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Freeze dried pomegranate juices of Moroccan fruits: main representative phenolic compounds.

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Abstract

Background: The pomegranate (Punica granatum) is an ancient perennial plant species of the Punicaceae family. Its seeds are consumed as food or as juice. Previous studies have noted that pomegranate juice encompasses many active compounds with beneficial effects. The main goals of this work were to study the phenolic component of freeze-dried and reconstituted pomegranate juices obtained from 13 pomegranate genotypes growing in Morocco.

Results: We analyzed several pomegranate juices by means of high-performance liquid chromatography and high-resolution-mass-spectrometry to determine phenolic compounds. 27 bio-phenols, belonging to four different classes (phenolic acids, hydrolyzable tannins, anthocyanins, and flavonoids), have been identified based on their accurate mass measurements and quantified. Some encouraging results were obtained. Even though the freeze-drying process introduced a marked degradation of bio-phenols, substantially lowering their levels in the reconstituted fruit juices, these latter are still rich enough in bio-phenols to compete with some fresh fruit juices. The reconstituted juices obtained by rehydration of the lyophilized material still differ one from another enough to enable a statistical classification based on their polyphenols content. A correlation analysis was applied to the polyphenol data to explore correlations and similarities between genotypes.

Conclusions: The results showed that freeze-drying and reconstitution of juices introduce some degradation to the polyphenol content, the overall polyphenolic pattern within the same

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cultivar, in two different harvesting years, is however maintained, suggesting composition stability of the freeze-dried juices produced in this time span.

Keywords: pomegranate; phenolic compounds; flavonoids; functional foods; mass spectrometry.

1. Introduction

The pomegranate (*Punica granatum*) is an ancient species of perennial plant of the Punicaceae family considered to be a miracle fruit.[1] Its seeds are consumed as food or as juice.[1] All over the world, pomegranate juice is widely known for its unique color, taste, and health benefits.[2] Previous studies have noted that pomegranate juice encompasses a wide range of classes of metabolites used in the health field, namely diabetes and obesity treatment, possessing also anti-inflammatory effects. The pomegranate juice is rich in amino acids, sugars and organic acids[3] that also contribute to its healthy properties. In addition to the juice, other parts of the fruit have been demonstrated valuable in medicinal applications.[1] The constituents of pomegranate indeed exhibit a wide range of bioactivities, such as anti-cancer, antimicrobial, antioxidant, and anti-inflammatory activities.[4-7] Pomegranate juice, however, is mostly known for its richness in dietary polyphenols with a potential antioxidant effect attributed mainly to its content of flavonoids, anthocyanins, and tannins.[8-14]

These phytochemicals are important factors for determining the quality of pomegranate juice.[1] Phenolic compounds are a large group of secondary metabolites, comprising at least one aromatic ring, one or more hydroxyl groups, and other constituents.[15] To date approximately 10,000 compounds have been characterized that are involved in many physiological processes that somehow reflect plant's interactions with the environment and their structure confers them very unique functions.[8,16] Phenolic compounds also contribute to the growth and development of the plant through various actions: are involved in the metabolism and transport of auxin,[17] in that of ethylene[18] and flavonoids can condition the formation of pollen grains.[19] The bio-phenols composition of pomegranate juice has been studied, showing variations among cultivars depending on the region of production and growing conditions.[20]

Several articles have reported the analysis of phenolic compounds in pomegranate juice and other parts of the fruit of different cultivars using colorimetric tests such as the Folin-Ciocalteu and Trolox tests.[21-27] These methods are not capable to deliver fine-grained information on the singular components of the phenolic class of compounds, which could be

attainable by more time-consuming and costly analyses such as Liquid Chromatography (LC)/Diode Array Device (DAD), LC/Tandem Mass Spectrometry (MSMS) and LC/High Resolution Mass Spectrometry (HRMS). This latest approach has been recently successfully applied for the determination of polyphenols in several matrices.[23,28]

From a commercial point of view, the use of concentrated fruit juices is widespread in the distribution chain as it allows to reduce costs and sometimes increases the shelf life of treated juices. Fruit juice concentrates are also easier to pack and transport. The concentration procedure of the juices implies the reduction of the water levels which in turn, inhibits the growth of microorganisms (leading to the aforementioned shelf-life increase).

To reduce the water content in juices, a wide range of methodologies, based on membrane filtration, freeze-drying, and thermal evaporation, are available.[29]

This latter approach is the most common but can lead to the depletion of several valuable molecules such as flavors, vitamins, antioxidants, and nutraceutical compounds.[29]

On the other hand, the freeze-drying procedure to obtain concentrated juices is considered one of the mildest approaches allowing for the preservation nutritional components of many foods, keeping them practically intact. Some losses in vitamins and other valuable bio-compounds (antioxidants and polyphenols) are however observed and this aspect should be taken in mind when choosing the best analytical procedures to determine them.[30] For this reason, we decided to carry out a series of analyses on pomegranate juices obtained from concentrated fruit juices, using sensitive and selective methods. This investigation aimed to quantify the residual polyphenols inside the concentrated juices and verify whether the residual amount of these minor components is still sufficient to guarantee the reconstituted juices' beneficial effects on health.

2. Materials and methods

In this study 13 cultivars of pomegranate were evaluated: seven were foreign varieties ('Dwarf semi-Evergreen', 'Gordo de Jativa', 'Mollar Osin Hueso', 'Negro Monstrioso', 'Ruby', 'Zherie d'Automne' and 'Zherie précoce') and six were local clones ('Bzou', 'Djeibi', 'Grenade rouge', 'Khikho', 'Ounk Hmam', 'Sefri') derived from surveys in different regions of Morocco and planted in a collection at the INRA experimental domain in Ain Taoujdate. This domain is in the Saïs plain at an altitude of 550 m, on moderately limestone clay-loam soil with an average of 3.04% $CaCO_3$ and rich in organic matter with an average of 2.51% on the surface. The average annual rainfall is 460 mm. The average temperature ranged from

9.67 ° C in January to 27.25 ° C in August. The orchard is irrigated at a rate of 2,500 m^3/ha in addition to the rainfall, from May to mid-October. All the genotypes were in the same geographical conditions and underwent the same horticultural practices. Foreign cultivars are among the most cultivated in the Mediterranean basin, hence the importance of their consideration in this work.

2.1. Fruit sampling

The fruits of Moroccan pomegranate varieties and the international cultivars were harvested during the first week of September and early October 2017/2018 in the INRA experimental farm. For each variety, four samples were collected. The plants were grown as a bush with three to five trunks spaced two meters apart within a row and four meters between rows. The fruits were harvested at commercial maturity, 15 weeks after fruit set, and were stored at room temperature for a few days, until use.

2.2. Juice extraction

The fruits were washed and peeled by hand. The arils were pressed using a mechanical press (juicer) to obtain juice. Before analysis, the juices obtained from the four samples harvested per cultivar were reunited, centrifuged for five min at room temperature to remove the pulp, and then stored at -20 ° C until analysis.

To perform LC/MS analyses four up to ten ml accurately weighted, of each sample were posed in test tubes. After a preheating phase lasting 20 minutes, the samples were freeze-dried at -60 °C for 48 hours at a pressure of 0.011 mbar, using a Christ Alpha 1-2 LD PLUS apparatus.

Once completed the freeze-drying process, the tubes were weighed to accurately determine the water loss for each sample. The final weights of the freeze-dried juices were, on average, 17% of the original weight. The freeze-dried samples were finally stored at -18 °C.

2.3 Antioxidant properties evaluation: DPPH assay

The evaluation of antioxidant properties is reported as the first step to evaluate the interactions among the antioxidant compounds.

The free radical scavenging activities of fresh fruit juices were evaluated for their ability to quench the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical[31,32] and monitoring its absorbance

decrease. Inhibition of free radical DPPH• was expressed as TEAC (Trolox Equivalent Activity Capacity).[33,34]

The free scavenging activity was evaluated using a method described by Brand-Williams[32] with a modification in the reaction time. Briefly, freeze-dried fruits (0.5 g) were mixed with ten mL of methanol/water (80:20 v/v, 1 % HCl), sonicated at 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 15,000 rpm for ten minutes. At this stage, ten µL of the supernatant were mixed with 40 µL of methanol and added to 950 µL of DPPH solution. The mixture was shaken vigorously and placed in a dark room for ten minutes. The decrease in absorbance was measured at 515 nm in UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France).

2.3.Phenolic compounds determination by LC/HRMS

Polyphenol determination has been performed by adopting a recent approach consisting of coupling of a UPLC separation system with a high-resolution mass spectrometer equipped with a soft ionization electrospray (ESI) source. Mass spectrometry is an analytical technique able to provide information of quantitative and qualitative nature on an ample range of substances.[31,35-38] For the largest and delicate species, a soft ionization process is required that could gently lead even polar and large species into the gas phase.[39-40] While the LC/MSMS approaches could lead to obtain structural information on the investigated analytes,[41-44] the UPLC/ESI/HRMS approach is reported to be the most appropriate for the accurate determination of bio-phenolic species. By means of this, admittedly costly approach, it is possible to markedly reduce analysis interferences[45] allowing the isolation of the analytes based on their accurate m/z values. Our experimental design prevised to restore from the freeze-dried juices the original fruit juice by adding an appropriate water volume before the instrumental determination.

In an analytical balance, Mettler Toledo ML104 (Switzerland) was accurately weighed about 125 mg of each freeze-dried fruit juice using 2.5 ml Eppendorf tubes. Each juice sample was diluted with 1,5 ml of deionized water, and, to reduce dissolving times, the tubes were posed in an ultrasonic bath for ten minutes. The solutions were filtered through a syringe filter 0.45 µm before being posed in the vials for the following HPLC/MS analysis.

The polyphenols determination was performed on a UHPLC (Dionex UltiMate 3000 Rapid Separation LC) system by Thermo Fischer Scientific (Bremen, Germany) equipped with an autosampler, coupled to a quadrupole Orbitrap mass spectrometer (Q-Exactive; Thermo

Scientific), configured with a heated electrospray ion source. Ionization of polyphenols was performed in the mixed positive-negative-ion mode under the following conditions optimized in order to reduce ESI drawbacks,[46,47]: sheath gas flow rate 35 (arbitrary units); auxiliary gas unit flow rate 4 (arbitrary units); spray voltage 3.5 kV; S lens RF 30 V; capillary temperature 320 °C; auxiliary gas heater temperature 250 °C The Chromatographic conditions were like that of Di Stefano et al.[23] with slight modifications. In brief, the elution was performed using a C-18 column Thermo Hypersyl Gold 2.1mm x 5 cm, 1,9 µm particle size. The elution gradient (at 400 µl/min) was the following: mobile phase A: water 0,1% formic acid; mobile phase B: methanol, 0,1% formic acid; isocratic elution, 2 min 2% (B); gradient elution 3 min up to 10% (B), gradient elution 5 min up to 35% (B); gradient elution 4 min up to 100% (B), isocratic elution 4 min 100% (B), step 0 min up to 2% (B); isocratic elution 5 min 2% (B). The mass resolving power was 35 000, full width at half maximum (at m/z 200), and the scan range was 100–800m/z. The scan rate was set at $1s^{-1}$ and the automatic gain control target was set at 1×10^5 ions for a maximum injection time of 200 ms.

Quantitation was performed adopting the following pure standards: quercetin, gallic acid, l-mandelic acid, gentisic acid, chlorogenic acid, naringenin, resveratrol, cyanidin 3-glucoside, cyanidin 3-5 diglucoside. A standard solution (10 ml) containing 5 ppm of each of the aforesaid analytical standards was made, using methanol as solvent. From this solution were obtained other five calibration solutions at 1ppm, 200 ppb, 100 ppb, 50 ppb, and 10 ppb of each analyte.

When reference compounds were not available, the quantitation was based on structurally related substances. In particular cyanidin 3-O-glucoside (m/z 447.093 $[M - 2H]^-$) in negative mode is the reference compound for the determination of vanillic acid hexoside, ferulic acid hexoside, ellagic acid pentoside, phlorizin, ellagic acid deoxyhexoside, kaempferol 3-O-glucoside, quercetin 3-O-hexoside, hexahydroxydiphenoyl (HHDP) hexoside, digalloyl hexoside, corilagin, lagerstannin. Cyanidin 3-O-glucoside is the reference compound in positive mode for pelargonidin-3-O-glucoside, delphinidin-3-O-glucoside, delphinidin-3-O-hexoside, and cyanidin-3-O-hexoside.

Here follow the chromatograms of the standard mixture at 500 ppb (Figure 1) and of a reconstituted pomegranate juice (Figure 2).

2.4.Statistical analysis

To perform statistical evaluation, data were normalized ($\mu = 0$ and $\sigma = 1$) to transform all characters to a comparable scale. The SPSS v22 software was used for the data analysis. Analysis of variance was performed to check for significant differences among the samples collected. Principal component analysis (PCA) was conducted using the correlation coefficient. In addition, two 3D scatterplots were created using the first three components of the PCA as axes of the 3D graphs, using the OriginLab v. 2020 software. Likewise, a heatmap correlation that groups the results of the two years was generated by the same software.

3. Results and discussion

In the literature phenolic composition of pomegranate juices has been recently reported.[48] In this study, among 53 phenolic compounds were identified: 20 hydrolyzable tannins, 15 phenolic acid derivatives, 12 uncolored flavonoids, four lignans, and two organic acids, but only 17 analytes are in common to all the cultivars.

It is noteworthy that the concentration of phenolic compounds could differ according to the extraction protocol and the portion of fruit used during the extraction procedures. Indeed, the DPPH test showed that the juice obtained from the whole fruit and/or the rind contained a higher amount of total antioxidant compounds than that produced from the arils alone.[49] Moreover, also the total content of hydrolyzable tannins[9] was higher in the juice obtained from the whole fruit. This was attributed to the passage of polyphenols from the membrane and rind of the pomegranate to juice during the pressing of whole fruits.[49]

Conversely, the highest total monomeric anthocyanins (14 mg cyanidin-3-glucoside equivalent / 100 ml raw juice) and the lowest flavonoid amount (23 mg catechin equivalent / 100 ml) were detected in the juice obtained from the whole fruit.

This decrease in flavonoid levels has been attributed to the formation of intensely colored flavanol anthocyanins adducts.[50]

Our results are reported in Table 1, which shows the levels of the phenolic compounds for the juice samples related to the harvesting years 2017 and 2018. The average, minimum and maximum concentrations according to the cultivars of Moroccan pomegranates are presented in the same table.

For the year 2017, the ‘Negro Monstrioso’ genotype was the cultivar with the highest content of total flavonoids, total tannins, total anthocyans, hydroxycinnammic acid, cyanidin-3-glucoside, HHDP-hexoside, quercetin 3-O-hexoside, and pelargonidin-3-O-glucoside. These results were also confirmed in 2018, when this genotype presented the maximum values,

among the other cultivar juices, for the following seven phenolic compounds: cyanidine3-5-diglucoside, cyanidin-3-glucoside, HHDP-hexoside, kanokoside A, pelargonidin-3-O-glucoside, kaempferol-3-O-glucoside, and delphinidin-3-O-glucoside.

The genotype 'Gordo de Jativa', in the year 2017, is characterized by the highest concentration of the following three phenolic compounds: ellagic acid pentoside, ellagic acid deoxyhexoside, and phlorizin, these results were almost confirmed in 2018, when 'Gordo de Jativa' shown the highest levels of ellagic acid pentoside and ellagic acid deoxyhexoside. The 'Ruby' genotype was characterized in the 2017 harvest by the highest levels of DPPH, digalloyl hexoside, and corilagin. Unfortunately, these levels were not confirmed in the 2018 harvest. chlorogenic acid, gentitisic acid, and ferulic acid hexoside are the most representative compounds in the 'Zherie précoce' genotype, at 0.03 ppm, 0.04 ppm, and 2.05 ppm, respectively. These results were partially confirmed in the 2018 harvest.

Our data evidenced that the polyphenol content in pomegranate fruit juice undergoes a marked reduction due to the freeze-drying process. The residual amount, in the reconstituted juices, ranges from 0.5 to 5% of the initial polyphenol content but remains comparable to the levels found in fresh fruits other than pomegranates[51,52] and probably is still high enough to justify healthy effects. To the best of our knowledge, other studies on freeze-dried and reconstituted pomegranate fruit juices are not present in literature and a comparison is not possible. Some studies on pomegranate freeze-dried seeds evidenced a far less severe loss in antioxidant compounds. In particular, Al Sanabani et al.[53] reported a marked reduction (70%) of anthocyanins content with respect to the fresh seeds. Flavonoids and phenols showed a less severe hit by the freeze-drying process (content reduction of about 20%). Other studies conducted in freeze-dried fruit juices evidenced that the freeze-drying process is the most recommended to obtain a reduced loss in antioxidants.[30]

In the literature has been reported that cyanidin-3,5-diglucoside was the predominant anthocyanin representing between 37 and 53% of total anthocyanins for the five pomegranate cultivars of global commercial interest[54] but anthocyanin proportions can change considerably following genetic factors.[55,56] In our study the residual concentration of cyanidin-3,5-diglucoside, in a reconstituted juice, ranges from 0.0719 ppm up to 0.23 ppm, conversely the cyanidin-3-glucoside, habitually present in a slightly lower extent in pomegranate juices, ranges from 0.18 up to 37 ppm. This could indicate either better stability of the mono-glucoside or the conversion of the di-glucoside in the mono glucoside moiety due to the fruit juice processing and conservation. Similar values of cyanidins derivatives are reported for the

European plum (33.850 ppm), Olives (72.352 ppm), Red raspberry (0.279-4.949 ppm), and Cherries (13.865 – 143.269 ppm).[52]

On the other hand, reading the results for the year 2018 shows that the 'Zherie d'Automne' genotype exhibited the lowest phenolic profile for the phenolic compounds. Likewise, the Ruby genotype recorded the lowest levels of Quercetin, cyanidin-3-5-diglucoside, gentisic acid, quercetin 3-O-hexoside, and phlorizin.

The HPLC analysis performed along with the most abundant substances retrieved is reported in Figure 1 which shows a typical HPLC chromatogram for juice, recorded at different m/z. Phenolic profiles and peak areas varied among cultivars, as fruit maturity, processing and storage can also significantly influence the content of the bio-phenols content. It has also been shown that variety, geographical origin, and growing conditions are also key factors. For this study, the pomegranate samples were collected from cultivars grown under homogeneous and well-controlled climatic conditions.

3.1. ANOVA and multivariate analysis of variance of all studied variables over pomegranate juice

Several compounds (22) belonging to phenolic acids (derivatives of hydroxycinnamic acids and hydroxybenzoic acids) and flavonoids (flavonols, flavones, and anthocyanidins) were detected in all the samples. Table 2 shows the different phenolic compounds detected. Flavonoids and phenolic acids are ubiquitous in plants at widely varying concentrations. These phytochemicals are generally classified as food antioxidants.[57]

Analysis of the results (ANOVA) showed highly significant differences among the genotypes studied at $p < 0.001$, showing significant differences among cultivars. Likewise, for the year factor the analysis showed highly significant results except for the phenolic compounds gentitisic acid; HHDP-hexoside; phlorizin; vanillic acid hexoside). Regarding the interaction between genotypes per year, this analysis also showed significant differences at $p < 0.001$ except for phlorizin (Table 2). The results presented in Table 2 also show a very significant impact of genotype and year. In addition, the interaction between genotype and variety was statistically significant.

Several studies have underlined the importance of these descriptors in exploring the potential correlations between them and certain antioxidant compounds, mainly phenols (anthocyanins, tannins, catechins) and carotenoids (lycopene, beta-carotene).

3.2. Principal component analysis (PCA)

A principal component analysis was carried out to define the variables contributing the most to the total variance and therefore, the most involved in the discrimination of genotypes. PCA is the widely used multivariate technique for reducing the size of numerical data sets and facilitates understanding of similarities and differences between objects by generating two or three-dimensional models without losing much information.[58] This analysis aims to define the main factors allowing to limit the number of discriminating variables to classify the cultivars according to their phenolic compositions and their respective contents. In this work, only variables with loading greater than 0.6 were considered significant for each major component. Indeed, for the year 2017, total variance of 56.77% was explained by the two three components, against 72.452% for the year 2018 (Table 3). Thus, in 2017 the first component was explained by 8 variables which are, total tannins (r = 0.87); total anthocyanins (r = 0.935); quercetin (r = 0.88); hydroxycinnammic acid (r = 0.63); cyan-3-glucoside (r = 0.98); HHDP-hexoside (r = 0.91); quercetin 3-O-hexoside (r = 0.98) and pelargonidin-3-O-glucoside (r = 0.92). These variables represent more than 36.36% of all the variables studied and explain more than 29.78% of the total variability observed (Table 3).[59]

The first component of the year 2018 was explained by 13 variables, justifying about 60% of all the variance. These variables are DPPH, total flavonoids, total anthocyanins, cyanidin 3-5-diglucoside, cyanidin-3-glucoside, HHDP-hexoside, quercetin 3-O-hexoside, phlorizin, kanokoside A, pelargonidin-3-O-glucoside, kaempferol-3-O-glucoside, delphinidin-3-O-glucoside, and vanillic acid-hexoside, which means that these attributes showed the greatest variation between genotypes and had the greatest impact on their discrimination.

The second component expresses 14.6% (in 2017) and 20.3% (in 2018) of the total inertia and was strongly influenced by DPPH, kanokoside A, kaempferol-3-O-glucoside, vanillic acid hexoside (in 2017 samples) and by quercetin, hydroxycinnammic, chlorogenic acid, mandelic acid, gallic acid, ellagic acid deoxyhexoside, corilagin (in 2018 samples).

Finally, the third principal component expresses 12.4% in 2017 and 13.0% in 2018 of the total inertia and is represented by only two compounds for the two years which are ellagic acid deoxyhexoside and phlorizin (in 2017 samples) and gallic acid and vanillic acid hexoside (in 2018 samples).

The PCA scatter plots were drawn based on the first three principal components, PC1, PC2, and PC3 for the two years (figures in Supplemental Information SI 1-a,b and SI 2-a,b). The distribution of cultivars varies depending on the year. Comparison by year can also give

information on the stability of polyphenols in genotypes. The information on cultivar/year approaches could lead to some discrimination.

For the year 2017 (Figure SI 1-a), cultivars 'Gordo de Jativa', 'Ruby', and 'Negro Monstrioso' were classified far from other genotypes due to their characteristic profiles. Indeed, the genotype, 'Gordo de Jativa', combined the highest concentrations of ellagic acid-pentoside and ellagic acid deoxyhexoside. The 'Ruby' genotype is denoted by the highest concentrations of DPPH; digalloyl hexoside and corilagin. The genotype 'Negro Monstrioso' showed the highest concentrations of 10 phenolic compounds and the highest anthocyanins concentration, indicating that this cultivar contained a great source of antioxidant compounds. On the other hand, the three genotypes 'Khikho', 'Mollar Osin Hueso', and 'Sefri' generally were characterized by the lower values of total anthocyanins, cyanidin-3-glucoside, ellagic acid deoxyhexoside, quercetin, ellagic acid pentoside; phlorizin, chlorogenic acid, gentitisic acid, ferulic acid hexoside, and digalloyl hexoside. The lowest values of total anthocyanins were in 'Khikho' (2017) and 'Zherie précoce' (2018) genotypes, confirming that these two varieties were not suitable for juice production due to browning and problems of color alteration (slightly colored fruits). From our investigation results, the other genotypes had relatively similar profiles. Regarding the scatter diagram for the year 2018 (Figure SI 2-a), we noticed a huge dispersion of genotypes. Even this year, the 'Negro Monstrioso' genotype stands out for its high content of polyphenols.

3.3. Correlation between variables

To identify the relationships among the phenolic compounds studied, all the descriptors used were involved in a bivariate correlation using Pearson's model (heatmap correlation, Figure SI 3). The data for the year 2017 are marked at the top, while those for 2018 are at the bottom of Figure SI 3. The most significant positive correlations are marked in dark red. Likewise, the highest negative and significant correlations are marked in dark blue.

Regarding the results for the year 2017, strong positive correlations ($p > 0.8$) are observed mainly for delphinidin-3-glucosides, cyanidin-3-glucoside, pelargonidin-3-O-glucoside, kaempferol 3-glucoside and they were all statistically linked one with the others. Looking at the aglycones a positive correlation was revealed between phlorizin, hydroxycinnamic acid, chlorogenic acid, mandelic acid, and gallic acid. Phlorizin also correlates with quercetin 3-O-hexoside. Regarding the year 2018 several of the correlations found in 2017 are confirmed, as it appears from the specularity of the Pearson correlation plot with respect to the diagonal

separating the harvesting years. There was also a positive relation between tannins concentration and HHDP hexoside and between total flavonoids and total anthocyanins, cyanidin-3-5-diglucoside, cyanidin-3-glucoside, kaempferol-3-O-glucoside, and ferulic acid hexoside.

Negative correlations are less strong as the lowest p-value found was -0.79 and negatively correlates corilagin and mandelic acid levels. This inverse correlation indicates that on the increase of the concentration of one analyte, the correlated one decrease proportionally.

Significant and strongly correlated traits are useful to predict other traits characterizing the same samples and such correlation must be taken into consideration to reduce the number of variables/traits in a discrimination analysis of genotypes.[60]

4. Conclusions

In the present study, 13 pomegranate genotypes were characterized by 27 phenolic compounds. The results showed a vast variance among pomegranate cultivars in terms of phenolic composition. The multivariate statistical approach revealed interesting associations between several compound traits, which could be useful for cultivar breeding and selection. Polyphenols are important components of pomegranate juice, due to their antioxidant properties. As also reported in the literature[61-63] the freeze-drying process does influence bio-phenols levels in fruit juices, which are substantially lowered. On the other hand, the reconstituted pomegranate juices are still rich enough in bio-phenols to compete with some fresh fruit juices. The juices obtained by reconstitution of the lyophilized material still differ one from the other and are distinguishable based on the polyphenols content. Finally, even if it should be better verified following the trend during several years, it appears that the fruit juice relative composition in bio-phenols remained stable in the two following harvesting years.

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Figure Legends:

Figure 1: HPLC/HRMS chromatograms of pure standard solutions of each phenolic analyte at 500 ppb each.

Figure 2: Typical extracted ion chromatographic traces for the pomegranate reconstituted juices, obtained for various m/z values. Not Detectable compounds (ND) traces have also been reported.

Table 1. Descriptive data of the phenolic compounds detected in the juices of pomegranate genotypes

	cultivar	DPPH	T Ph	Fla	Tan	Ant	Que	Cya	Hyd	Cyan-3	Chlo	Gen	Man	Gall
		%	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
2017	Bzou	41.67	3791.67	245.83	104.38	84.05	0.23	0.05	0.05	0.75	0.02	0.04	0.03	0.05
	Djeibi	49.96	2093.75	261.46	52.00	140.72	0.23	0.03	0.07	1.47	0.02	0.04	0.03	0.23
	Dwarf semi Evergreen	60.00	9000.00	350.00	82.97	100.00	0.03	0.18	0.07	1.30	0.02	0.03	0.03	0.82
	Gordo de Jativa	90.82	3644.89	198.96	382.81	80.15	0.02	0.03	0.04	0.49	0.02	0.04	0.02	1.86
	Grenade rouge	44.30	2698.05	327.43	134.95	132.73	0.06	0.03	0.06	1.06	0.02	0.03	0.03	0.18
	Khikho	50.57	1477.27	219.79	150.19	31.02	0.03	0.04	0.05	0.18	0.02	0.04	0.03	0.32
	Mollar Osin Hueso	70.00	8400.00	250.00	166.07	130.00	0.02	0.03	0.05	0.19	0.02	0.03	0.03	0.12
	Negro Monstrioso	49.00	1500.00	500.00	692.66	400.00	0.49	0.05	0.07	8.39	0.02	0.04	0.03	0.35
	Ounk Hmam	34.99	755.68	430.73	94.60	38.87	0.09	0.05	0.04	0.55	0.02	0.04	0.17	0.79
	Ruby	91.08	2059.66	310.94	120.83	91.34	0.05	0.03	0.05	0.55	0.02	0.03	0.03	0.50
	Sefri2	80.00	2000.00	300.00	58.74	100.00	0.03	0.03	0.05	0.37	0.01	0.03	0.02	0.75
	Zherie d'Automne	66.65	2882.10	292.71	55.14	111.88	0.04	0.03	0.05	0.89	0.02	0.03	0.03	2.40
	Zherie précoce	57.78	4074.81	420.31	95.13	66.51	0.03	0.06	0.05	0.26	0.03	0.04	0.03	0.43
	Maximum	91.08	9000.00	500.00	692.66	400.00	0.49	0.18	0.07	8.39	0.03	0.04	0.17	2.40
	Minimum	34.99	755.68	198.96	52.00	31.02	0.02	0.03	0.04	0.18	0.01	0.03	0.02	0.05
	Mean	60.52	3413.68	316.01	168.50	115.94	0.10	0.05	0.05	1.27	0.02	0.04	0.04	0.68
	Deviation	18.21	2542.13	89.08	179.15	91.65	0.14	0.04	0.01	2.18	0.01	0.01	0.04	0.70
2018	Bzou	61.36	6780.00	318.86	85.84	90.37	0.15	0.05	0.04	1.97	0.02	0.03	0.03	0.28
	Djeibi	70.55	4880.00	312.14	83.47	125.85	1.25	0.18	0.06	14.87	0.01	0.03	0.04	0.44
	Dwarf semi Evergreen	72.82	7570.00	346.97	133.44	141.16	0.44	0.18	0.04	19.31	0.02	0.03	0.02	0.32
	Gordo de Jativa	65.61	9100.00	375.59	230.82	117.34	0.50	0.06	0.05	5.56	0.01	0.04	0.03	0.30
	Grenade rouge	75.07	8440.00	332.48	65.57	176.70	0.21	0.20	0.04	15.03	0.02	0.05	0.03	0.48
	Khikho	73.83	3990.00	230.41	150.51	45.00	0.13	0.04	0.05	0.53	0.01	0.04	0.03	0.35
	Mollar Osin Hueso	76.03	8820.00	275.76	234.01	112.80	0.58	0.03	0.06	4.27	0.01	0.05	0.03	0.48
	Negro Monstrioso	50.91	1630.00	573.17	254.81	433.39	0.20	0.23	0.04	37.42	0.02	0.04	0.03	0.16
	Ounk Hmam	80.02	9540.00	289.03	85.96	76.26	0.07	0.04	0.04	0.48	0.02	0.03	0.02	0.24
	Ruby	75.81	5540.00	361.45	44.59	141.22	0.02	0.03	0.05	6.01	0.02	0.03	0.03	0.21
	Sefri2	72.60	7680.00	314.03	112.31	111.08	0.15	0.18	0.04	18.81	0.02	0.04	0.02	0.35
	Zherie d'Automne	66.73	1960.00	308.34	35.77	84.39	0.08	0.04	0.04	0.63	0.01	0.02	0.03	0.26
	Zherie précoce	73.65	2480.00	262.31	137.91	66.24	0.39	0.05	0.04	3.06	0.02	0.04	0.03	0.40
	Maximum	80.02	9540.00	573.17	254.81	433.39	1.25	0.23	0.06	37.42	0.02	0.05	0.04	0.48
	Minimum	50.91	1630.00	230.41	35.77	45.00	0.02	0.03	0.04	0.48	0.01	0.02	0.02	0.16
	Mean	70.38	6031.54	330.81	127.31	132.45	0.32	0.10	0.05	9.84	0.02	0.04	0.03	0.33
	Deviation	7.67	2817.29	83.05	72.83	97.07	0.33	0.08	0.01	10.86	0.00	0.01	0.00	0.10

DPPH= total antioxidant activity % (measured in the fresh juice); for the other results the values are expressed in ppm, abbreviations:, T ph= Total phenols, Fla=Total flavonoids; Tan= Total tannins; Ant= Total antocyans; Que= Quercetin; Cya= Cyanidin; Hyd= Hydroxycinnamic acid; Cyan-3=cyanidin 3-glucoside; Chlo=Chlorogenic acid; Gen=Gentisic acid; Man=Mandelic acid; Gall=Gallic acid; HHDP= HHDP-glucoside; Ell=Ellagic acid; Qu3=Quercetina 3 hexoside; Edh= Ellagic acid deoxyhexoside; Phl = Phlorizin; Kan = Kanokoside; Pel = Pelargonidin-3-O-glucoside; Kae= Kaempferol-3-O-glucoside; Del=Delphinidin 3-O-glucoside; Fer=Ferulic acid hexoside; Dig= Digalloyl-hexoside; Van= Vanillic acid; Lag=Lagerstannin; Cor=Corilagin

… Table 1 continued

cultivar	HHDP ppm	Ell ppm	Qu3 ppm	Edh ppm	Phl ppm	Kan ppm	Pel ppm	Kae ppm	Del ppm	Fer ppm	Dig ppm	Van ppm	Lag ppm	Cor ppm
Bzou	71.46	2.63	0.00	7.09	0.30	190.97	0.19	0.61	0.02	0.36	0.57	2.35	0.80	0.32
Djeibi	46.41	1.64	0.01	5.89	0.37	193.28	0.40	1.55	0.29	1.35	0.65	4.33	0.24	0.08
Dwarf semi Evergreen	64.38	2.33	0.00	5.04	0.23	180.30	0.18	1.18	0.02	1.55	1.46	2.15	0.94	0.26
Gordo de Jativa	241.66	8.77	0.00	26.19	0.40	131.05	0.22	0.24	0.03	0.64	0.97	1.29	0.05	0.01
Grenade rouge	102.25	1.45	0.00	4.17	0.33	218.20	0.28	0.93	0.02	0.50	1.07	2.05	2.15	0.43
Khikho	64.21	0.66	0.00	1.52	0.16	75.28	0.03	0.04	0.13	0.73	0.32	1.55	0.54	0.09
Mollar Osin Hueso	58.25	0.41	0.00	2.34	0.04	57.10	0.03	0.06	0.03	0.30	0.31	1.42	0.02	0.08
Negro Monstrioso	473.82	1.83	0.31	5.30	0.33	0.00	0.91	0.00	0.02	0.24	0.40	0.22	1.36	0.25
Ounk Hmam	100.06	2.65	0.00	12.43	0.17	122.68	0.18	0.45	0.15	0.56	0.51	3.45	0.18	0.14
Ruby	98.49	2.87	0.00	8.71	0.21	147.31	0.14	0.44	0.02	1.60	3.67	1.96	1.97	1.32
Sefri2	51.61	0.44	0.01	1.62	0.17	45.69	0.15	0.19	0.02	0.20	0.21	0.66	0.62	0.29
Zherie d'Automne	38.09	1.23	0.00	4.27	0.26	97.69	0.30	1.00	0.29	0.45	0.90	4.92	1.02	1.05
Zherie précoce	82.95	2.20	0.00	7.38	0.20	179.85	0.14	0.20	0.14	2.05	0.79	2.62	0.29	0.13
Maximum	473.82	8.77	0.31	26.19	0.40	218.20	0.91	1.55	0.29	2.05	3.67	4.92	2.15	1.32
Minimum	38.09	0.41	0.00	1.52	0.04	0.00	0.03	0.00	0.02	0.20	0.21	0.22	0.02	0.01
Mean	114.89	2.24	0.03	7.08	0.24	126.11	0.24	0.53	0.09	0.81	0.91	2.23	0.78	0.34
Deviation	119.51	2.13	0.08	6.49	0.10	67.10	0.22	0.49	0.10	0.61	0.90	1.35	0.70	0.40
Bzou	58.62	1.00	0.25	2.29	0.31	105.32	0.46	1.41	0.84	0.36	0.73	1.11	0.78	0.32
Djeibi	88.50	6.82	0.77	18.14	0.46	247.29	4.06	9.41	4.21	0.76	0.68	4.45	1.20	0.17
Dwarf semi Evergreen	103.43	3.37	0.38	6.07	0.29	185.44	1.68	13.75	4.09	0.40	4.10	3.28	1.44	2.08
Gordo de Jativa	197.23	11.54	0.28	19.89	0.37	107.20	1.12	4.16	0.45	0.23	1.98	1.30	0.28	0.05
Grenade rouge	63.57	1.86	0.77	4.68	0.40	227.25	2.88	14.99	3.49	0.49	0.60	2.79	0.73	0.05
Khikho	101.96	0.79	0.04	2.77	0.18	99.58	0.16	0.49	0.26	0.76	0.81	2.87	1.87	0.23
Mollar Osin Hueso	268.26	2.02	0.25	10.07	0.25	137.58	0.86	3.19	0.94	0.66	0.45	3.30	0.04	0.00
Negro Monstrioso	334.60	3.73	1.35	9.27	0.48	351.03	4.30	34.56	16.93	0.31	1.25	4.39	2.49	0.23
Ounk Hmam	66.87	1.04	0.05	1.37	0.23	129.42	0.20	0.74	0.25	0.34	1.05	2.87	1.65	1.60
Ruby	32.99	0.81	0.03	1.02	0.15	44.13	0.47	4.49	1.53	0.21	0.72	0.55	0.42	0.47
Sefri2	103.90	3.74	0.62	6.77	0.37	286.01	3.78	13.29	8.33	0.48	2.93	5.30	5.52	1.77
Zherie d'Automne	30.09	0.38	0.06	0.88	0.14	46.49	0.21	0.80	0.19	0.25	0.19	0.91	0.14	0.04
Zherie précoce	133.39	6.17	0.26	9.09	0.20	170.55	0.38	2.01	0.36	1.67	1.30	3.44	0.93	0.23
Maximum	334.60	11.54	1.35	19.89	0.48	351.03	4.30	34.56	16.93	1.67	4.10	5.30	5.52	2.08
Minimum	30.09	0.38	0.03	0.88	0.14	44.13	0.16	0.49	0.19	0.21	0.19	0.55	0.04	0.00
Mean	121.80	3.33	0.39	7.10	0.30	164.41	1.58	7.95	3.22	0.53	1.29	2.81	1.34	0.56
Deviation	91.93	3.21	0.39	6.19	0.11	92.29	1.59	9.61	4.76	0.39	1.11	1.48	1.45	0.74

DPPH= total antioxidant activity % (measured in the fresh juice); for the other results the values are expressed in ppm, abbreviations:, T ph= Total phenols, Fla=Total flavonoids; Tan= Total tannins; Ant= Total antocyans; Que= Quercetin; Cya= Cyanidin; Hyd= Hydroxycinnamic acid; Cyan-3=cyanidin 3-glucoside; Chlo=Chlorogenic acid; Gen=Gentisic acid; Man=Mandelic acid; Gall=Gallic acid; HHDP= HHDP-glucoside; Ell=Ellagic acid; Qu3=Quercetina 3 hexoside; Edh= Ellagic acid deoxyhexoside; Phl = Phlorizin; Kan = Kanokoside; Pel = Pelargonidin-3-O-glucoside; Kae= Kaempferol-3-O-glucoside; Del=Delphinidin 3-O-glucoside; Fer=Ferulic acid hexoside; Dig= Digalloyl-hexoside; Van= Vanillic acid; Lag=Lagerstannin; Cor=Corilagin

Table 2. Mean Square, ANOVA and multivariate analysis of all studied variables over pomegranate juice

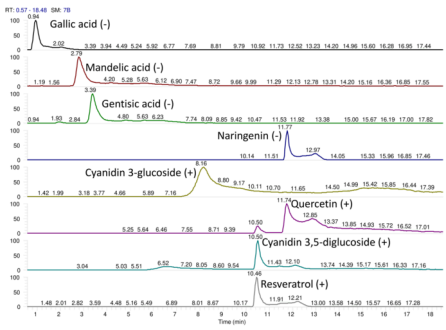
	Cultivar		**Year**		**Cultivar per Year**	
	Mean Square	ANOVA p-value	Mean Square	ANOVA p-value	Mean Square	ANOVA p-value
Quercetin	0.054	0.002	0.406	0.000	0.061	0.002
Cyanidine3-5-diglucoside	0.012	< 0.001	0.036	0.001	0.009	0.003
Hydroxycinnammic acid	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001
Cyanidine-3-glucoside	167.976	< 0.001	1,051.233	< 0.001	116.741	< 0.001
Chlorogenic acid	0.032	< 0.001	< 0.001	< 0.001	0.049	< 0.001
Gentitisic acid	0.098	< 0.001	0.002	0.752	0.075	< 0.001
Mandelic acid	0.001	< 0.001	0.002	< 0.001	0.003	< 0.001
Gallic acid	0.487	< 0.001	1.969	< 0.001	0.907	< 0.001
HHDP-hexoside	43,440.983	< 0.001	881.407	0.569	10,501.180	< 0.001
Ellagic acid pentoside-	32.527	< 0.001	27.028	< 0.001	10.419	< 0.001
Quercetin 3-O-hexoside	0.253	< 0.001	2.167	< 0.001	0.185	< 0.001
Ellagic acid deoxyhexoside	167.703	< 0.001	4.070	0.001	74.984	< 0.001
Phlorizin	0.045	< 0.001	0.029	0.080	0.012	0.227
Kanokoside A	11,366.945	< 0.001	7,013.657	0.005	17,694.687	< 0.001
Pelargonidin-3-O-glucoside	2.503	< 0.001	21.209	< 0.001	2.039	< 0.001
Kaempferol-3-O-glucoside	81.805	< 0.001	845.336	< 0.001	118.665	< 0.001
Delphinidin-3-O-glucoside	25.396	< 0.001	129.319	< 0.001	29.707	< 0.001
Ferulic acid hexoside	0.848	< 0.001	1.730	< 0.001	0.393	< 0.001
Digalloyl hexoside	2.466	< 0.001	0.395	0.019	2.135	< 0.001
Vanillic acid hexoside	4.116	< 0.001	0.264	0.196	5.780	< 0.001
Lagerstannin	4.179	< 0.001	0.318	< 0.001	1.693	< 0.001
Corilangin	0.707	< 0.001	0.088	< 0.001	0.784	< 0.001
Effect		Wilks' Lambda Value	F	Hypothesis df	Error df	Significance
Intercept		< 0.001	9,902.819[a]	22.000	27.000	< 0.001
Cultivar		< 0.001	50.102	374.000	421.292	< 0.001
Year		< 0.001	4,233.674[b]	22.000	27.000	< 0.001
Cultivar*year		< 0.001	75.472	242.000	294.929	< 0.001

[a.] Design: Intercept + cultivar + year + cultivar per year

[b.] Exact statistic

Table 3. phenolic compounds detected in pomegranate juice on the first three PCA components

Component Matrix						
	Principal components (PC) (2017)			Principal components (PC) (2018)		
	1	2	3	1	2	3
DPPH	-0.256	-0.600	0.457	-0.703	-0.020	0.556
Total phenols	-0.247	-0.085	-0.142	-0.519	0.040	0.329
Total flavonoids	0.589	0.306	-0.168	0.797	-0.203	-0.529
Total tannins	0.878	-0.335	0.248	0.436	0.382	-0.345
Total anthocyanins	0.935	-0.001	-0.053	0.866	-0.150	-0.386
Quercetin	0.887	0.262	-0.032	0.222	0.753	0.284
Cyanidine-3-5-diglucoside	0.027	0.304	-0.030	0.855	-0.128	0.377
Hydroxycynnammic acid	0.637	0.488	-0.186	-0.122	0.874	-0.004
Cyanidine-3-glucoside	0.982	0.069	-0.007	0.955	-0.190	0.044
Chlorogenic acid	-0.040	0.420	0.029	0.128	-0.710	0.060
Gentisic acid	0.431	0.596	0.199	0.219	0.339	0.262
Mandelic acid	-0.081	0.105	-0.123	0.215	0.789	-0.254
Gallic acid	-0.195	-0.174	0.547	-0.154	0.611	0.648
HHDP-hexoside	0.911	-0.225	0.296	0.613	0.336	-0.343
Ellagic acid pentoside	0.000	-0.211	0.902	0.329	0.541	-0.069
Quercetin-3-O-hexoside	0.982	-0.062	-0.049	0.969	0.048	0.057
Ellagic acid deoxyhexoside	-0.032	-0.200	0.860	0.403	0.763	0.009
Phlorizin	0.387	0.371	0.688	0.844	0.256	0.148
Kaempferol hexoside A	-0.448	0.658	0.326	0.924	-0.063	0.346
Pelargonidin-3-O-glucoside	0.924	0.228	0.156	0.891	0.095	0.341
Kaempferol-3-O-glucoside	-0.238	0.746	0.162	0.949	-0.213	-0.058
Delphinidin-3-O-glucoside	-0.236	0.538	0.000	0.945	-0.243	-0.054
Ferulic acid hexoside	-0.295	0.519	0.267	-0.044	0.529	0.583
Digalloyl hexoside	-0.206	0.082	0.479	0.224	-0.386	0.343
Vanillic acid hexoside	-0.445	0.684	0.113	0.646	0.032	0.653
Lagerstannin	0.266	0.162	0.078	0.422	-0.436	0.498
Corilagin	-0.150	0.064	0.224	-0.040	-0.694	0.491
% of Variance	29.784	14.566	12.427	39.140	20.315	12.996
Cumulative %	29.784	44.350	56.777	39.140	59.455	72.452



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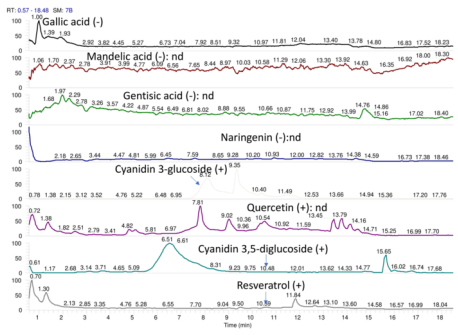

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