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Ultra-HPLC–MS^n (Poly)phenolic Profiling and Chemometric Analysis of Juices from Ancient *Punica granatum* L. Cultivars: A Nontargeted Approach

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ABSTRACT: This study deals with the qualitative characterization of the phenolic profile of pomegranate juices obtained from ancient accessions. Composition data, together with genetic, morphological, and agronomical parameters, may lead to a full characterization of such germplasm, with the aim of its retrieval and biodiversity valorization. Environmental adaptation, indeed, may contribute to an enrichment of the phenolic content in pomegranate, with important effects on its nutritional properties. More than 65 punicalagins, ellagic acid derivatives, flavonoids, anthocyanins, and phenylpropanoids were simultaneously detected from four centuries old *Punica granatum* L. ecotypes from northern Italy and compared with those of *P. granatum* cv. Dente di Cavallo, a widely cultivated Italian cultivar, using a simple ultra-HPLC (uHPLC) separation and MS^n linear ion trap mass spectrometric characterization. Fingerprinting phytochemical discrimination of the accessions was obtained by chemometric analysis despite their limited geographical distribution, confirming the great intraspecific variability in pomegranate secondary metabolism. The combined recourse to uHPLC–MS^n qualitative fingerprinting and multivariate analysis may represent a useful tool for the discrimination and selection of pomegranate germplasm with specific properties related to polyphenolic content.

KEYWORDS: *pomegranate, ellagitannins, polyphenols, mass spectrometry, ultra-high-performance liquid chromatography, germplasm screening*

INTRODUCTION

Polyphenol-rich dietary habits have been positively correlated to various health benefits, including a reduced or delayed incidence of degenerative and metabolic diseases such as cancer, cardiovascular disorders, diabetes, cataracts, and osteoporosis.[1−4] As a consequence, health-conscious consumers and producers interested in this surging agrofood branch have turned their attention to polyphenol-rich fruits and vegetables to increase the dietary intake of these substances.[5] Nowadays, *Punica granatum* L. is one of those species enjoying such growing interest, after having become an out-fashioned fruit with limited commercial appeal, mostly due to the time and patience needed to remove the rind and the tiny seeds. In particular, the juice obtained from pomegranates is experiencing a soaring success in the marketplace, spurred by several features, including a favorable combination of novelty, cheap availability, color, unique taste, and health properties.[6,7] The latter are strictly related to the phytochemical composition of this fruit, which is both complex and unique and encompasses the presence of anthocyanins (monoglycosides and diglycosides of cyanidin, delphinidin, and pelargonidin), ellagic acid and ellagitannins (mainly punicalagins and punicalins), gallic acid and gallotannins, proanthocyanidins, flavanols, and lignans, whose combination is deemed responsible for a wide range of health-promoting biological activities exerted both directly or after an assimilation mediated through colonic biotransformation.[8−11] However, despite the impressive growth of its market, pomegranate is still an underutilized species if compared to other domesticated fruit plants. For example, an accurate evaluation of its germplasm from a phytochemical standpoint is not available, and only a few studies have screened its intraspecific chemodiversity in Spanish, Iranian, Turkish, and Tunisian accessions, most of them providing only a partial description of the phytocomplex of pomegranate juice.[12−19] The Italian germplasm in particular has been scarcely studied, despite the wide distribution of *P. granatum* in many rural areas and the presence of various cultivars, in particular from Sicily. At present, available studies provide limited phytochemical fingerprinting

Table 1. *P. granatum* L. Accessions Used in This Study

ID	ecotype/cultivar name	province (region)	characteristics	pH
ME1	Venturini	Parma (Emilia Romagna)	large fruits and dark-rose juice, thick skin	2.71
ME3	L. Costanza 2	Parma (Emilia Romagna)	large fruits and dark-rose juice, thick skin	2.75
ME5	L. Costanza 4	Parma (Emilia Romagna)	large fruits and dark-rose juice, thick skin	2.76
ME8	Marzapello 2	Parma (Emilia Romagna)	small fruits and dark-rose juice, thin skin	2.58
ME9	Dente di Cavallo	Catania (Sicily)	large fruits and rose-red juice, thick skin	3.82

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Table 2. Mass Spectral Characteristics of (Poly)phenolic Compounds Detected in Negative Ionization Mode and Relative Occurrence in Juice Samples[a]

compd	t_R (min)	$[M - H]^-$ (m/z)	MS^2 ion fragments (m/z)	MS^3 ion fragments (m/z)	juice sample				
					ME1	ME3	ME5	ME8	ME9
vanillic acid	6.05	167	152 (100), 123 (94), 108 (14)	167 → 152: 108	+	+	+	+	+
gallic acid	2.59	169	125		+	+	+	+	+
syringaldehyde	8.38	181	137 (1), 166 (100)	181 → 166: 151 (100)	+	+	+	+	+
ferulic acid	8.58	193	134 (25), 149 (100), 178 (54)	193 → 149: 107 (1), 134 (100)	+	+	+	+	−
naringenin-like	9.01	271	177 (21), 165 (4), 151 (100), 125 (3), 107 (3)	271 → 151: 83 (1), 107 (100)	+	+	+	+	+
pinocembrin	11.15	255	107 (5), 145 (14), 151 (38), 169 (10), 183 (8), 187 (13), 211 (70), 213 (100), 214 (17)	255 → 213: 141 (10), 145 (23), 169 (29), 185 (100)	+	+	+	+	+
protocatechuic acid pentoside	6.37	285	109 (5), 152 (21), 153 (100), 241 (7)	285 → 153: 109	+	+	+	+	+
hydroxybenzoic acid hexoside	5.89	299	239 (70), 209 (26), 179 (67), 137 (100), 113 (5), 93 (4)	299 → 137: 93	+	+	+	+	+
ellagic acid	8.19	301	284 (4), 257 (16), 229 (6), 185 (3)		+	+	+	+	+
protocatechuic acid hexoside	3.29	315	109 (11), 153 (100), 177 (5), 195 (23), 225 (4), 255 (33)	315 → 153: 109	−	+	−	−	+
coumaric acid hexoside	6.9	325	265 (15), 235 (7), 205 (7), 187 (46), 163 (100), 145 (85), 119 (8)	325 → 163: 119	+	+	+	+	+
vanillic acid hexoside	5.82	329	269 (5), 239 (2), 209 (16), 181 (2), 167 (100), 152 (2)	329 → 167: 108 (10), 123 (100), 152 (74)	+	+	+	+	+
vanillic acid hexoside	6.34	329	269 (34), 239 (15), 209 (94), 181 (9), 167 (100), 152 (1)	329 → 167: 108 (3), 123 (100), 152 (35)	+	+	+	+	+
galloyl glucoside	1.66	331	271 (81), 211 (25), 193 (11), 169 (100), 125 (8)	331 → 169: 125	+	+	+	+	+
galloyl glucoside	2.11	331	313 (1), 271 (100), 193 (1), 169 (59), 125 (5)	331 → 169: 125					
				331 → 271: 169 (14), 211 (100)	+	+	+	+	+
galloyl glucoside	2.94	331	271 (100), 241 (5), 193, 169 (25), 125 (2)	331 → 271: 211 (100), 169 (9)	+	+	+	−	+
caffeic acid hexoside	6.49	341	135 (3), 161 (19), 179 (100), 203 (5)	341 → 179: 135	+	+	+	+	+
ferulic acid hexoside	7.1	355	175 (24), 193 (100), 217 (45), 235 (9), 265 (5)	355 → 193: 134 (100), 149 (48), 165 (8)	+	+	+	+	+
pinoresinol	8.55	357	136 (31), 151 (100), 311 (13), 342 (7)	357 → 151: 123 (1), 136 (100)	+	+	+	+	+
vanillic acid derivative	6.84	363	152 (9), 165 (14), 167 (83), 181 (8), 195 (36), 315 (100)	363 → 167: 108 (14), 123 (100), 152 (96)	+	+	+	+	+
ferulic acid derivative	7.45	389	134 (3), 149 (3), 165 (6), 193 (79), 195 (17), 341 (100)	389 → 193: 134 (20), 149 (100), 178 (56)	+	+	+	+	+
syringic acid derivative	7.99	391	179 (48), 182 (7), 193 (51), 197 (100), 211 (32), 317 (60)	391 → 197: 138 (9), 153 (24), 182 (100)	+	+	+	+	+
syringaresinol	8.79	417	151 (12), 166 (31), 181 (100), 371 (12), 402 (38)	417 → 181: 166 (100)	+	+	+	+	+
ellagic acid pentoside	7.99	433	300 (100), 301 (85)	433 → 300: 185 (4), 229 (10), 244 (27), 257 (34)	+	+	+	+	+
ellagic acid rhamnoside	8.03	447	300 (100), 301 (49)	447 → 300: 185 (9), 200 (9), 229 (14), 257 (16)	+	+	+	+	+
eriodictyol hexoside	9.19	449	151 (3), 169 (8), 287 (100), 313 (5), 331 (5)	449 → 287: 107 (1), 135 (1), 125 (3), 151 (100)	+	+	+	+	+
ellagic acid glucoside	7.35	463	300 (37), 301 (100), 302 (9)	463 → 301: 185 (4), 229 (7), 257 (16)	+	+	+	+	+
taxifolin hexoside	8.16	465	151 (6), 303 (100), 313 (28)	465 → 303: 125 (4), 151 (100), 177 (4), 285 (10)	+	+	+	+	−
myricetin hexoside	7.73	479	151 (1), 179 (4), 316 (68), 317 (100), 318 (8)	479 → 317: 151 (30), 179 (100), 192 (25), 271 (11), 272 (12), 285 (11)	+	+	+	+	+
HHDP glucoside	1.30	481	275 (8), 301 (100)	447 → 301: 185 (17), 201 (5), 213 (6), 229 (63), 257 (94), 299 (7)	+	+	−	−	−
digalloyl glucoside	2.57	483	169 (26), 193 (2), 313 (24), 331 (100), 332 (10)	483 → 331: 125 (10), 169 (89), 193 (100), 211 (40), 271 (67), 313 (30)	+	+	+	+	+
digalloyl glucoside	6.06	483	169 (15), 193 (2), 313 (18), 331 (100), 332 (8)	483 → 331: 125 (7), 169 (100), 193 (80), 211 (29), 271 (55), 313 (21)	+	+	+	+	+
syringetin hexoside	7.71	507	315 (45), 327 (100), 345 (43), 489 (2)	507 → 327: 167 (5), 268 (3), 283 (13), 295 (9), 296 (11), 297 (7), 312 (100)	+	+	+	+	+

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Table 2. continued

compd	t_R (min)	$[M - H]^-$ (m/z)	MS^2 ion fragments (m/z)	MS^3 ion fragments (m/z)	juice sample				
					ME1	ME3	ME5	ME8	ME9
cyclolariciresinol hexoside	7.98	521	311 (2), 327 (3), 341 (3), 345 (3), 359 (100)	521 → 359: 344 (100)	+	+	+	+	+
secoisolariciresinol hexoside	8.30	523	329 (6), 343 (4), 347 (8), 361 (100), 362 (9)	523 → 361: 122 (7), 165 (28), 179 (22), 313 (21), 331 (16), 343 (16), 346 (100)	+	+	+	+	+
secoisolariciresinol hexoside	8.43	523	329 (5), 343 (4), 347 (8), 361 (100), 362 (4)	523 → 361: 122 (12), 165 (44), 179 (24), 313 (30), 331 (13), 343 (12), 346 (100)	+	+	+	+	+
syringic acid derivative	7.25	555	197 (3), 393 (100), 481 (14), 495 (3)	555 → 393: 165 (17), 182 (11), 195 (30), 197 (100), 345 (12) 393 → 197: 153 (46), 182 (100)	+	+	+	+	+
kaempferol rutinoside	8.55	593	229 (3), 257 (4), 285 (100)	593 → 285: 151 (12), 163 (20), 197 (22), 199 (17), 213 (26), 223 (15), 229 (31), 239 (19), 240 (15), 241 (44), 256 (13), 257 (100), 267 (57)	+	+	+	+	+
quercetin rutinoside	8.07	609	179 (1), 271 (3), 300 (20), 301 (100), 343 (9), 563 (6)	609 → 301: 151 (70), 179 (100), 257 (15), 273 (10)	+	+	+	+	+
galloyl HHDP glucoside	2.09	633	231 (3), 249 (16), 275 (20), 301 (100), 302 (5), 331 (3), 463 (3), 481 (3)	633 → 301: 185 (20), 229 (37), 257 (100), 284 (15)	+	+	+	+	−
galloyl HHDP glucoside	4.05	633	249 (17), 275 (18), 301 (100), 302 (7), 481 (2)	633 → 301: 185 (30), 229 (37), 257 (100), 284 (10), 300 (12)	+	+	+	+	−
galloyl HHDP glucoside	7.15	633	275 (14), 301 (100), 302 (9), 463 (28)	633 → 301: 185 (17), 229 (35), 273 (12), 257 (100), 284 (8)	+	+	+	+	+
galloyl HHDP glucoside (lagerstannin C)	1.57	649	275 (5), 301 (100), 497 (61)	649 → 301: 185 (2), 229 (8), 257 (19), 284 (4), 301	+	+	+	+	+
galloyl HHDP glucoside (lagerstannin C)	5.45	649	301 (85), 497 (100)	643 → 497: 301 643 → 301: 185 (3), 229 (7), 257 (21)	+	+	+	+	−
α-punicalin	3.11	781	575 (7), 601 (100), 721 (15)	781 → 601: 243 (4), 271 (39), 299 (100), 300 (8), 301 (4)	+	+	+	+	+
β-punicalin	3.35	781	575 (5), 601 (100), 602 (13), 721 (16)	781 → 601: 243 (3), 271 (35), 299 (100), 300 (4), 301 (3)	+	+	+	+	+
bis-HHDP glucoside (pedunculagin I)	5.58	783	275 (16), 301 (100), 481 (49), 765 (4)	783 → 301: 229 (40), 257 (100), 284 (9)	−	−	−	+	−
bis-HHDP glucoside (pedunculagin I)	6.15	783	275 (19), 301 (100), 451 (21), 481 (55), 631 (14), 765 (13)	783 → 301: 229 (37), 257 (88), 284 (23)	−	−	−	−	−
bis-HHDP glucoside (pedunculagin I)	7.07	783	275 (17), 301 (59), 481 (15), 763 (14), 765 (100)	783 → 765: 229 (10), 275 (14), 299 (24), 301 (58), 595 (15), 597 (86), 613 (100), 721 (21), 747 (81)	+	+	+	+	−
bis-HHDP glucoside (pedunculagin I)	7.57	783	301 (13), 755 (12), 765 (100)	783 → 765: 595 (62), 597 (100), 613 (100), 721 (14), 747 (7)	+	−	+	−	−
digalloyl HHDP glucoside (pedunculagin II)	6.69	785	249 (16), 275 (23), 301 (100), 331 (11), 419 (12), 483 (70), 615 (16), 633 (34), 767 (33)	785 → 301: 229 (38), 257 (100), 283 (12)	+	+	+	+	−
digalloyl HHDP glucoside (pedunculagin II)	7.03	785	249 (11), 275 (17), 301 (100), 313 (12), 419 (12), 483 (100), 615 (11), 633 (23), 767 (12)	785 → 483: 223 (7), 295 (13), 313 (100), 331 (55)	+	+	+	+	−
digalloyl HHDP glucoside (pedunculagin II)	7.36	785	249 (22), 275 (23), 301 (100), 313 (9), 419 (32), 483 (24), 615 (47), 617 (16), 633 (9), 767 (16)	785 → 301: 229 (31), 257 (97), 284 (20)	+	+	+	+	+
ellagic acid derivative	6.90	799	299 (7), 301 (79), 479 (100), 781 (54)	799 → 479: 273 (78), 299 (58), 435 (100), 451 (88), 461 (83)	+	+	+	+	−
ellagic acid derivative	5.91	805	463 (17), 481 (9), 625 (30), 643 (100)	805 → 643: 283 (17), 355 (22), 463 (100), 481 (77), 505 (11), 517 (17), 625 (4)	+	+	+	+	+
pedunculagin III (galloylpunicalin)	6.02	933	601 (38), 721 (100), 781 (19), 915 (16)	933 → 721: 449 (6), 601 (100)	+	+	+	+	−
trisgalloyl HHDP glucose isomer	6.56	951	781(2), 783(2), 907(100)	951 → 907: 299 (11), 301 (11), 481 (15), 605 (44), 745 (30), 763 (46), 781 (34), 782 (53), 783 (100), 845 (11), 889 (55)	+	+	+	+	-
galloyl HHDP DHHDP glucoside (granatin B)	7.70	951	301 (4), 613 (8), 933 (100)	951 → 933: 301 (77), 445 (58), 463 (25), 613 (31), 765 (19), 897 (24), 915 (100)	+	+	+	+	+
ellagic acid derivative	8.42	953	617 (1), 909 (1), 935 (100)	953 → 935: 617 (100), 749 (21), 767 (15), 891 (14), 917 (83)	+	+	+	+	−

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Table 2. continued

compd	t_R (min)	$[M-H]^-$ (m/z)	MS^2 ion fragments (m/z)	MS^3 ion fragments (m/z)	juice sample				
					ME1	ME3	ME5	ME8	ME9
punicalagin isomer	5.95	1083	575 (8), 601 (30), 721 (11), 807 (47), 1021 (20), 1065 (100)	1083 → 1065: 721 (100), 959 (37), 977 (47), 1003 (59), 1021 (65), 1047 (57)	+	+	+	+	–
α-punicalagin	6.42	1083	575 (16), 601 (70), 721 (12), 781 (100)	1083 → 781: 575 (6), 601 (100), 721 (11)	+	+	+	+	+
β-punicalagin	6.72	1083	575 (10), 601 (63), 721 (8), 781 (100)	1083 → 781: 575 (9), 601 (100), 721 (11)	+	+	+	+	+
digalloyl gallagyl glucoside	6.83	1085	451 (41), 631 (14), 633 (6), 783 (100), 933 (7)	1085 → 783: 451 (100), 631 (30), 723 (10)	–	–	–	–	–
punicalagin-like	2.76	1101	601 (6), 781 (100), 1057 (13)	1101 → 781: 575 (6), 601 (100), 721 (16)	–	–	–	+	+

[a]Key: +, detected; –, not detected. Abbreviations: HHDP, hexahydroxydiphenoyl; DHHDP, dihexahydroxydiphenoyl. The relative ionic abundance for each ion is reported in parentheses.

data, do not describe ancient accessions, and are focused only on cultivars from southern Italy.[20−24]

With more than 500 known pomegranate varieties and an ecological behavior that emphasizes intraspecific diversity, *P. granatum* offers a large phenotypic variability, represented in a wide range of fruit size, color, pulp content, fruit cracking, and drought resistance. This diversity reverberates also in the secondary metabolism, and recent studies have highlighted different polyphenolic fingerprints in leaves, bark, peels, pericarp, pith, seeds, and, consequently, juice.[9] In some cases, the reported differences within groups of wild or domesticated accessions have relapses on sensorial, organoleptic, and morphological traits that can exceed 700%.[25] For example, in a collection of 35 Spanish varieties, each accession displayed a peculiar organic acid profile; 6-fold differences within specific organic acids and a 2-fold difference in total acidity were reported.[17] In this regard, populations adapted to difficult ecological conditions attract considerable attention, as their polyphenolic content is usually higher and may thus be used as a reservoir for genetic traits related to such properties. At the same time, the growing interest in the development of elite cultivars enriched in health-promoting polyphenols must confront the limitations of consumers' acceptance. In fact, the strong positive correlation between phenolic content and health benefits may be hindered by astringency induced by an excess of the same compounds, a bottleneck that makes the juice less desirable. Therefore, breeding projects for elite pomegranate cultivars and varieties are paying increasing attention to a proper phytochemical balance, which is nowadays included in traditional strategies previously focused only on size, shape, color, pulp content, seed softness, resistance to cracking, and drought.[26] As polyphenolic content represents a key factor in contemporary breeding of elite cultivars, there is a consequent need for comprehensive fingerprinting tools tailored for pomegranate germplasm. Moreover, provided that it can guarantee comprehensive information with limited effort, a comprehensive description of intraspecific polyphenolic chemodiversity in pomegranate is deemed to be a crucial starting point to assist the selection of new varieties with enhanced biological, nutritional, and technological properties. Traditional approaches for evaluating the phytochemical profile of plant matrices are usually based on a list of target analytes, whose accurate quantitation is made difficult by the extreme complexity of some materials and by the lack of analytical standards for many plant secondary metabolites. Nontargeted approaches may offer reliable tools in this regard, allowing the assignment of special features by combining high-throughput analytical techniques and statistical data analysis.[27]

This work, mainly aimed at ultra-HPLC (uHPLC)−MS^n fingerprinting and chemometric discrimination of polyphenolic compounds in four ancient pomegranate ecotypes and an established Italian cultivar, is part of a multidisciplinary project for the retrieval and exploitation of ancient pomegranate cultivars from northern Italy, which can be considered for both direct commercialization and breeding purposes.

The profiling approach was chosen to combine quick separation and high-throughput potential, collecting a high amount of data in the shortest time possible, a combination of capital relevance to comprehensively evaluate a large germplasm. Moreover, the total polyphenolic content and total antioxidant activity of the juices were evaluated and correlated with the chemical composition to obtain a full characterization of the potential nutraceutical properties of the samples.

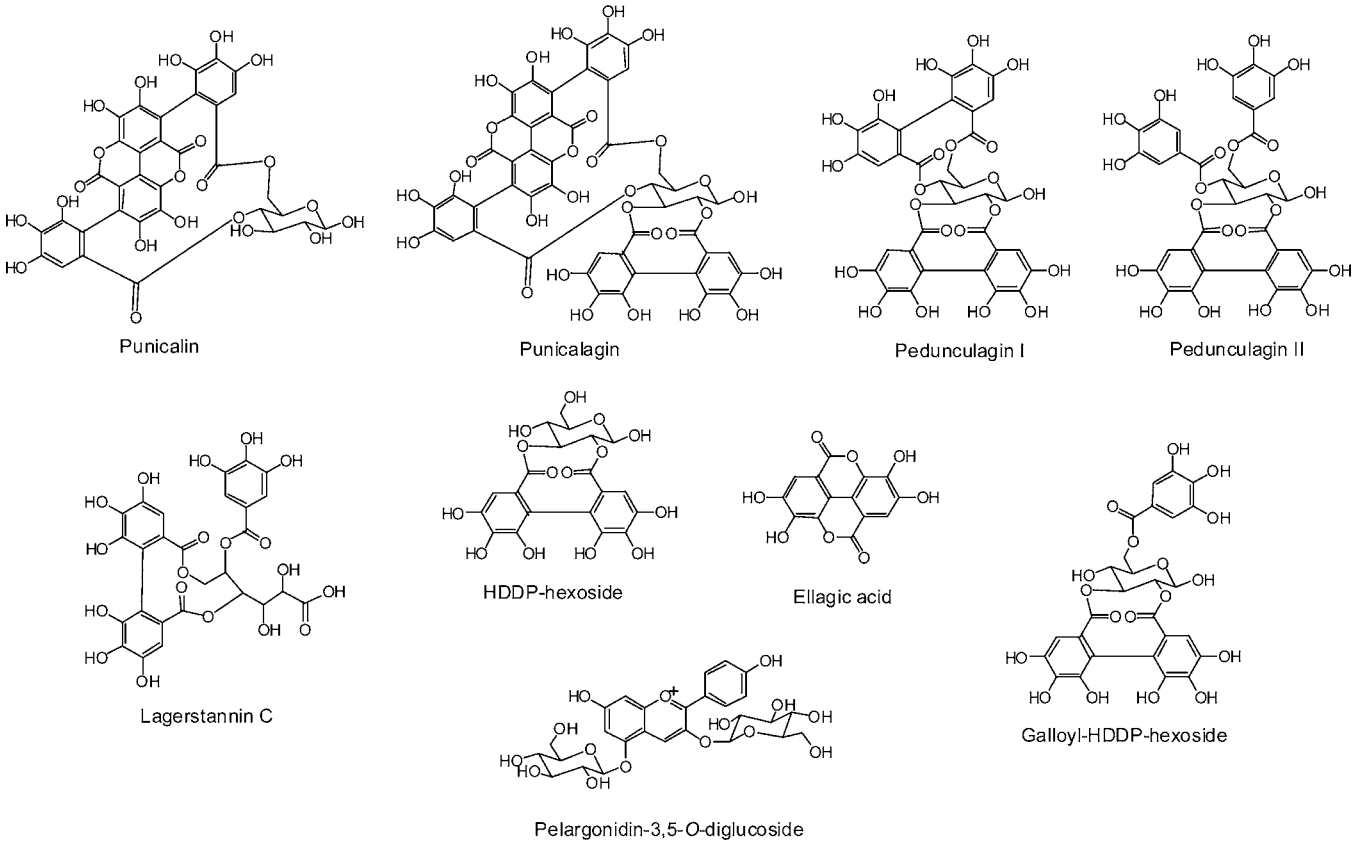

Figure 1. Chemical structures of the main (poly)phenolic components of pomegranate juice.

MATERIALS AND METHODS

Solvents and Chemicals. The Folin−Ciocalteu reagent was purchased from Merck (Darmstadt, Germany), while 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri-2-pyridyl-*s*-triazine (TPTZ), anhydrous sodium carbonate, and iron(III) chloride hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO). All solvents (analytical grade) were from Carlo Erba Reagents (Milano, Italy). High-purity water was produced in our laboratory by using an Alpha-Q system (Millipore, Marlborough, MA).

Study Area and Plant Materials. Four ancient accessions of *P. granatum* were collected and studied in 2011. The plants were located in a relatively small area of Parma province (Emilia-Romagna region) (44°69′ N, 10°02′ E) in northern Italy. This region is located outside the traditional area of pomegranate cultivation, and it can be characterized by harsh winter temperatures. However, some microareas of the Apennines are characterized by a relatively mild climate, more similar to that of central Italy, thus showing pedoclimatic conditions favorable to pomegranate cultivation. All the studied trees were located in the hill area at an altitude of 150 m asl with southeast and southwest exposures, thus being sheltered from cold winds. The accessions were chosen as representative samples of the pomegranate local population, after a preliminary agronomical, morphological, and genetic evaluation (data not shown). In particular, the selected genotypes were characterized using molecular markers and showed distinct genetic profiles both between them and with respect to the Italian cultivar Dente di Cavallo (data to be published).

Five fruits from each accession were collected at ripening in October, which is the common ripening period in Italy;[10,23] this stage was established with reference to a color index, according to which the ground color must be homogeneous over the whole epicarp surface. Moreover, five fruits of the commercial cultivar Dente di Cavallo were collected at ripening in a Sicilian orchard (Catania province) (37°30′ N, 10°5′ E), under a Mediterranean climate, and sent by courier to be used as samples for comparison. All samples were named with the name of the location of retrieval or by cultivar name and coded with an alphanumerical code (ID) (Table 1).

Pomegranate Juice Preparation and Sampling. The juice of each pomegranate was obtained by placing the arils 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 25 or 50 mL (Falcon), filtered and stored, after passage in liquid nitrogen, and then kept frozen at −80 °C until analysis. Suitable aliquots of pomegranate juice samples from each accession were centrifuged at 3000 rpm and then filtered with a 0.45 μm nylon filter, before being directly analyzed by LC−MS without further processing.

pH Measurements. pH values were measured by a 62 standard pH meter (Radiometer, Copenhagen, Denmark) equipped with an electrochemical sensor (Hamilton, Bonaduz, Switzerland).

uHPLC−MS Analyses. The juices were analyzed according to a method recently developed in our laboratories.[28] Briefly, phenolic compounds were determined using an Accela uHPLC 1250 equipped with a linear ion trap mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA), fitted with a heated electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc.). Separations were performed using a BlueOrchid-1.8 C18 column (50 × 2 mm) (Knauer, Berlin, Germany). Three MS experiments were performed, two in negative mode and one using positive ionization (for anthocyanins), under the same chromatographic and ionization conditions reported by Mena et al.[28]

Colorimetric Assays. The total polyphenol content was assayed using the Folin−Ciocalteu method as follows: A 790 μL volume of Milli-Q water, 10 μL of sample appropriately diluted with MeOH, and 50 μL of Folin−Ciocalteu reagent were added to a 1.5 mL Eppendorf microtube and vortexed.[29] After exactly 1 min, 150 μL of 200 g L^{-1} sodium carbonate was added, and the mixture was vortexed again and allowed to stand at room temperature in the dark for 120 min. The absorbance was recorded at 750 nm using a Hewlett-Packard 8453 UV−vis spectrophotometer (Waldbronn, Germany). The results are expressed as milligrams per milliliter (gallic acid was used as the standard). The total antioxidant capacity was assayed using the ferric reducing

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Table 3. Mass Spectral Characteristics of Anthocyanins and Relative Occurrence in Juice Samples[a]

compd	t_R (min)	$[M]^+$ (m/z)	MS^2 ion fragments (m/z)	juice sample				
				ME1	ME3	ME5	ME8	ME9
delphinidin 3,5-*O*-diglucoside	4.12	627	465, 303	+	+	+	+	+
cyanidin 3,5-*O*-diglucoside	4.56	611	287, 449	+	+	+	+	+
pelargonidin 3,5-*O*-diglucoside	4.86	595	433, 271	+	−	−	+	+
delphinidin 3-*O*-glucoside	5.15	465	303	+	+	+	+	+
cyanidin 3-*O*-glucoside	5.72	449	287	+	+	+	+	+
pelargonidin 3-*O*-glucoside	6.35	433	271	+	+	+	+	+

[a]Key: +, detected; −, not detected.

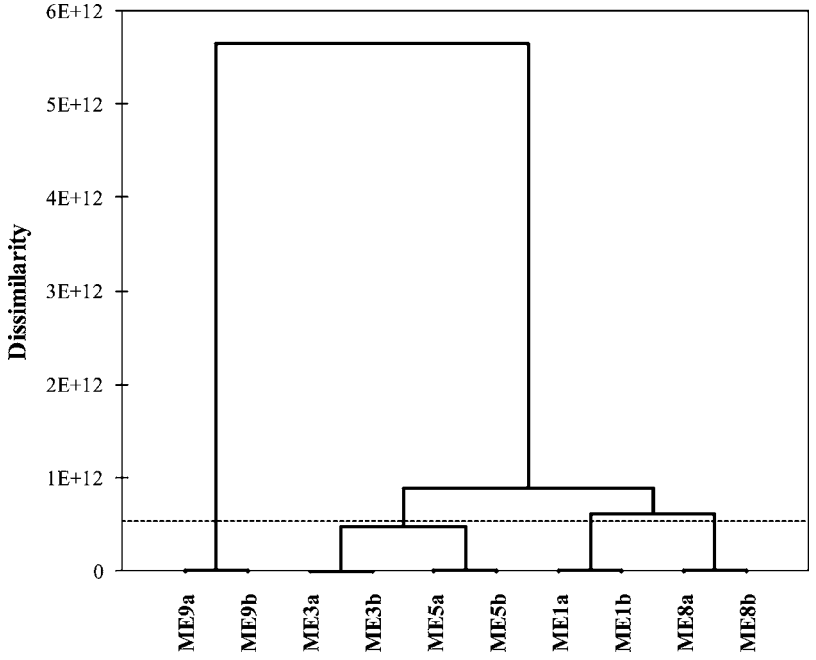

Figure 2. Dendrogram of the hierarchical cluster analysis among accessions (letters a and b denote the sample analyzed in duplicate). Clusters above the dashed lines show differences higher than 90%.

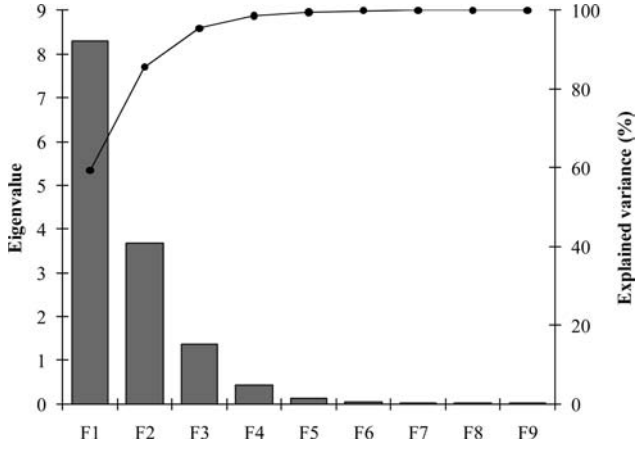

Figure 3. Screen plot obtained from the PCA (F1−F9 denote the nine principal components).

antioxidant power (FRAP) assay, based on the reduction of the Fe^{3+}−2,4,6-tripyridyl-*s*-triazine (TPTZ) complex to the ferrous form at low pH.[30] The reaction was monitored by measuring the absorbance (A) at 593 nm. Briefly, 10 μL of diluted sample was mixed with 30 μL of water and 260 μL of working FRAP reagent; then, after 30 min of incubation at 37 °C, the absorbance at 593 nm was recorded using a plate reader (Tecan Group Ltd., Männedorf, Switzerland). FRAP values were obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Trolox and expressed as millimoles per liter of Trolox.

Table 4. Variable Contribution (%) to the Main Principal Components[a]

compd	F1	F2	F3
PunII	9.881	0.989	0.693
PunI	9.190	3.328	5.973
ElAcHex	10.527	0.032	4.137
GalHex	2.417	17.927	6.572
ElAc	8.975	6.370	0.668
Pul	8.261	4.824	0.431
GrB	9.292	5.063	0.678
ElAcPent	10.219	3.347	0.261
ElAcRhamn	9.510	5.301	0.003
CyanHex	6.499	10.810	3.089
CyanDiHex	2.407	9.441	32.747
DelphDiHex	0.988	11.985	33.406
DelphHex	5.639	9.276	8.669
PelHex	6.194	11.307	2.675

[a]Abbreviations: PunII, punicalagin II; PunI, punicalagin I; ElAcHex, ellagic acid glucoside; GalHex, galloyl glucoside; ElAc, ellagic acid; Pul, punicalin; GrB, granatin B; ElAcPent, ellagic acid pentoside; ElAcRhamn, ellagic acid rhamnoside; CyanHex, cyanidin 3-glucoside; CyanDiHex, cyanidin 3,5-glucoside; DelphDiHex, delphinidin 3,5-diglucoside; DelphHex, delphinidin 3-glucoside; PelHex, pelargonidin 3-glucoside.

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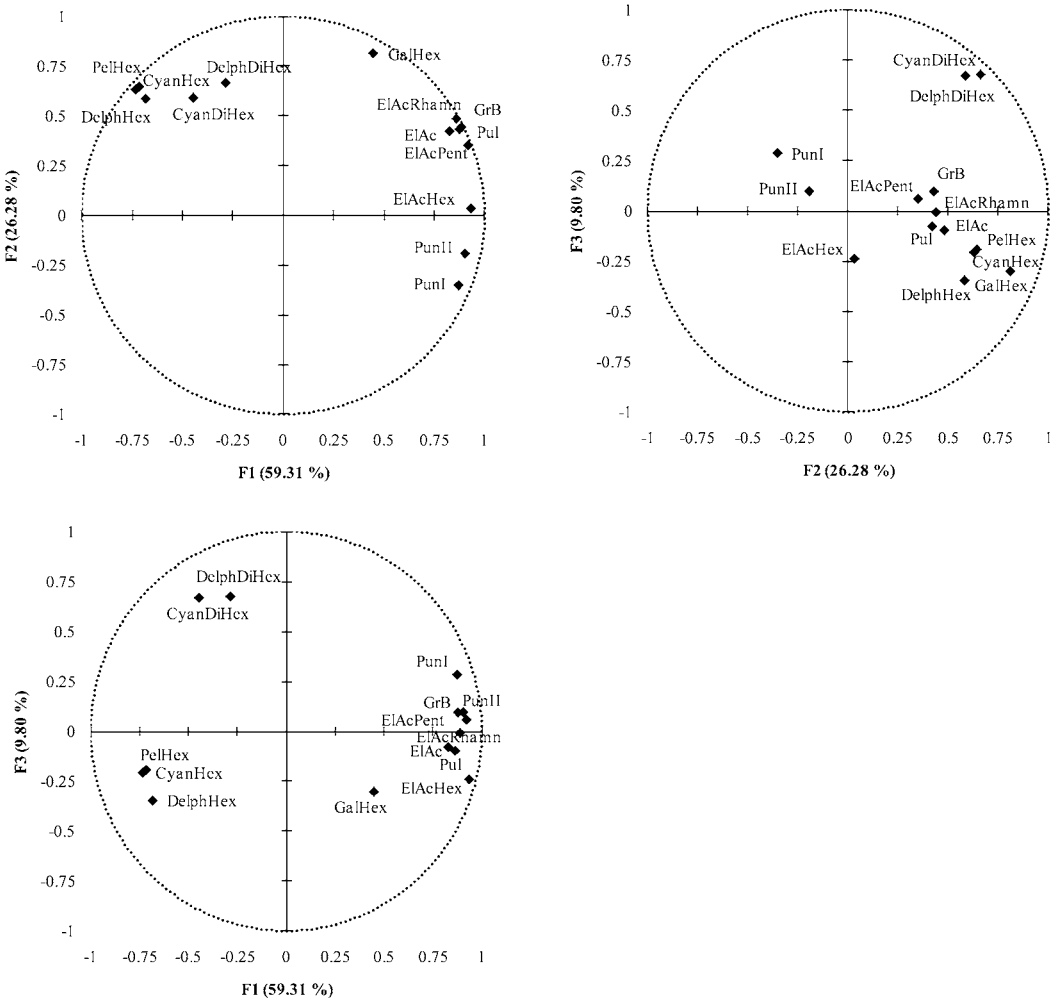

Figure 4. Loading plots obtained from the PCA of the pomegranate juices.

Statistical Analysis. Data were processed using the SPSS 19.0 software package (Chicago, IL). An analysis of variance (ANOVA) and a multiple range test (Tukey's HSD test) were carried out. Spearman's correlation analysis was performed to corroborate relationships between selected parameters. Principal component analysis was carried out using the analytical data as variables, without solution rotation. Cluster analysis was applied to the standardized data to obtain hierarchical associations employing the Euclidean distance and Ward's method as the dissimilarity measure and amalgamation rule, respectively.

RESULTS AND DISCUSSION

Pomegranate Juice Analysis. In this study, four pomegranate juices (ME1, ME3, ME5, ME8), obtained from ancient cultivars, and one juice (ME9), obtained from a commercial cultivar (Dente di Cavallo), were compared. Trees of the ancient cultivars are located in the Parma province (Emilia-Romagna region, northern Italy) and thus experience peculiar pedoclimatic conditions which may influence their phytochemical composition, whereas the commercial cultivar has its origin in a typical Mediterranean cultivation area (Sicily, southern Italy). Juices were obtained by hand-squeezing of arils to avoid the extraction of compounds from nonedible tissues of the fruit.[31]

The chemical composition of the juices was evaluated by a uHPLC–MS^n fingerprinting technique paired with chemometric classification to evaluate its potentiality for the rapid identification of peculiar compositional characteristics.

More specifically, two MS experiments were performed in negative ion mode, while one was performed in positive ionization. The two experiments in negative ionization were chosen after optimization trials focused on covering the different ionization and fragmentation capacities of the polyphenolic structures of pomegranate juice. The experiment performed in positive mode was focused on the anthocyanin profile. Analyses were carried out by using full-scan and MS^2/MS^3 data-dependent experiments.

The combination of these three experiments allowed the tentative identification of a total of 68 compounds, reported in Table 2 and whose identification was performed as previously described.[27] Ellagic acid related phenolics were the main class of identified compounds, but a broad number of anthocyanins, noncolored flavonoids, and phenolic acids were also found. Among the 68 identified phenolics, 48 were found to occur in all the considered samples, although at different levels.

As already reported in the literature, hydrolyzable tannins are the most abundant antioxidant polyphenolic compounds in pomegranate juices and include gallotannins, ellagitannins, and gallagyl esters, such as punicalagin and punicalin (Figure 1).[9,31]

The anthocyanin content (Table 3) was found to vary significantly among ecotypes, in agreement with the literature.[13] In particular, the juice obtained from cultivar Dente di Cavallo was richer in anthocyanins compared to juices from ancient ecotypes.

Although quantitative determination of the bioactive compounds is possible, analytical standards for most of them are

usually not commercially available, and quantification has generally been performed expressing the results as equivalents of a chemically related reference compound. Nonetheless, when mass spectrometry detection is used, slight differences in the structure may lead to significant differences in ionization ability; thus, the quantitation of a class of compounds on the basis of a reference standard can be cumbersome. As an alternative, we applied a nontargeted fingerprinting approach and a chemometric analysis of the chromatographic data. This approach allowed for juice clusterization according to their phytochemical profile, thus producing relevant information for the evaluation of their putative nutraceutical potential. Moreover, the method evaluated in this study combined a quick separation and a high-throughput strategy, collecting a high amount of data in a very short time. A single run was completed in 20 min, thus offering an excellent time-to-data ratio, which is an important factor when large batches of samples have to be screened, as in germplasm screening.[27]

Chemometric Classification. In this study, two unsupervised pattern recognition methods were applied, starting from the most abundant data obtained by uHPLC−MS^n analysis.

For both statistical methods, the ionic abundance values of the most representative phenolic compounds were used as variables. An ionic abundance cutoff value of 1×10^5 was used as the selection criterion to avoid misinterpretation; in addition, only compounds occurring in all the samples were considered for classification.

Cluster analysis was performed to obtain hierarchical associations among accessions as a dendrogram (Figure 2). Two major groups of clusters were formed on the basis of existing similarities with regard to the analyzed parameters. The first group included ancient accessions, while the second one only grouped cultivar Dente di Cavallo. The groups are highly dissimilar, pointing out clear differences among accessions, in agreement with preliminary morphological and genetic analyses (data not shown).

Concerning the ancient cultivar group, two subclusters were identified, corresponding to accessions ME3−ME5 and ME1−ME8, respectively. In particular, accessions ME3 and ME5 were classified as identical according to their phenolic composition, and this classification is consistent with the strong morphological similarity of the fruits and plants. Accessions ME1 and ME8 are also classified as dissimilar, although to a lower extent, with slight differences in phenolic composition. This dissimilarity is in agreement with genetic data obtained from preliminary studies (data not shown) and with morphological results, since ME8 has smaller fruits and thicker skins, probably on account of the peculiar growing conditions.

As far as principal component analysis (PCA) is concerned, the first three principal components explain 95.4% of the total variance among pomegranate cultivars, as reported in the screen plot (Figure 3). A comparison of scores and loadings for F1, F2, and F3 allows the identification of the compounds having a greater influence on the ranking of juices. The variable percentage contributions to the main principal components are reported in Table 4. Loading and score plots are reported in Figures 4 and 5, respectively.

The clusterization obtained by PCA was very similar to that reported before for the hierarchical cluster analysis. Again, ME9 juice was found to significantly differ from samples obtained from ancient accessions. Among the latter, ME8 juice showed an interesting profile with higher ellagitannin content, probably ascribable to the harsh growing conditions. In particular, ellagic acid derivatives and tannins showed positive eigenvalues on F1,

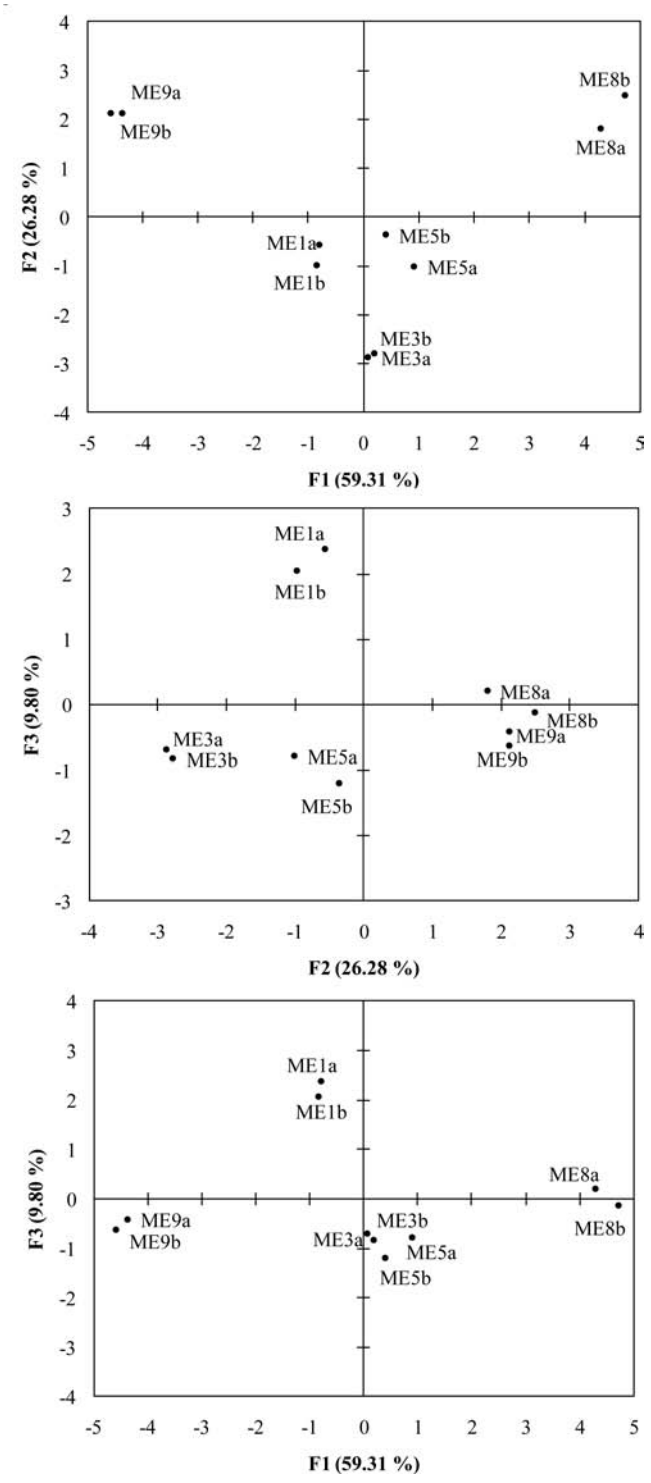

Figure 5. Score plots obtained from the PCA of the pomegranate juices.

while anthocyanins showed negative eigenvalues. Among these phenolics, punicalagin isomers and ellagic acid glucoside, rhamnoside, and pentoside showed the higher contribution to F1. Concerning the second component, the main positive contribution was due to galloyl glucoside and to anthocyanins; only punicalagins gave negative eigenvalues on F2. Finally, delphinidin 3,5-diglucoside and cyanidin 3,5-diglucoside showed the higher contribution to F3.

The PCA analysis allowed a clusterization of the juices according to their main characteristics. In general, juices obtained

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from ancient cultivars were particularly rich in ellagitannins, while the reference juice ME9 was particularly rich in anthocyanins. Samples ME8 and ME9 are mainly separated on F1 on account of the content of ellagitannins and anthocyanins, the former being more abundant in ME8. ME3 is differentiated from ME8 and ME9 along F2 for the higher content of punicalagin isomers. Finally, M1 showed a particularly high value on F3 on the basis of the higher levels of cyanidin and delphinidin 3,5-diglucoside compared to the other samples. This fact can be ascribed to varietal characteristics, ME3 growing conditions and morphological parameters being similar to those of the ME1 accession, although the latter does not show the same anthocyanin profile.

Total Phenolic Compounds and Antioxidant Capacity Evaluation. To fully characterize the properties of the juices obtained from ancient pomegranate accessions and to compare them with the commercial one, the total phenolic content (TPC) and total antioxidant capacity (TAC) were also evaluated. The Folin−Ciocalteu method, employed for the determination of TPC, showed remarkable and significant differences among juices, with values ranging between 1.6 and 3.7 mg of GAE/mL, as reported in Table 5.

Table 5. Antioxidant Capacity (FRAP) and TPC of Pomegranate Juices Obtained from Different Accessions

accession	FRAP (mM Trolox)	TPC (mg of GAE/mL)
ME1	12.50 ± 0.76 d[a]	1.65 ± 0.03 d
ME3	11.94 ± 0.61 d	1.60 ± 0.08 d
ME5	16.20 ± 0.89 b	2.10 ± 0.06 b
ME8	27.73 ± 0.69 a	3.73 ± 0.06 a
ME9	14.82 ± 0.10 c	1.94 ± 0.02 c
ANOVA p value	***[b]	***

[a]Means (n = 3) within a column followed by different letters are significantly different at $p < 0.05$ according to the Tukey HSD multiple range test. [b]Nonsignificant ($p > 0.05$). Significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

These results were consistent with other previously published works about pomegranate juice, although no specific studies were performed on the accessions considered in this work.[32] As far as TAC is concerned, the FRAP assay was chosen in this study (see Table 5). FRAP data, reported as Trolox equivalents (mM) were in the range of 11.9−27.7 mM, showing good agreement with the literature.[32]

In this study, ME8 showed a significantly higher TAC as well as a significantly higher TPC in comparison to other juices, in agreement with the chemometric classification obtained by PCA and by hierarchical cluster analysis. TPC and FRAP showed a very strong positive correlation (two-tailed Spearmans test, ρ = 0.899, $p < 0.001$), as reported in Figure 6. Similarities in sample rating with respect to FRAP and TPC are due to the highly significant correlation between them. From a nutritional viewpoint, the ME8 sample could easily contribute to increases in the dietary TAC intake to levels that have been demonstrated to reduce systemic inflammation.[33]

From the data reported, it appears that ancient pomegranate accessions show a peculiar polyphenolic profile and could constitute interesting sources of specific compounds which have been described as potentially beneficial toward several pathogenic processes in humans.[11] In particular, ME8 accession could be considered very interesting for its specific phenolic composition, being particularly rich in ellagic acid derivatives, and further studies should be addressed to investigate the genetic or environmental basis leading to such accumulation of phenolic compounds. Although because of their morphological characteristics (e.g., small fruits, thick skins), ME8 fruits are unlikely to be used for direct consumption, this ecotype may be successfully employed for the preparation of nutraceutical products or for industrial blending of juices. Moreover, the use of ancient accessions for breeding purposes could also be envisaged to

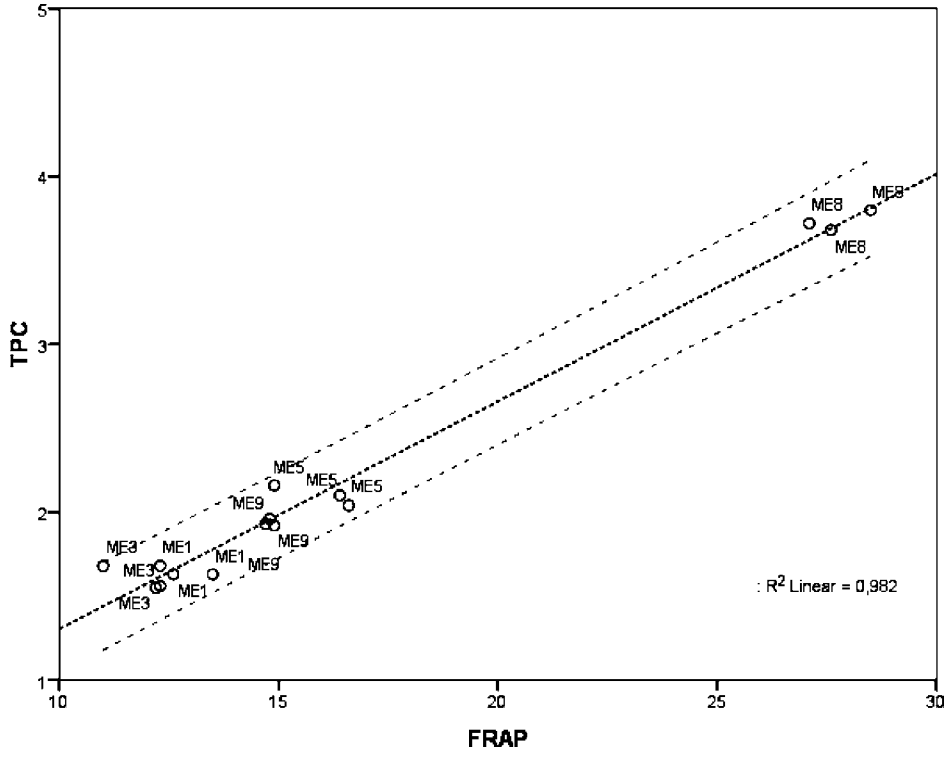

Figure 6. Statistical correlation between TPC and FRAP (two-tailed Spearmans test, ρ = 0.899, $p < 0.001$, samples analyzed in triplicate).

obtain new pomegranate varieties with increased phenolic content. In this context, the combined recourse to HPLC−MS^n qualitative fingerprinting and multivariate analysis may represent a useful tool for the discrimination and selection of pomegranate germplasm with specific properties related to (poly)phenolic content.

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Notes

The authors declare no competing financial interest.

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