Volatile profile of Italian and Montenegrine pomegranate juices for geographical origin classification

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Please replace the sentence with "In particular, aliphatic esters as ethyl caproate, ethyl caprate and ethyl laurate can be formed from fatty acid metabolism during the ripening stage, as other compounds such as alcohols as 1-nonanol, acids and aldehydes as dodecanal "

Original Paper

Volatile profile of Italian and montenegrine pomegranate juices for geographical origin classification

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Abstract

In the present study, we have characterized for the first time the volatile fraction of 20 pomegranate juices from fruits harvested in Northern Italy and southern Montenegro, by means of headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry technique (GC–MS). The volatile profile accounted for 57 compounds belonging mainly to three chemical classes: alcohols, aldehydes and terpenes. Thanks to advance chemometric analysis, the samples were clusterized according to the geographical origin, and the volatiles responsible for differentiation were identified, indicating that the use of volatile profile for discriminating between pomegranate ecotypes grown in different geographical areas is a promising approach.

AQ1

Overall, the chemical information acquired represents a very relevant tool for the retrieval and exploitation of minor varieties and in support of biodiversity of these promising geographical areas for pomegranate cultivation.

Keywords

Punica granatum L.
HS-SPME/GS–MS
Phytochemical characterization
Aroma profile

Abbreviations

GC–MS	Gas chromatography–mass spectrometry
LIR	Linear retention indices
HS-SPME	Headspace solid-phase micro-extraction

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s00217-020-03619-4) contains supplementary material, which is available to authorized users.

Introduction

Pomegranate (*Punica granatum* L.) is a temperate climate species native of the Central Asia; from this area, it spread to the neighboring regions and, in the course of millennia, in other parts of the world, among them the Mediterranean Basin. Pomegranates are cultivated in large parts of the world, including Latin America, southern Europe, Asia and Africa [1]. Due to the good adaptation to abiotic stress conditions, typical of the Mediterranean Basin, pomegranate has spread in this geographical area over times, leading to the appearance of a multitude of new, local individuals [2]. Pomegranate has a large genetic patrimony, represented by over 500 described cultivars, and by a wide amount of wild plants, whose germplasm is so far only partially explored and exploited [1].

Pomegranate production suffered for years of a low retail attractiveness, due to the inconvenient consumption. Over the last decade, however, the pomegranate EU market has rapidly increased, driven by a tremendous hike in the consumer demand of fruits perceived as healthy and tasty, i.e., the so-called “superfruits” [3].

AQ2

At the same time, scientific and agronomic interest has grown as well, with a particular focus on the unmatched profile in bioactive compounds of pomegranate fruit, leading to unique nutritional properties and sensorial profile. In addition to direct consumption and as preserves, pomegranate has indeed a great market potential as an ingredient in food supplements and functional food production, as well as in the so-called nutraceuticals and cosmeceuticals industry [1, 4, 5].

For this reason, besides the mainstream production of market cultivars, a complete characterization of local germplasm is essential in pomegranate species. Following the increasing interest around this cultivation, several retrieval programs have been set up for the conservation, identification and investigation of local varieties [2, 6]. In Italy, pomegranate was cultivated since ancient times, particularly in southern areas [7]. Despite the wide distribution of *P. granatum* in many rural areas of the country and the presence of various cultivars, in particular from Sicily, the Italian germplasm has been scarcely studied. To date, only few available studies describe Italian genotypes, prevalently focused on plants from center and Southern Italy [5, 8, 9, 10] and, to our knowledge, only two regards ancient accessions of Northern Italy [11, 12]. In Montenegro, the pomegranate has long traditional cultivation and wild pomegranate thrives in the calcareous karst areas along the Adriatic Coast, around Skadar Lake and its spread spans the valleys of the Moraĉa and Zeta rivers deep in the continental part [13]. However, local ecotypes have not been investigated so far, and nothing is known about their phytochemical profile.

Among phytochemicals, the composition and concentration of volatile compounds vary depending on different varieties, growing regions, cultural practices, environmental conditions, maturity stages and postharvest storage of fruit and extraction procedure [14, 15, 16]. On this regard, relatively little is yet known so far, and the study of organoleptic attributes of *P. granatum* deserves more attention to disclose the unique and complex mixture of different compounds, responsible for the flavor signature of single fruit varieties [4].

In this framework, the aim of this work was to describe and compare for the first time the volatile profiles of pomegranate juices derived from fruits of two populations from Northern Italy and southern Montenegro, offering therefore relevant chemical information for the retrieval and exploitation of minor varieties and in support of biodiversity.

Materials and methods

Sampling

Twenty pomegranate ecotypes from Northern Italy, in the Parma province (western of Emilia Romagna region, hereafter referred to as EP) and from southern Montenegro (hereafter referred to as MP), in the area near Adriatic Coast and in the Zeta valley, near Skadar Lake, were considered for this study.

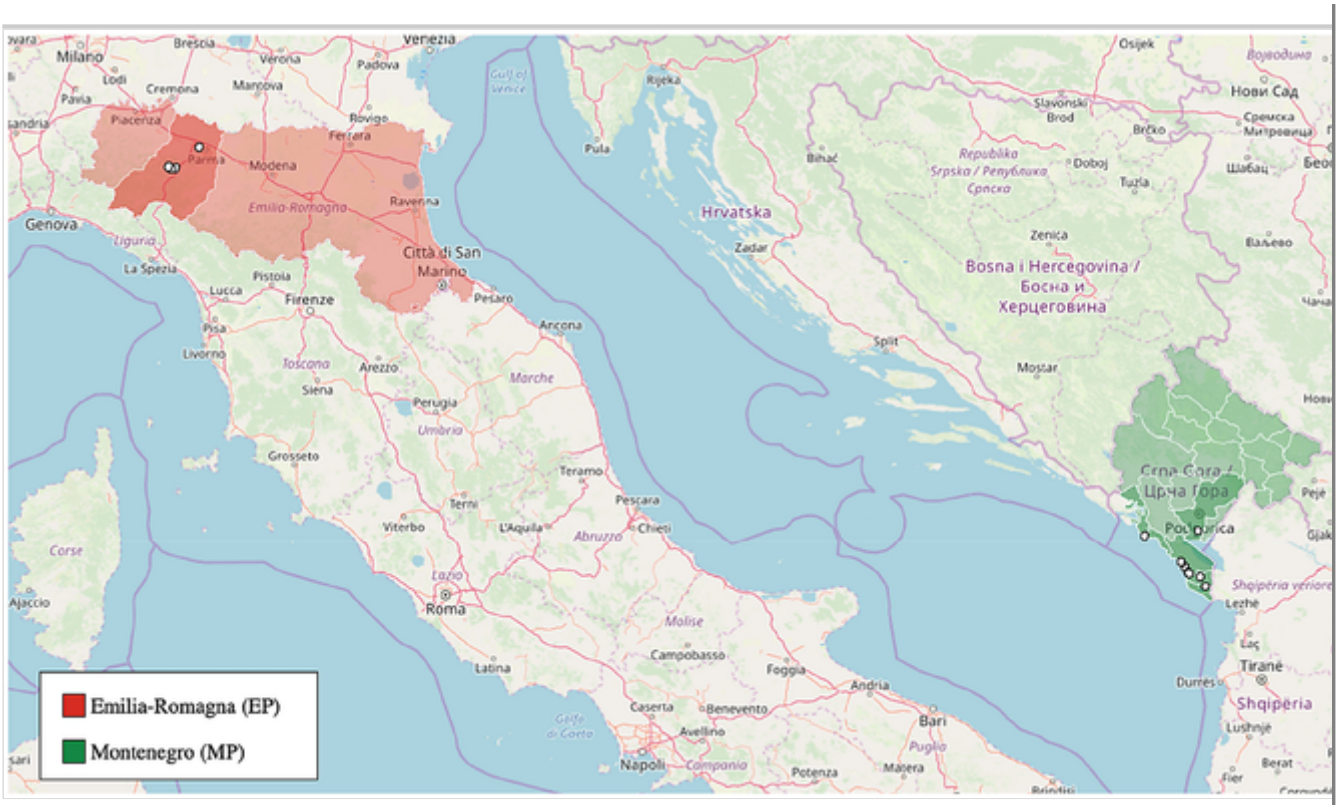
For EP, we chose ten ancient accessions that showed interesting morphological and chemical characteristics in our previous studies [11, 12]. For MP, we chose four accessions in the spots of naturally grown wild pomegranates, together with four accessions belonging to the most prevalent pomegranate cultivars (Slatki Barski, Šerbetaš—sweet variety, Dividiš, Dividiš Meke Kore—sour variety) and two local fresh-squeezed juices of unknown cultivar.

In addition, the cultivar Wonderful, an appreciated variety worldwide marketed, was considered as the cultivar of reference due to its standardized characteristics [17].

All accessions were named with the denomination of the plant location or by cultivar name and tagged with an alpha-numerical code (ID). The localization of ecotypes studied is shown in Fig. 1.

Fig. 1

Geographic distribution of the pomegranate accessions studied in Emilia-Romagna region in Northern Italy (EP), red color, and in southern Montenegro (MP), green color. The white spots indicate ecotypes studied: ME1 (Venturini), ME2 (Lago Costanza1), ME3 (Lago Costanza2), ME4 (Lago Costanza3), ME6 (Reggiolo), ME7 (Marzapello1), ME8 (Marzapello2), for EP population; and MO1 (Slatki Barski), MO2 (Dividiš), MO3 (Dividiš Meke Kore), MO5 (Šerbetaš), MO6 and MO7 (local juices), MO8, MO9, MO10 and MO11 (wild ecotypes), for MP population



Five fruits from each accession were randomly collected at ripening in October 2015, based on the ripening period in Italy and Montenegro [11, 13]. The juice of each pomegranate was obtained by placing the seeds on a metal sieve and manually gently pressing them. Then a subsample of mixed juice of five fruits was put into individual conical tubes of 15 mL, filtered and stored, after passage in liquid nitrogen, and then kept frozen at − 80 °C until analysis.

Headspace solid-phase micro-extraction (HS-SPME) and GC–MS analyses

The volatile profile of the different pomegranate juices was characterized by means of HS-SPME/GC–MS technique following the protocol reported by Ricci et al. (2018) with slight modifications [18]. In particular, 1.5 mL of pomegranate juice was used for the analyses and placed in a 20 mL glass vial and 5 µL of an aqueous toluene standard solution was added (100 mg/L). The sample was equilibrated for 15 min at 40 °C and, then, the headspace was extracted by inserting an SPME fiber coated with 50/30 µm of divinylbenzene–carboxen–polydimethylsiloxane (DVB/carboxen/PDMS; Supelco, Bellefonte, PA, USA) for 30 min at the same temperature. After that, the desorption was performed by inserting the fiber into the GC injector at 250 °C for 2 min. All the analyses were conducted on a Thermo Scientific Trace 1300 gas chromatograph coupled to a Thermo Scientific ISQ single quadrupole mass spectrometer equipped with an electronic impact (EI) source. The separation of the analytes was accomplished on a SUPELCOWAX 10 capillary column (Supelco, Bellefonte, PA, USA; 30 m

× 0.25 mm × 0.25 μm) applying a temperature gradient starting from 50 °C for 3 min, increasing temperature of 5 °C/min until 200 °C and maintaining the final temperature for 12 min, with a total run time of 45 min. Injector and transfer line temperatures were set at 250 °C. Splitless mode was chosen as injection mode, keeping the valve closed for 2 min. Helium was used as carrier gas with a total flow of 1 mL/min. The detection was performed in full scan acquisition mode in a range of 40–500 m/z.

The detected gas-chromatographic signals were identified by the comparison of their registered mass spectra with those present in the instrument library (NIST 14). In addition, linear retention indices (LRIs) were calculated on the basis of the retention times of a C8–C20 alkane solution analyzed applying the same conditions used for sample analysis, and compared with literature. The semi-quantification of the identified compounds was performed on the basis of the use of an internal standard (toluene). All the samples were analyzed twice.

Statistical analysis

All data were obtained as a relative concentration of each detected volatile, calculated on the basis of a reference standard (toluene). Multivariate analysis was performed using MetaboAnalyst 4.0 [19]. Data underwent quality check and normalization (log-transformation followed by Pareto scaling). Then, the data underwent volcano plot analysis, unsupervised followed by supervised multivariate analysis, and hierarchical clustering.

Results

Characterization of the volatile profile of different pomegranate juices

The characterization of the volatile fraction of pomegranate juices from Northern Italy and southern Montenegro was performed by HS-SPME/GC–MS. Overall, 57 volatile compounds were identified and semi-quantified based on internal standard addition, as reported in Table 1. The international standard cultivar Wonderful was considered for comparison, as it is the most widely grown and consumed pomegranate cultivar globally and it is a worldwide appreciated for its sweet–sour taste, and it is also one of the more studied cultivar concerning its volatile composition [3, 4, 17].

Table 1

Identification of GC–MS signals and their relative flavor notes

Peak number/chemical class	Identification	Flavor note	LRI calc	Identification method	R
Aldehydes					
p1	Hexanal	Herbal	1107	MS + LRI	[1
p2	Heptanal	Fresh, aldehydic	1212	MS + LRI	[2
p3	2-Hexenal	Sweet, almond	1247	MS + LRI	[1
p4	Octanal	Aldehydic	1314	MS + LRI	[3
p5	2-Heptenal	Green, fatty	1351	MS + LRI	[3
p6	Nonanal	Waxy	1415	MS + LRI	[3
p7	2-Octenal	Green	1452	MS + LRI	[3
p8	Furfural	Sweet, woody, almond	1493	MS + LRI	[3
p9	Decanal	Sweet, aldehydic	1515	MS + LRI	[3
p10	Benzaldehyde	Fruity	1543	MS + LRI	[3
p11	2-Nonenal	Green	1557	MS + LRI	[3
p12	5-Methylfurfural	Bready	1583	MS + LRI	[3
p13	2-Dodecenal	Green, citrus	1686	MS	
p14	2,4-Nonadienal	Fatty	1727	MS + LRI	[3
p15	Dodecanal	Soap, waxy	1736	MS + LRI	[3
p16	2,5-Furandicarboxyaldehyde		1947	MS + LRI	[3
Esters					
p17	Isoamyl acetate	Sweet, fruity, banana	1141	MS + LRI	[1
p18	Ethyl caproate	Sweet, fruity, pineapple	1255	MS + LRI	[3
p19	Hexyl acetate	Fruity, green, apple	1292	MS + LRI	[3
p20	Ethyl caprylate	Fruity, winey	1460	MS + LRI	[3

Peak number/chemical class	Identification	Flavor note	LRI calc	Identification method	R
p21	Ethyl caprate	Sweet, waxy	1622	MS + LRI	[3
p22	Methyl salicylate	Wintergreen mint	1776	MS + LRI	[1
p23	Ethyl salicylate	Wintergreen mint, sweet	1813	MS + LRI	[3
p24	Ethyl laurate	Sweet, waxy	1847	MS + LRI	[3
Terpenes and derivatives					
p25	β-Myrcene	Peppery, spicy	1182	MS + LRI	[1
p26	Limonene	Citrus	1218	MS + LRI	[1
p27	Terpene not specified		1216	MS	
p28	Eucalyptol	Eucalyptus, herbal	1230	MS + LRI	[2
p29	γ-Terpinene	Terpenic	1265	MS + LRI	[3
p30	o-Cymene	Citrus	1292	MS	
p31	Dihydromyrcenol	Fresh, citrus	1475	MS + LRI	[3
p32	β-Linalool	Floral	1566	MS + LRI	[1
p33	β-Caryophyllene	Sweet, woody	1608	MS + LRI	[1
p34	Terpinen-4-ol	Peppery, woody	1624	MS + LRI	[3
p35	Menthol	Peppermint	1666	MS + LRI	[3
p36	α-Terpineol	Pine, terpenic	1686	MS + LRI	[1
p37	p-Cymen-8-ol	Fruity, cherry	1873	MS + LRI	[2
p38	Geranyl acetone	Green, fruity	1882	MS	
Alcohols					
p39	Isoamyl alcohol	Alcoholic, whiskey	1233	MS + LRI	[1
p40	1-Hexanol	Herbal	1371	MS + LRI	[1

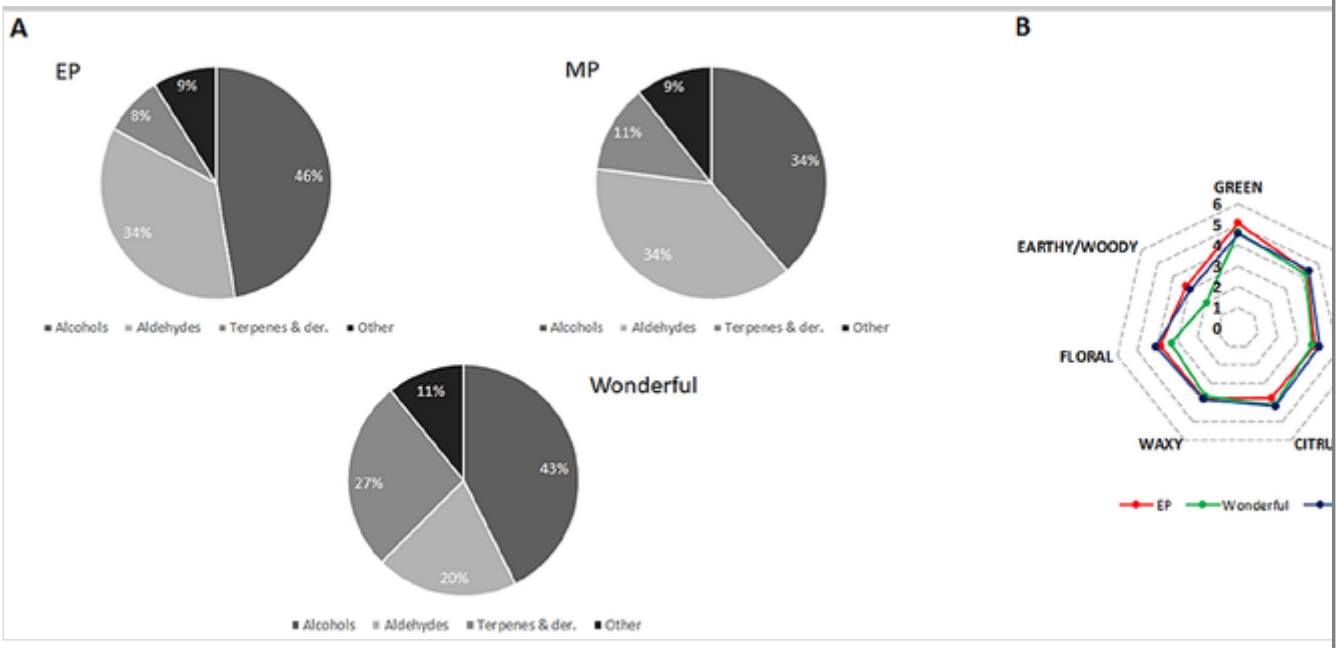
Peak number/chemical class	Identification	Flavor note	LRI calc	Identification method	R
p41	(E)-3-Hexen-1-ol	Green, leafy	1376	MS + LRI	[
p42	(Z)-3-Hexen-1-ol	Green, leafy	1389	MS + LRI	[
p43	1-Octen-3-ol	Earthy	1464	MS + LRI	[
p44	2-Ethyl-1-hexanol	Citrus	1496	MS + LRI	[
p45	2-Nonanol	Waxy, green, creamy	1527	MS + LRI	[
p46	1-Octanol	Waxy, green	1573	MS + LRI	[
p47	1-Nonanol	Fresh, fatty, floral	1675	MS + LRI	[
p48	Phenylethyl alcohol	Floral, rose	1887	MS + LRI	[
Ketones					
p49	2-Heptanone	Fruity, spicy	1211	MS + LRI	[
p50	4-Methyl-2-heptanone		1230	MS + LRI	[
p51	2-Octanone	Soap	1308	MS	
p52	2,3-Octanedione	Asparagus	1347	MS + LRI	[
p53	Sulcatone	Citrus	1364	MS + LRI	[
p54	2-Nonanone	Fruity	1408	MS + LRI	[
Others					
p55	2-Pentylfuran	Fruity	1254	MS + LRI	[
p56	Styrene	Sweet balsam, floral	1279	MS + LRI	[
p57	4-Methyl-benzaldehyde, acetophenone (co-elution)	Fruity, spicy/floral, powdery	1668	MS	

Overall, linear alcohols and aldehydes deriving from lipid degradation, as well as terpene compounds, are the most represented compounds in all the analyzed samples, as already reported for pomegranate juice [9, 10]. However, as reported

in Fig. 2a, relevant differences can be observed in volatile profiles obtained for Emilia (EP) and Montenegrine (MP) samples compared to the international standard cultivar Wonderful. While the latter is rich in terpene compounds (27% in “Wonderful” versus 8% and 11% in EP and MP, respectively), ecotypes from both area studied are more characterized by lipid-derived aldehydes (34% in EP and MP versus 20% in “Wonderful”). In addition, EP and “Wonderful” showed a higher content of linear alcohols (46% and 43%, respectively) compared to MP (34%). Low amounts of different esters, ketones and hydrocarbons were also measured in all the considered ecotypes (Supplementary tables S1 and S2), but no evident differences were noted among the samples. These categories were grouped and considered as “other” in Fig. 2a.

Fig. 2

a Relative abundance (expressed as a percentage) of main classes of volatiles found in EP and MP. **b** Overall flavor perceptions of EP, MP and cultivar Wonderful juice in relation to the volatiles and their typical odor note



AQ3

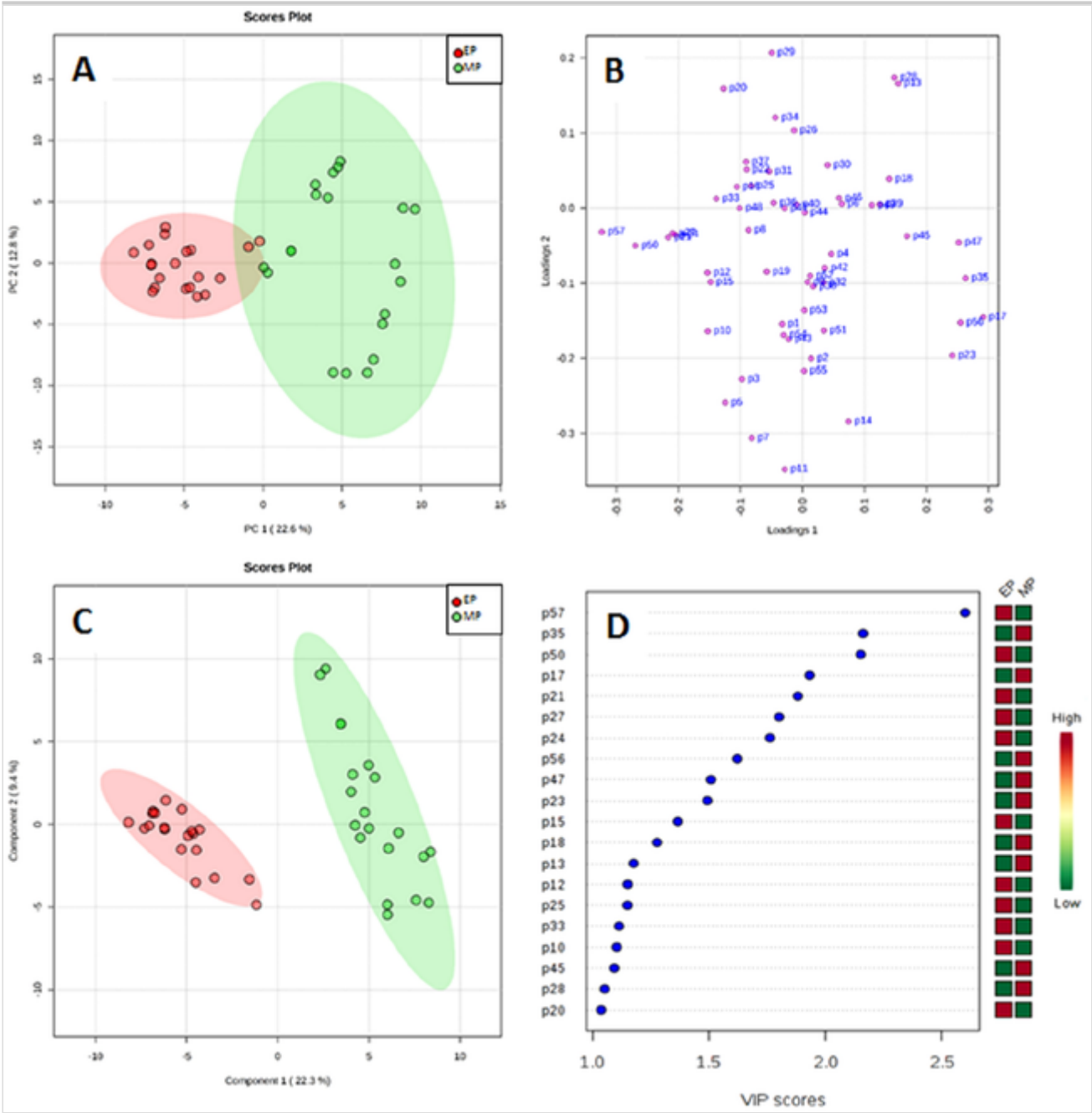
To describe the overall sensorial character of EP and MP pomegranate juice compared to cultivar Wonderful juice, volatile compounds were grouped according to their aromatic note description family. Data are reported as a spider plot in Fig. 2b. It can be noticed that the overall profile of EP and MP is similar, while the international standard cultivar Wonderful showed a lower floral and earthy/woody impact, probably due to the lower content in lipid oxidation-derived volatile compounds.

Unsupervised and supervised dimension reduction and cluster analysis of volatiles

To better visualize the differences between EP and MP juices, multivariate analysis was performed on the dataset originated from volatile profiling. Unsupervised principal component analysis (PCA) was performed on the dataset, followed by supervised partial least squares discriminant analysis (PLS-DA), along with the VIP (variable importance in projection) plot, as reported in Fig. 3.

Fig. 3

Principal component analysis (PCA), scores plot of the pomegranate juices colored according to the groups (EP, red and MP, green) **a** and loadings plot **b**, partial least squares discriminant analysis (PLS-DA), scores plot of the pomegranate juices colored according to the groups (EP, red and MP, green) **c**, and variable importance in projection (VIP) plot **(D)**. Ellipses are based on a 95% confidence region. PLS-DA cross-validation data: Accuracy 1.0 on both PC1 and PC2, R2 0.88929 and 0.95388 on PC1 and PC2, respectively; Q2 0.83013 and 0.90159 on PC1 and PC2, respectively. Loading plots (B and D) show different variables and are numbered according to their peak numbers, as reported in Tables 1, 2. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article



AQ4

A good separation between groups was already achieved with unsupervised analysis, suggesting the capability of volatile compound dataset to successfully differentiate the EP (red) and MP (green) samples (Fig. 3a, b). Although only 35.4% of the total variability was explained by the first two principal components (PC1 and PC2), as expected based on the large variability of the sample set, the cross-validation test returned satisfactory parameters.

According to the volcano plot, calculated considering $\alpha \leq 0.01$ and fold-change EP/MP ≥ 2 (see Table 2), EP and MP samples mainly differ in the amount of volatiles coming from the lipid oxidative degradation pathway, while the terpene profile is similar with the only exception of a higher concentration level of menthol in MP.

Table 2

Significant feature based on volcano plot analysis (fold-change EP/MP > 2; FDR-adjusted alpha: 0.01)

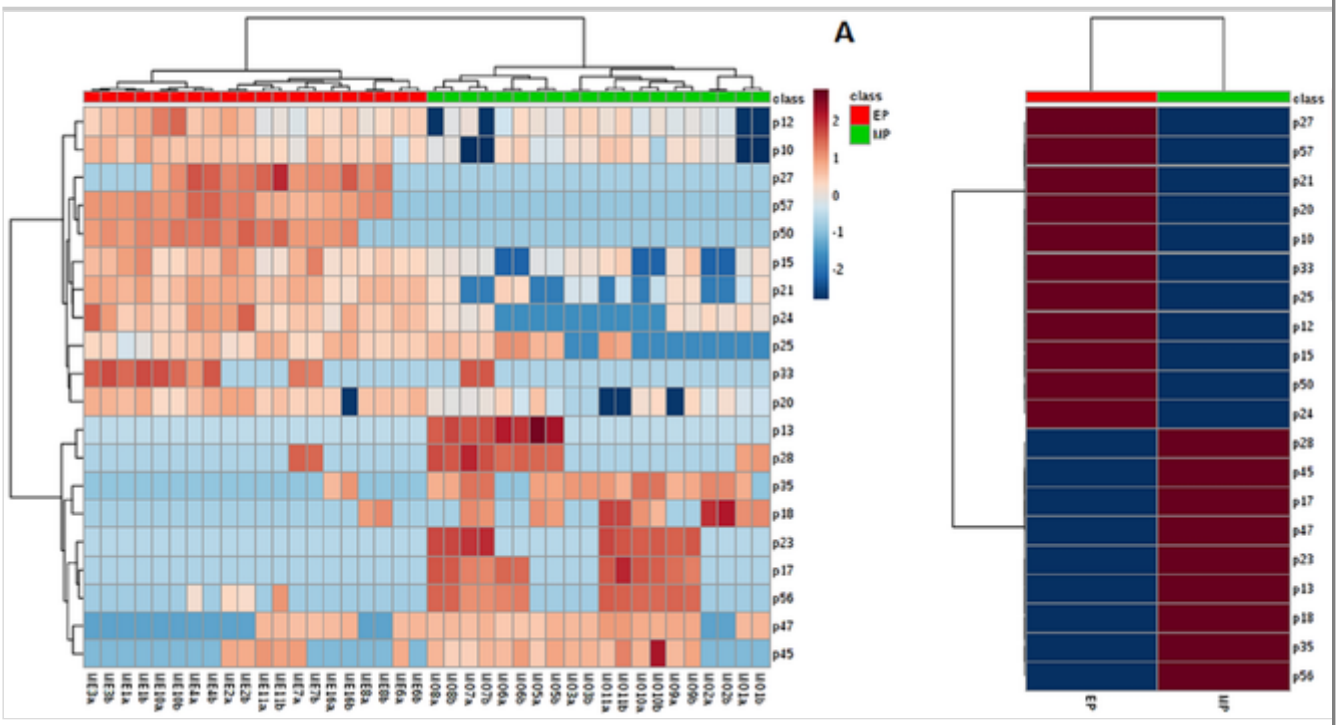
Peak number	Volatile compounds	Volcano plot	
		log2(FC)	*p* value
p57	4-Methyl-benzaldehyde, acetophenone	8.4902	3.75E-13
p50	4-Methyl-2-heptanone	6.896	5.73E-09
p35	Menthol	− 3.8387	2.93E-07
p21	Ethyl caprate	2.8671	1.98E-06
p27	Terpene not specified	6.2172	1.98E-06
p24	Ethyl laurate	3.005	2.30E-06
p17	Isoamyl acetate	− 9.2099	4.65E-05
p15	Dodecanal	1.9771	0.00045472
p23	Ethyl salicylate	− 6.8278	0.00064385
p18	Ethyl caproate	− 3.6328	0.002046
p47	1-Nonanol	− 1.4786	0.002046
p56	Styrene	− 4.9791	0.002046
p12	5-Methylfurfural	1.7756	0.0022898
p10	Benzaldehyde	1.2808	0.0047657
p13	2-Dodecenal	− 6.0896	0.0047657
p45	2-Nonanol	− 1.9514	0.0092279
p20	Ethyl caprylate	1.719	0.0096122

Based on the top features obtained by the PLS-DA VIP plot, a hierarchical clustering and a heat map analysis were then performed, as reported in Fig. 4. In agreement with PLS-DA, the map clearly indicates a good clusterization of EP and MP according to the cultivation area and based on volatile profile. As a confirmation, the most significant compounds are consistent with those already identified by volcano plot analysis.

Fig. 4

a Hierarchical clustering analysis and heat map visualization obtained using the 20 top features listed by VIP PLS-DA model (distance: Euclidean; clustering algorithm: Ward). The color scale represents the scaled abundance of each

variable, with red indicating high abundance and blue indicating low abundance. The compounds (Class) represented in the heat map are numbered according to their peak numbers, as detailed in Table 1. **b** Hierarchical clustering analysis and heat map visualization obtained considering the total population EP and MP, using the 20 top features listed by VIP PLS-DA model (distance: Euclidean; clustering algorithm: Ward). The compounds (Class) represented in the heat map are numbered according to their peak numbers, as detailed in Table 1



Discussion

The investigation and characterization of local germplasm may support the identification of genotypes with the highest nutritional and sensorial value compared to standard cultivars. These genotypes are usually well adapted to local climatic conditions and therefore could be successfully used in breeding programs. For this reason, our work focused on ecotypes from two underexploited, but promising geographical areas for pomegranate cultivation, Emilia-Romagna in Italy and in southern Montenegro.

Emilia Romagna region, Northern Italy, is located outside the traditional area of pomegranate cultivation, and it is characterized prevalently from a continental clime with harsh winter temperatures, in particular in the western region. However, it includes areas having microclimate similar to that areas of Central Italy, thus showing pedoclimatic conditions favorable to pomegranate cultivation. At the moment, there are not pomegranate cultivations in this area, but ancient pomegranate trees, surviving for hundreds of years and adapted to

local conditions, are still present in this territory [11] and are the evidence of a past cultivation of this species in this area.

Montenegro is characterized by continental climate in the mountainous outback (northern part) and by typical Mediterranean climate (characterized by long, warm summers and mild winters, with large amounts of precipitation) on the coastal region and moderate Mediterranean in their hinterland. The pomegranate cultivation has a centuries old tradition, even due to the favorable environmental conditions, in particular in the coast and around Skadar Lake. Moreover, there is a large population of wild pomegranate shrubs, suggesting that this area could be a wider gene pool for this species [13].

Results presented herein described the differences in the volatile profile, and therefore in flavor, among pomegranate ancient and/or wild ecotypes collected in Emilia-Romagna and in southern Montenegro, also in comparison with the standard international cultivar Wonderful. Taken altogether, our data are in agreement with the literature on the major impact due to alcohols on the overall pomegranate aroma [3, 16, 20, 21].

The C5, C6 and C8 alcohols and carbonyl compounds that dominate both EP and MP aroma profile such as (*E*)- and (*Z*)-3-hexen-1-ol, 1-hexanol, hexanal, and hexenal are responsible for the green note, and are known to derive from linoleic and linolenic acids. These compounds have been already reported in pomegranate [22, 23] and usually decline over storage, inducing a decrease of the green note in stored pomegranate juice. Alcohols and aldehydes were the first and the second most abundant chemical categories in EP and MP pomegranate juices, as reported for other cultivars belonging to Turkey [16], while in the commercial samples pertaining to cultivar Wonderful, alcohols were the first most abundant chemical classes, followed by terpenes. The concentration of terpenes and their derivatives were slightly higher with respect to that of aldehydes.

Terpenes such as α-terpineol, linalool, limonene, γ-terpinene, menthol, β-myrcene and eucalyptol have been already reported in other studies as contributors of the overall pomegranate aroma [20]. Consistently with previous studies, our data confirmed that, although fundamental for consumer's acceptance, terpene compounds only account for a lower percentage of the pomegranate juice volatile fraction [17, 22, 24].

Different esters, ketones, hydrocarbons and other compounds were measured in all the considered samples in minor amounts with respect to aldehydes, alcohols and terpenes. Interestingly, among esters, ethyl salicylate was found only in

juices derived from Montenegro, while methyl salicylate was detected in all the samples analyzed (Supplementary tables S1 and S2). These two compounds could be formed in pomegranate starting from cinnamic acids [25]. Styrene was found in higher quantities in MP samples, especially in juices derived from wild accessions (Table S2), while it was almost absent in EP samples. This molecule was identified recently for the first time in the aromatic profile of different Turkish varieties [16], but no indication about its formation was reported. On the other hand, among ketones, 4-methyl-2-heptanone was measured only in samples pertaining to Emilia-Romagna region. Sensorial traits of fruits are affected by both the genetic background and the pedoclimatic conditions; so, the adaptation of pomegranate genotypes to their geographical area may lead to interesting sensorial properties, and unique bioactive profiles, as in the two pomegranate populations studied. A number of studies have so far reported the differences in sensorial profiles of pomegranate ecotypes from different areas [3, 7, 16, 21].

Observing the results obtained for the samples analysed, it appears evident that the volatile profiles were similar in term of composition among the different juices, but the abundance of specific compounds may be diverse depending on the two pomegranate populations in the study. This is well evidenced by cluster analyses performed (PCA and PCA-DA). The volatile compounds responsible for this differentiation were 4-methylbenzaldehyde and acetophenone (p57), 4-methyl-2-heptanone (p50), ethyl caprate (p21), ethyl laurate (p24), dodecanal (p15), 5-methylfurfural (p12), β-myrcene (p25) and a terpenic compound not well identified (p25). All these volatiles showed higher concentrations in EP samples with respect to the values calculated in MP juices. On the contrary, more abundant quantities of menthol (p35), isoamylacetate (p17), styrene (p56), 1-nonanol (p47), ethyl salycilate (p23), ethyl caproate (p18) and 2-dodecanol (p13) contributed to distinguishing and characterizing MP juices.

Based on these data, it is possible to speculate that EP juices were richer in some molecules that can confer sweet and waxy aromatic notes, such as ethyl caprate, ethyl laurate and dodecanal, spicy and peppery sensations (*β*-myrcene) and fruity and floral notes (4-methylbenzaldehyde and acetophenone), while MP juices showed an abundance in compounds related to fresh (menthol, 1-nonanol, ethyl salicylate), fruity (isoamylacetate, ethyl caproate, 2-dodecanol) and balsam (styrene) notes with respect to all the other samples. Esters are often correlated with fruity, waxy and sweet aromatic notes and their concentration in a fruit may depend on different factors, such as the ripening stage [26]. Moreover, aliphatic esters as ethyl caproate, ethyl caprate and ethyl laurate can be formed during ripening from fatty acid metabolism, as other compounds such as alcohols as 1-nonanol, acids and aldehydes as dodecanal [27].

AQ5

AQ6

To highlight and verify the main differences between Italian and Montenegrine pomegranate juices by means of PCA and PLS-DA analyses, a hierarchical clustering and heat map was built (Fig. 4) using as variables the 20 most significant in the first statistical elaborations. Also in this case, a good separation between the two geographical origins considered was achieved. The main distinctions were based on the same variables discussed above, but in addition other parameters helped to differentiate samples, as the concentrations of benzaldehyde (p10, fruity notes), ethyl caprylate (p20, fruity and winey notes), and *β*-caryophyllene (p33, sweet and woody notes) were most abundant in EP juice, while the content of eucalyptol (p28, eucalyptus and herbal notes) and 2-nonanol (p45, waxy, green and creamy notes) were higher in MP samples. *β*-Caryophyllene is considered together with other terpenes as β-pinene and limonene, a key compound in describing the flavor of pomegranate fruit [4], while benzaldehyde already detected in pomegranate volatile profile [16] is characteristic of cherry [28].

Conclusion

This study reported the volatile profile characterization of pomegranate juices prepared from fruits collected in Northern Italy and southern Montenegro.

The aromatic profile was composed of 57 different molecules pertaining mainly to three chemical classes: alcohols, aldehydes and terpenes.

To our best knowledge, this is the first attempt of characterizing *Punica granatum* L. species typical from these geographical area, offering therefore relevant chemical information for the retrieval and exploitation of minor varieties and in support of biodiversity.

Interestingly, chemometric analysis allowed to discriminate among pomegranates from Emilia-Romagna and Montenegro on the basis of volatile profiles. Although the analysis could account only for 33% of the sample variability, the cross-validation test was successful, indicating that the use of volatile profile for discriminating between pomegranate ecotypes, grown in different geographical areas, is a promising approach. Further studies should be performed to correlate genetic background and volatile metabolome, and to validate the model on different harvesting years.

Publisher's Note

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary file1 (DOCX 42 kb)

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Figure 1 Geographic distribution of the pomegranate accessions studied in Emilia Romagna region in Northern Italy (EP), red color, and in Southern Montenegro (MP), green color. The white spots indicate ecotypes studied: ME1 (Venturini), ME2 (Lago Costanza1), ME3 (Lago Costanza2), ME4 (Lago Costanza3), ME6 (Reggiolo), ME7 (Marzapello1), ME8 (Marzapello2), for EP population and MO1 (Slatki Barski), MO2 (Dividiš), MO3 (Dividiš Meke Kore), MO5 (Šerbetaš), MO6 and MO7 (local juices), MO8, MO9, MO10 and MO11 (wild ecotypes), for MP population.

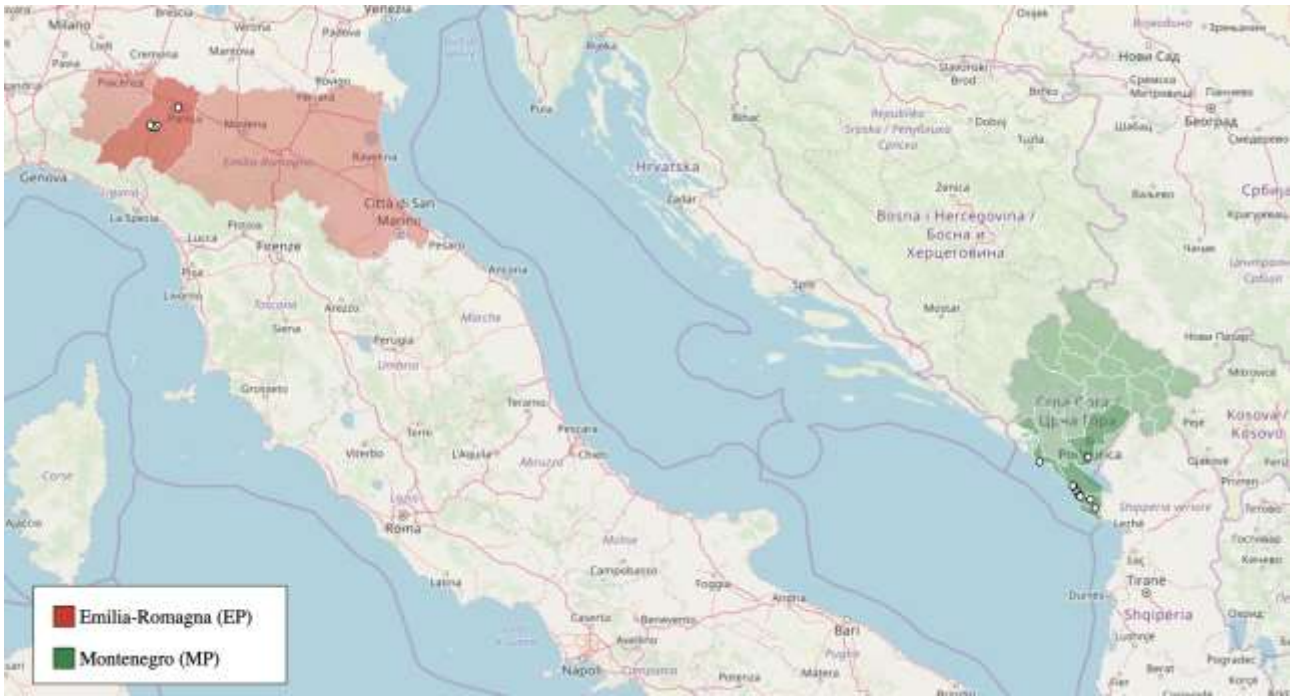

Figure 2 (A) Relative abundance (expressed as a percentage) of main classes of volatiles found in EP and MP. (**B)** Overall flavour perceptions of EP, MP and cultivar Wonderful juice in relation to the volatiles and their typical odour note.

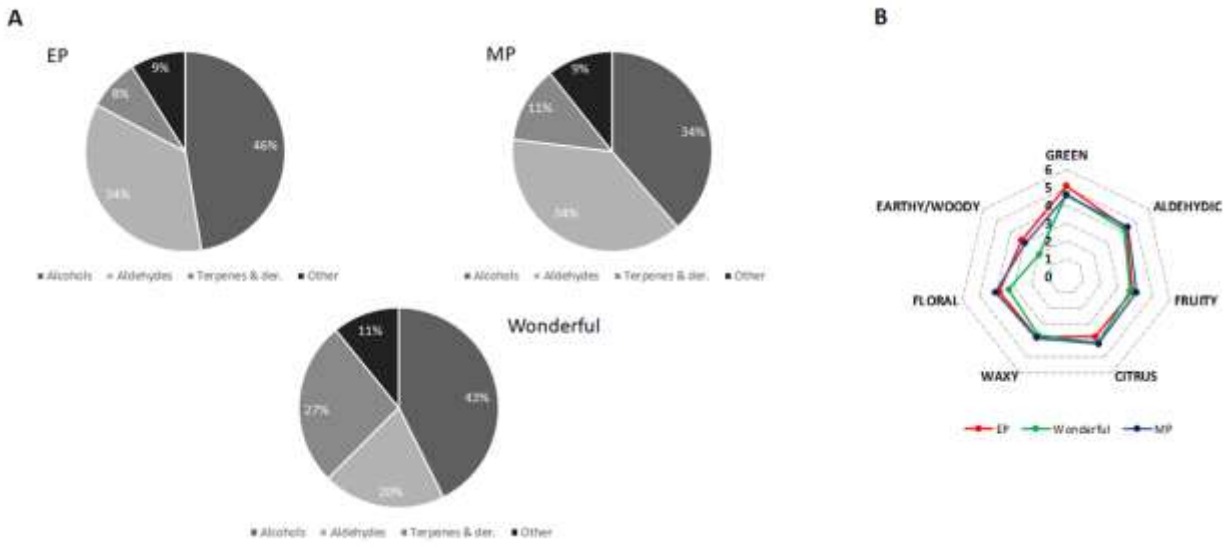

Figure 3 Principal component analysis (PCA), scores plot of the pomegranate juices colored according to the groups (EP, red and MP, green) **(A**) and loadings plot (**B)**, partial least squares discriminant analysis (PLS-DA), scores plot of the pomegranate juices colored according to the groups (EP, red and MP, green) (**C**), and variable importance in projection (VIP) plot **(D**). Ellipses are based on a 95% confidence region. PLS-DA cross validation data: Accuracy 1.0 on both PC1 and PC2, R2 0.88929 and 0.95388 on PC1 and PC2, respectively; Q2 0.83013 and 0.90159 on PC1 and PC2, respectively. Loading plots (B and D) show different variables and are numbered according to their peak numbers, as reported in Tables 1 and 2. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

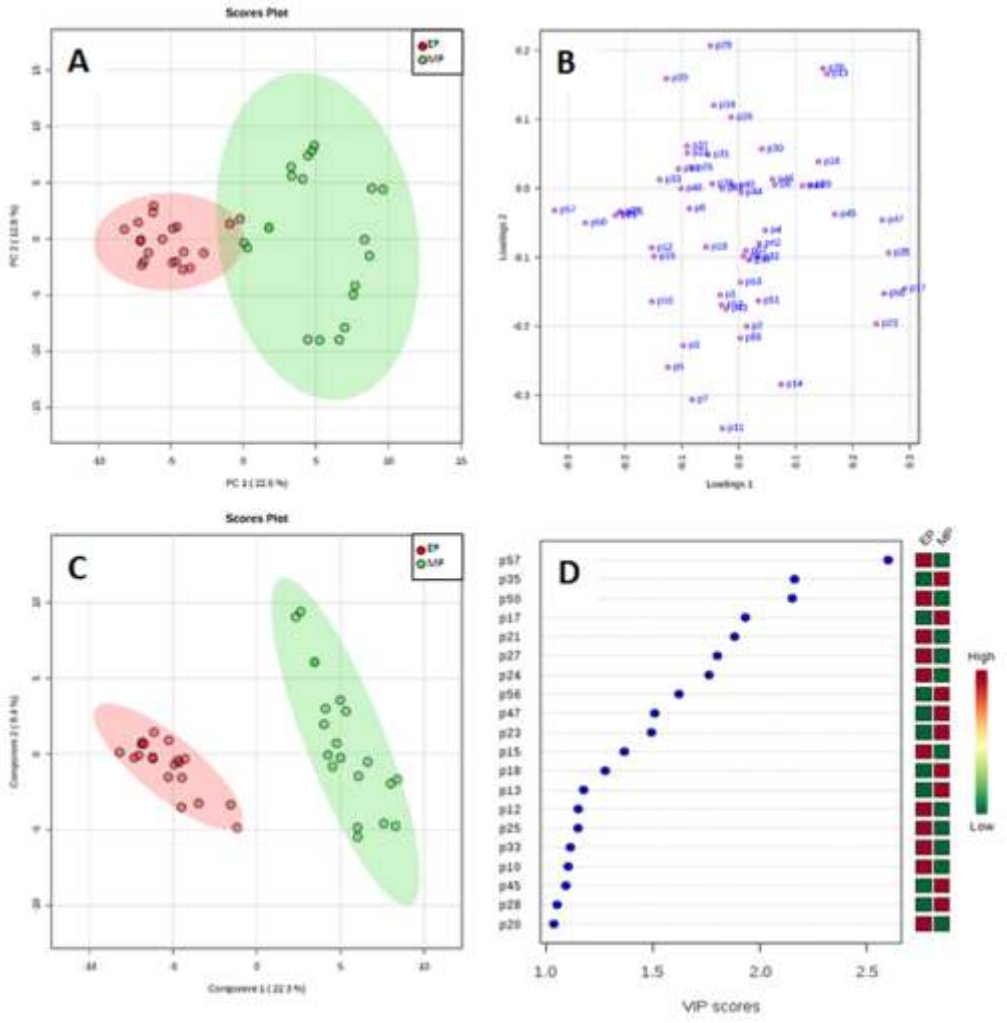

Figure 4 (A) Hierarchical clustering analysis and Heat-Map visualization obtained using the 20 top features listed by VIP PLS-DA model (distance: Euclidean; clustering algorithm: Ward). The color scale represents the scaled abundance of each variable, with red indicating high abundance and blue indicating low abundance. The compounds (Class) represented in the heat-map are numbered according to their peak numbers, as detailed in Table 1. **(B)** Hierarchical clustering analysis and Heat-Map visualization obtained considering the total population EP and MP, using the 20 top features listed by VIP PLS-DA model (distance: Euclidean; clustering algorithm: Ward). The compounds (Class) represented in the heat-map are numbered according to their peak numbers, as detailed in Table 1.