of juices were freeze-dried. The freeze-dried material (10 mg DW) was dissolved in a 700 µL mixture of 1.5 mL of phosphate buffer (Fluka Chemika, Buchs, Switzerland; 90 mM; pH 6.0) in D_2O (Cambridge Isotope Laboratories, Andover, MA, USA)—containing 0.1% *w*/*w* trimethylsilylpropionic-2,2,3,3-d_4 acid sodium salt (TMSP, Sigma–Aldrich, St. Louis, MO, USA)—and CD_3OD (Sigma–Aldrich, St. Louis, MO, USA) (1:1). The mixture was vortexed at room temperature for 1 min. A volume of 600 µL was transferred to a 5 mm NMR tube and analyzed by NMR. The analyses were carried out on three replicates.

NMR spectra were recorded at 25 °C and at 500 MHz using a Varian Unity 500 spectrometer. CD_3OD was used as the internal lock. One-dimensional and two-dimensional NMR spectra were acquired using Varian standard pulse sequences. ^{1}H NMR spectra were acquired using a presaturation sequence to suppress the residual water signal.

^{1}H NMR spectra were scaled to total intensity and bucketed, and reduced to integral segments (0.04 ppm in width) with ACDLABS 12.0 ^{1}H NMR processor (ACDLABS). The regions at δ −0.02–0.02, 4.70–4.90, and 3.30–3.34 were excluded from the analysis (by indicating them as dark regions before integration) because of residual TMSP and solvent signals.

2.6. Statistical Analysis

All results are expressed as the mean ± standard error. The data of the investigated traits within each genotype and ripening stage were analyzed using analysis of variance (ANOVA) with Duncan's test using a significance level of $p \leq 0.05$, and the effects of the accession (A) and the ripening stage (S) were analyzed with a two-way ANOVA.

Principal component analysis (PCA) was applied to evaluate the principal components that contribute to the variation within the dataset, evaluating the differences in physico–chemical and qualitative traits of the three analyzed pomegranate accessions. All analyses were carried out using XLSTAT (XLSTAT, New York, NY, USA). Principal component analysis (PCA) for metabolomic traits was performed with SIMCA-P software (version 14.0, Umetrics, Umeå, Sweden) with scaling based on Pareto.

3. Results and Discussion

3.1. Morphological Characterization of Pomegranate Accessions

The morphological parameters of the three pomegranate accessions considered in this study are shown in Table 2. Fruit weight showed an increase during the four ripening stages, with the highest value for 'Di Benedetto' (392.19 g) and 'Santa Lucia' (323.22 g) showing an increase of 106.3% and 94.92%, respectively, at stage IV; the lowest value was observed in 'Arborea' at stage IV (295.25 g), with an increase during the ripening stage of 75%. During the I and II ripening stage, fruit weight was significantly different among the analyzed accessions.

High values of equatorial and polar diameters were recorded at stage IV in all three accessions. In particular, the highest values were exhibited by 'Di Benedetto' (97.33 mm and 78.38 mm). During the ripening of pomegranate fruits, the physiological and biochemical processes that occur lead to changes in size, color, and flavor, improving the fruit acceptability for the consumer [8]. It is reported that pomegranate genotypes of different geographical

origin show high variability in all of the analyzed traits [22,26], including differences in average fruit weight and whole fruit size. Pomegranate seeds and juice yield differences have also been reported between local accessions in the central area of the Apulia region (Southeastern Italy) [21]. Our results are comparable to those reported by Ferrara et al. for 13 Italian and Israeli pomegranate genotypes [22], which showed a fruit weight range from 192.6 g (for genotype 'MondTri') to 622.3 g (for genotype 'Wond'). Adiletta et al. [27] also reported similar morphological parameters, especially for the 'Roce' and 'Granato' genotypes.

In recent years, pomegranate fruit and juice consumption has strongly increased, leading also to an increase in the market demand [28]. Therefore, to investigate the potential for commercial utilization and the suitability for fresh consumption of the studied accessions, the weight of the pomegranate seeds and the juice yield were measured (Table 2).

Pomegranate seed weight followed an increasing trend during the four ripening stages in all of the accessions, with the highest values recorded for 'Santa Lucia', with an average of 147.21 g and undergoing, at stage IV, an increase of 79.21 %. 'Di Benedetto' and 'Arborea' accessions had corresponding averages of 136.89 g and 141.13 g, with increases in pomegranate seed weight of 76.43 % and 83.30 %, respectively, from stage I to IV.

The juice content is reported in Table 2, and for all three accessions an increase from stage I to IV is shown. 'Di Benedetto' and 'Arborea' had a juice content of 70% and 69.50 % at stage IV, respectively, while 'Santa Lucia' had a lower juice content of 63.25 % (Table 2). Our results are consistent with those reported in the literature, where an increase in pomegranate seed size could be attributed to enhanced fruit juiciness as the fruit matures [29]. It is important to highlight that optimizing the quality attributes of these fruit parts is the goal of growers, breeders, and processors [30,31].

3.2. Physico–Chemical Traits

The total soluble solids content, titratable acidity, pH, and the color of the epicarp were determined (Figure 1 and Table 3) for all of the four ripening stages. The highest content of total soluble solids (TSS) in the three accessions analyzed was detected in stage III (Figure 1A). At stage III, 'Santa Lucia', 'Di Benedetto', and 'Arborea' had TSS values of 15.9, 16.1, and 16.2 °Brix, respectively (Figure 1A), showing no significant statistical differences. The juice of fully ripened pomegranate fruits contained 12–16% sugar, principally glucose and fructose [32]. After stage III, TSS content significantly decreased in 'Di Benedetto' and 'Arborea' pomegranate accessions, affecting their quality, while this reduction was not observed in 'Santa Lucia'.

For all the pomegranate accessions, a lower TA content corresponded to a higher pH value. 'Santa Lucia' showed the lowest TA content and the highest pH value for all the considered stages, while the opposite trend was found for 'Di Benedetto' (Figure 1B,C). In all three accessions, titratable acidity tended to decrease as maturation continued, especially in the 'Di Benedetto' accession, undergoing a reduction of 59.08% at stage IV. Furthermore, two-way ANOVA analysis showed a significant interaction ($p < 0.001$) between accession and ripening stages for the dependent variables TSS, TA, and pH.

Similar results have also been reported by Nuncio-Jáuregui et al. [33], where, during three ripening stages of Spanish pomegranate accessions ('Mollar de Elche', 'Pinón Tierno de Ojós', and 'Borde de Albatera'), a statistically significant increase in TSS and decrease in TA content was observed. Several authors have reported a significant increase in the content of TSS during pomegranate ripening [34], probably due to an increase in sugar content [8]. In non-climacteric fruits, there is a decline in titratable acidity, mostly due to the catabolism of organic acids [33].

The consumers' perception of fruit quality is also influenced by the epicarp color; indeed, skin color is an important quality attribute in pomegranate marketing [35]. Table 3 shows the chromatic changes of the L^*, a^*, and b^* values of the three accessions during the ripening stages. 'Di Benedetto' fruits showed a more intense red color, as demonstrated by high a^* values (Table 2); on the contrary, for 'Arborea' pomegranates, lower a^* values were reported due to the greenish coloring of the epicarp, whereas in 'Santa Lucia' samples,

intermediate values of *a** were recorded (Table 2). Accession and ripening stage showed a significant interaction for the dependent variable *L** in the two-way ANOVA. In the literature, the characteristic red coloration in some pomegranate genotypes has been attributed to the increasing biosynthesis and accumulation of red anthocyanins in the fruit during the ripening process [32].

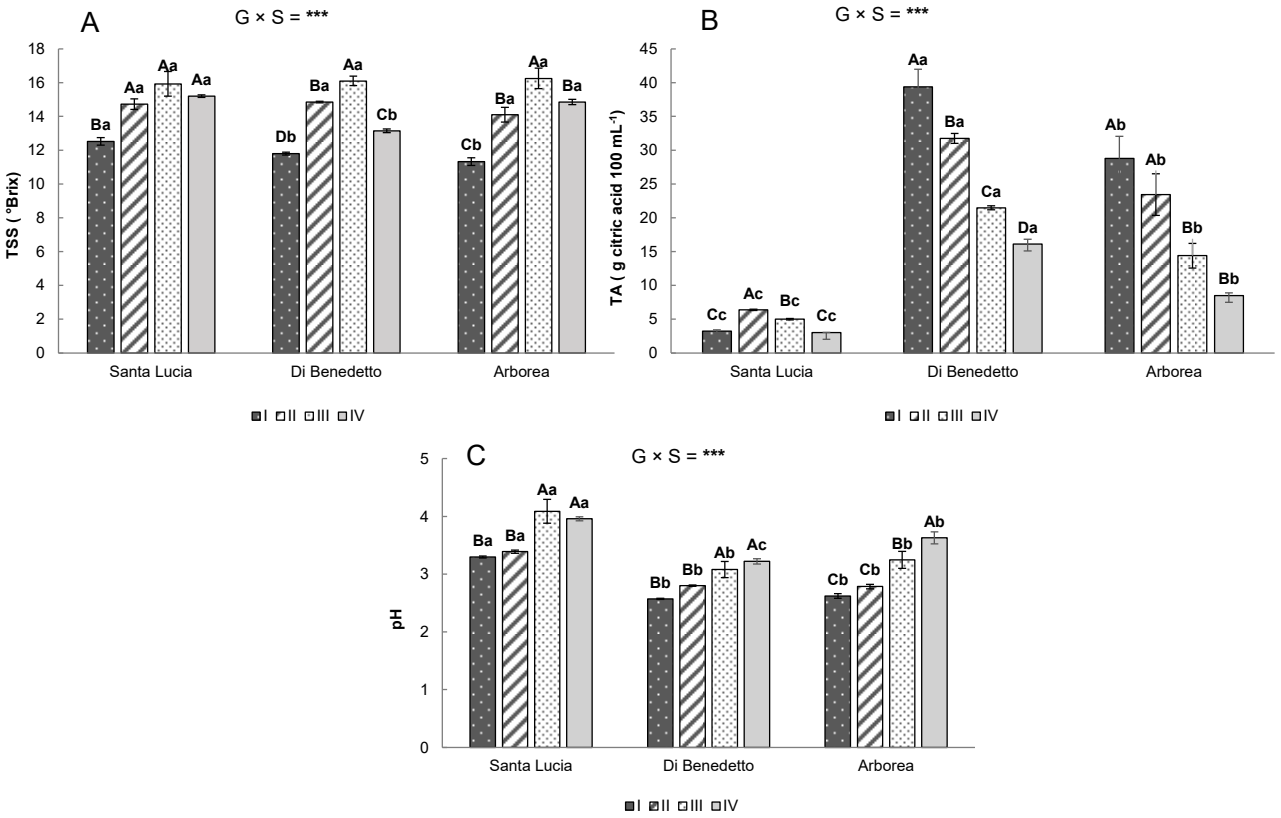

Figure 1. Total soluble solids (TSS) (**A**), titratable acidity (TA) (**B**), and pH (**C**) of three pomegranate genotypes ('Santa Lucia', 'Di Benedetto', and 'Arborea') during I–II–III–IV ripening stages. All the data are expressed as mean ± SE (standard error). Different uppercase letters indicate significant differences during ripening stages in the same accession, and different lowercase letters indicate significant differences in the same ripening stages for the three accessions, in accordance with Duncan's multiple range test ($p = 0.05$). Levels of significance for the two-way ANOVA (Genotype (G) × Stages (S)) are indicated as *** ($p < 0.001$).

3.3. Bioactive Compounds and Antioxidant Activity

Polyphenols, along with other phytochemicals, determine the antioxidant power of pomegranate fruits [26,36]. In our study, the accessions 'Santa Lucia', 'Di Benedetto', and 'Arborea' showed a decline in polyphenol content until the stage III, followed by an increase in stage IV (Figure 2A). Similarly, the antioxidant activity for the 'Di Benedetto' accession decreased into stage III, and subsequently increased in the last stage. In addition, in 'Arborea' fruits, antioxidant activity showed a more stable trend over time, whereas in 'Santa Lucia', a discontinuous trend was observed, with a maximum peak at stage IV and a minimum at stage III (Figure 2B). Flavonoids and anthocyanins are responsible for the color and fragrance of flowers and fruits, which are used to attract pollinators for consequent pollen and seed dispersion [37]. Anthocyanins are responsible for the attractive red and violet colors of pomegranate seeds and juice [38]. The flavonoid content underwent a statistically significant decrease during all stages after full bloom in all the accessions considered (Figure 2D). 'Santa Lucia' and 'Arborea' accessions showed a stable trend, whereas in 'Di Benedetto', an unstable trend, ranging from a maximum of 118.5 mg CA 100 mL^{-1} juice (stage I) to 29.1 mg CA 100 mL^{-1} juice (stage II), was recorded

(Figure 2). The anthocyanin content increased during ripening in all the accessions. In the first three stages after the bloom, 'Santa Lucia' had low levels of anthocyanins, followed by even lower levels in 'Di Benedetto' and 'Arborea'. The maximum value was recorded in stage IV of 'Santa Lucia' (26.8 mg/100 mL juice), while the minimum was recorded in the 'Di Benedetto' accession (10.3 mg/100 mL juice) (Figure 2C). Bioactive compounds and antioxidant activity were highly dependent on the accession and ripening stage (Figure 2).

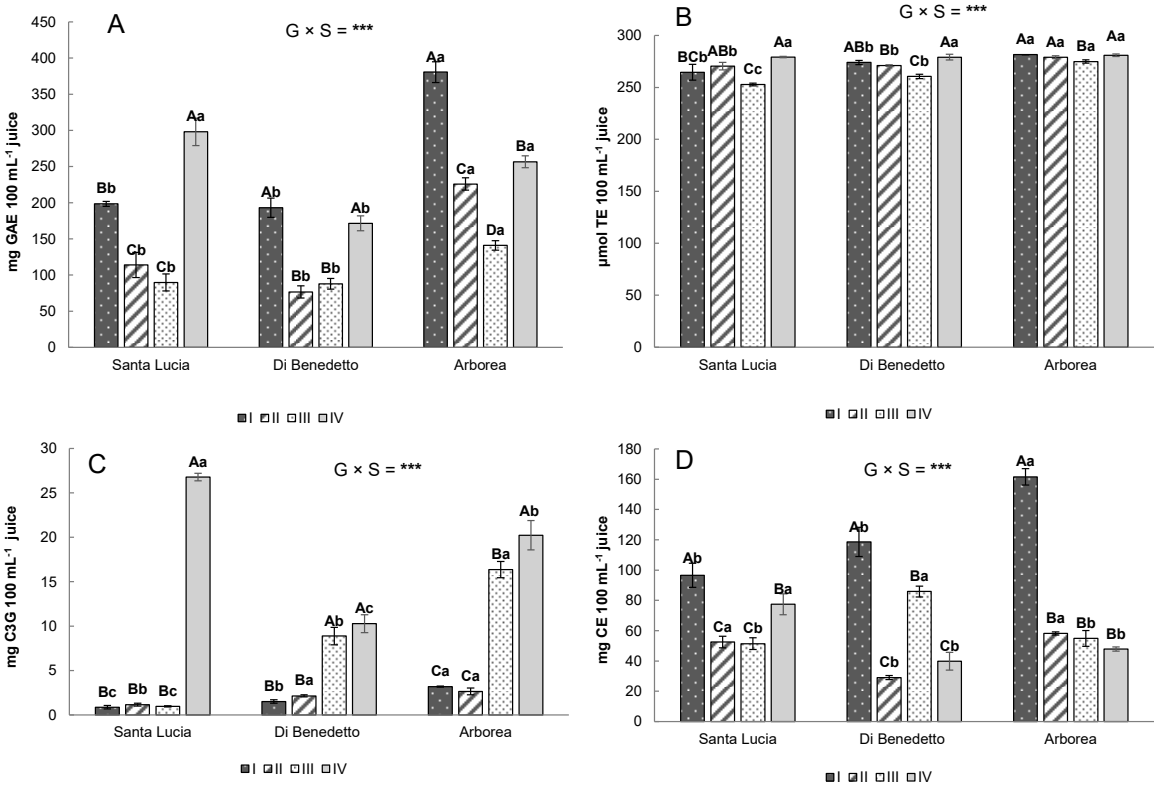

Figure 2. Total polyphenol (**A**), DPPH—antioxidant activity (**B**), anthocyanin content (**C**), and flavonoid content (**D**) of three pomegranate accessions ('Santa Lucia', 'Di Benedetto', and 'Arborea') during I–II–III–IV ripening stages. All the data are expressed as mean ± SE (standard error). Different uppercase letters indicate significant differences during ripening stages in the same accession, and different lowercase letters indicate significant differences in the same ripening stages for the three accessions, in accordance with Duncan's multiple range test ($p = 0.05$). Levels of significance for the two-way ANOVA (Genotypes (G) × Stages (S)) are indicated as *** ($p < 0.001$).

The content of bioactive compounds showed high variability, depending on genotype, growing season, cultivation techniques, and analysis methods [39,40]. Furthermore, polyphenol, anthocyanin, tannin, and flavonoid content all significantly affect antioxidant activities, depending on pomegranate cultivars and the different parts of the fruit [36]. The peel of pomegranate fruit contains higher total polyphenol and tannin content compared to other fruit parts, while higher total anthocyanin and flavonoid contents in the juice and seeds were detected. [40,41]. The total polyphenol content in the analyzed pomegranate accessions was higher compared to the values reported for ten Moroccan accessions (41.01 to 83.4 mg GAE 100 g^{-1} FW) [42]. The difference between accessions was also highlighted by Ferrara et al. [22] in nine local Italian and four Israelian genotypes. The total anthocyanin content reported in our study is in agreement with previous studies, where this value ranged from 213.1 to 585.8 mg L^{-1} in different pomegranate cultivars, and the juice properties were influenced by the extraction method [43,44]. In recent years, the pomegranate has become a functional food of increasing interest, both economically and within the scientific community, generating a significant increase in publications focused mainly on its characteristics, benefits, and nutritional composition [45].

Table 2. Morphological parameters (fruit weight, equatorial diameter, polar diameter, seed weight, and juice content) of pomegranate fruits of the three genotypes ('Santa Lucia', 'Di Benedetto', and 'Arborea') at different ripening stages (I–II–III–IV).

	Fruit Weight (g)			**Equatorial Diameter (mm)**			**Polar Diameter (mm)**		
Stages	**Santa Lucia**	**Di Benedetto**	**Arborea**	**Santa Lucia**	**Di Benedetto**	**Arborea**	**Santa Lucia**	**Di Benedetto**	**Arborea**
I	165.82 ± 5.05 Cb	190.06 ± 7.20 Da	168.71 ± 9.08 Bb	72.97 ± 1.04 Cb	78.13 ± 1.28 Ca	74.25 ± 1.29 Bb	61.70 ± 0.46 Ca	62.75 ± 1.07 Ca	62.14 ± 1.16 Ca
II	267.78 ± 22.24 Ba	242.69 ± 8.40 Cb	193.88 ± 15.94 Bc	84.67 ± 1.95 Ba	81.88 ± 1.52 Cb	75.88 ± 1.59 Bc	70.72 ± 1.30 Ba	65.69 ± 0.90 Cb	65.81 ± 1.32 Bb
III	324.00 ± 35.22 Aa	317.19 ± 19.59 Ba	290.50 ± 13.24 Ab	89.00 ± 3.36 Aa	90.31 ± 2.04 Ba	87.13 ± 1.51 Aa	77.19 ± 2.32 Aa	74.50 ± 1.60 Ba	76.56 ± 1.26 Aa
IV	323.22 ± 16.63 Aa	392.19 ± 14.87 Aa	295.25 ± 10.54 Ab	89.19 ± 1.53 Ab	97.63 ± 1.25 Aa	86.50 ± 1.18 Ac	75.03 ± 1.27 Aab	78.38 ± 1.11 Aa	73.38 ± 1.21 Ab
Significance									
G × S		***			***			***	
	Seed Weight (g)						**Juice Content (%)**		
Stages	**Santa Lucia**	**Di Benedetto**	**Arborea**				**Santa Lucia**	**Di Benedetto**	**Arborea**
I	82.20 ± 3.75 Ca	77.59 ± 3.72 Ca	76.99 ± 4.82 Ba				54.50 ± 1.32 Ba	51.50 ± 5.50 Ba	37.50 ± 9.85 Db
II	124.38 ± 13.42 Ba	104.69 ± 5.57 Bb	85.109 ± 6.54 Bb				68.75 ± 2.53 Aa	53.50 ± 6.96 Bb	48.00 ± 10.03 Cb
III	141.03 ± 14.96 ABa	124.79 ± 8.22 Aa	135.119 ± 7.51 Aa				66.75 ± 3.66 Aa	68.25 ± 7.17 Aa	61.50 ± 11.87 Ba
IV	147.31 ± 7.96 Aa	136.89 ± 7.60 Aa	141.129 ± 6.60 Aa				63.25 ± 4.90 Ab	70.00 ± 8.91 Aa	69.50 ± 12.96 Aa
Significance									
G × S		***						***	

All the data are expressed as mean ± SE (standard error). Different uppercase letters within each column indicate significant differences during ripening stages in the same accession, and different lowercase letters within each row indicate significant differences in the same ripening stages for the three accessions, in accordance with Duncan's multiple range test ($p = 0.05$). Levels of significance for the two-way ANOVA (Genotypes (G) × Stages (S)) are indicated as *** ($p < 0.001$).

Table 3. Color attributes (L^*, a^*, b^*) of three pomegranate genotypes ('Santa Lucia', 'Di Benedetto', and 'Arborea') at different ripening stages (I–II–III–IV).

	L*			***a****			***b****		
Stages	**Santa Lucia**	**Di Benedetto**	**Arborea**	**Santa Lucia**	**Di Benedetto**	**Arborea**	**Santa Lucia**	**Di Benedetto**	**Arborea**
I	48.62 ± 0.87 Cc	53.21 ± 1.41 Bb	56.56 ± 0.99 Ca	5.10 ± 1.37 Cab	30.42 ± 16.52 Aa	−0.78 ± 1.53 Dc	27.55 ± 0.76 Ab	32.73 ± 1.05 Ba	34.89 ± 0.94 Aa
II	58.53 ± 1.22 Ba	59.25 ± 1.15 Aa	59.56 ± 1.03 Ba	13.52 ± 1.63 Bb	28.81 ± 1.08 Aa	7.78 ± 1.71 Cc	68.82 ± 36.12 Aa	35.58 ± 0.72 Aa	36.93 ± 0.84 Aa
III	64.19 ± 1.31 Aa	52.06 ± 1.12 Bb	65.02 ± 0.71 Aa	15.81 ± 2.28 Ba	116.87 ± 76.83 Aa	15.01 ± 1.41 Ba	30.98 ± 0.81 Ab	26.23 ± 1.10 Cc	35.17 ± 0.33 Aa
IV	44.70 ± 1.21 Db	45.55 ± 1.11 Cb	61.21 ± 0.93 Ba	33.13 ± 0.78 Ab	36.41 ± 1.18 Aa	20.51 ± 1.16 Ac	22.28 ± 0.97 Aa	23.67 ± 0.86 Ca	79.92 ± 44.40 Aa
Significance									
G × S		***			ns			ns	

All the data are expressed as mean ± SE (standard error). Different uppercase letters within each column indicate significant differences during ripening stages in the same accession, and different lowercase letters within each row indicate significant differences in the same ripening stages for the three accessions, in accordance with Duncan's multiple range test ($p = 0.05$). Levels of significance for the two-way ANOVA (Genotypes (G) × Stages (S)) are indicated as ns (not significant and *** ($p < 0.001$)).

3.4. ^{1}H NMR Analysis

^{1}H NMR analysis was carried out, in which the spectra were processed and bucketed, and the data were analyzed by PCA. From the PCA score scatter plot (Figure 3), it is clear that the samples of the 'Di Benedetto' accession were differentiated from the other two accessions, since they were separated, along PC1, from the 'Santa Lucia' and 'Arborea' samples. The 'Di Benedetto' juice was indeed characterized by a much higher relative content of citric acid than the juice derived from the other two accessions. The separation along PC2 mirrored, instead, the time of collection for 'Di Benedetto' and 'Arborea'; meanwhile, for 'Santa Lucia', all the samples were clustered together, showing no relative change in metabolite content over time. The differences along PC1 were mainly ascribable to sugars, which showed a relatively higher abundance in 'Santa Lucia' and 'Arborea' compared to 'Di Benedetto'. During ripening, a decrease in the relative abundance of citric acid, and an increase in sugars, was observed. NMR-based metabolomics is a valid tool to highlight the similarities and dissimilarities between different pomegranate ecotypes, and to discriminate their different geographical origins [46]. The metabolomics analysis, in our case, allowed us to have an overview of the changes in the content of different metabolites, both between the different accessions and the different ripening stages. From the PCA of the metabolomics data (Figure 3), it was observed that the main metabolites that changed during ripening were sugars and citric acid. Citric acid levels decreased over time, while the sugar content increased. These changes were stronger for the accessions 'Di Benedetto' and 'Arborea', while for 'Santa Lucia', the changes over the different ripening stages were minimal. Furthermore, it was observed that the 'Di Benedetto' accession was, in general, characterized by a higher content of citric acid. The increase in sugar content and the decrease in organic acids obtained via metabolomic analysis was also confirmed by physico–chemical analysis (Figure 1A,B). The accumulation of sugars during the ripening stages is directly controlled by increasing the activities of sucrose synthase and sucrose phosphate synthase [47].

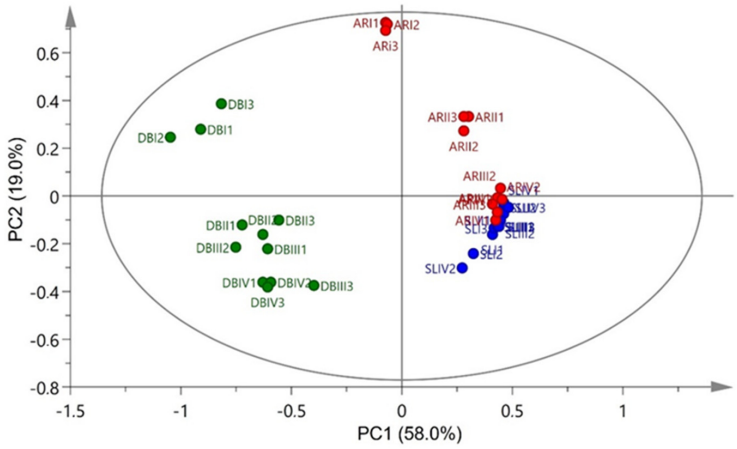

Figure 3. Principal component analysis of ^{1}H NMR data, score scatter plot of PC1 versus PC2. The ellipse represents the Hotelling T2 with 95% confidence. 'Di Benedetto' (DB); 'Arborea' (AR); 'Santa Lucia' (SL). Numbers I–IV refer to the stages, as indicated in Table 1. Numbers 1–3 indicate different replicates.

The accumulation of organic acids, such as citric and malic acids, in mesocarp cells depends both on the genotype and on environmental stresses [13,48]. Different organic acids, the most abundant being citric and malic acids, are reported from fruits, leaves, and seeds of pomegranates. Furthermore, ascorbic, fumaric, oxalic, quinic, succinic, and tartaric acids are reported, although they are usually detected in lower quantities compared to citric and malic acid [3]. As described by Chater and collaborators, the level of compounds, such as citric acid, glucose, and fructose, are important to discriminate the different pomegranate accessions [49]. Moreover, the cultivation technique also affects the production of metabolites; in fact, Villa-Ruano and collaborators reported that pomegranate juice obtained

from conventionally grown plants possesses higher concentrations of acetone, aspartic acid, and ethanol, whereas those grown organically contain higher amounts of acetic acid, alanine, arginine, fumaric acid, GABA, galactose, glutamine, histidine, isoleucine, lactic acid, leucine, malic acid, mannose, methionine, phenylalanine, proline, sucrose, threonine, trigonelline, tyrosine, and valine [50]. Legua and coworkers reported that the cultivars 'Piñón duro de Albater' and 'Wonderful' possessed a higher amount of citric acid than the other 17 evaluated pomegranate cultivars [42]. Usually, the highest concentration of organic acids, such as citric, fumaric, tartaric, and malic acid, is registered in unripe fruits, as reported in the grape berry ripening process [51].

In the metabolomics analysis, no significant changes in the phenolic component were observed, neither for the ripening stages or the accessions. However, it must be underlined that the phenolic component is negligible compared to that of the primary metabolites; therefore, target analyses of phenolic-enriched fractions would provide a tool to further explore this aspect in the future. In the present study, colorimetric assays showed that there was a general decrease in the phenolic content in the first three stages, followed by an increase in the last ripening stage that was accompanied by an increase in polyphenolic content and antioxidant capacity (Figure 3).

3.5. Principal Component Analysis

Principal component analysis (PCA) was used to highlight the differences in the three pomegranate genotypes ('Santa Lucia', 'Di Benedetto', and 'Arborea'), concerning the changes in pomological, qualitative, and chemical traits. The first two principal components (PCs) disclosed 100% of the cumulative variance (Figure 4), with PC1 explaining 57.65% and PC2 42.35%. The PC1 was positively correlated with nutraceutical parameters and juice content, while PC2 was negatively correlated with TSS and pH. Several studies demonstrated that multivariate data analysis is a valid tool to highlight similarities and differences between all analyzed traits [27,52,53]. The nutraceutical and antioxidant traits showed a correlation with 'Arborea' genotype, while juice content, colorimetric index, and titratable acidity were correlated positively with 'Di Benedetto' fruits. 'Santa Lucia' pomegranate showed the highest values of TSS and pH.

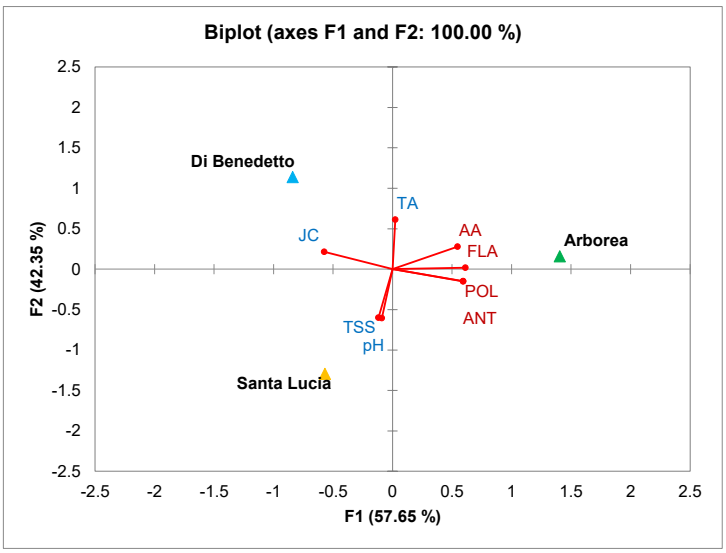

Figure 4. Principal component analysis (PCA) of total polyphenol (POL), antioxidant activity (AA), anthocyanin (ANT) content, flavonoid (FLA) content, juice content (%) (JC), and of qualitative parameters (TSS, TA, and pH) in 'Santa Lucia', 'Di Benedetto', and 'Arborea' pomegranate genotypes.

4. Conclusions

This study provides useful information regarding the morphological, qualitative, and metabolomic features of three pomegranates genotypes, 'Di Benedetto', 'Santa Lucia',

and 'Arborea', during four ripening stages. Significant intra- and inter-variability was observed among all analyzed genotypes during ripening, and the third ripening stage was optimal for obtaining fruits with a good balance between sugar and organic acid contents, as well as nutraceutical and agronomic traits of the pomegranate. Metabolomic analysis of pomegranate fruits also revealed a clear distinction between genotypes based on organic acid and sugar content. 'Di Benedetto' can be considered a promising genotype, with an advantage in the fresh market due to its fruit weight. On the other hand, 'Santa Lucia' is a subacid genotype with low acid content and is rich in nutraceutical compounds. This genotype can therefore be used for juice production. Significant qualitative changes occurred during the ripening stage, influencing taste and flavor. Understanding the bio-agronomic and nutraceutical traits of local pomegranate accessions, grown on different continents, could pave the way for breeding programs and germplasm management.

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