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Polyphenols as Chemotaxonomic Markers in Italian "Long-Storage" Tomato Genotypes

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ABSTRACT: "Long-storage" tomato (*Solanum lycopersicum* L.) is a niche product typical of the Mediterranean area, traditionally cultivated under no water supply, the fruits of which combine a good taste with excellent nutritional properties. High-performance liquid chromatography coupled with diode array detection and electron spray-mass spectrometry (HPLC/DAD/ESI-MS) was used to identify the phenolic profile in 10 landraces of long-storage tomato, grown under a typical semiarid climate, as compared to a processing tomato hybrid cultivated in the same environment, under both well-irrigated and unirrigated conditions. Sixteen different secondary metabolites, belonging to the classes of cinnamoylquinic acids and flavonoids, were identified. Quantitative analyses were also performed to monitor the changes in the phenolic content along the batch. The results highlighted that landraces originating from the same area exhibit different fruit morphologies but own a similar biochemical profile. Moreover, the two controls (well irrigated and unirrigated) are placed into the same cluster, suggesting that these secondary metabolites in tomato fruits may be more genetics-dependent than environment-dependent. Given the analysis of phenols nowadays represents a useful tool to assess the genetic variability in tomato, these compounds could be adopted as chemotaxonomic markers in the traceability of this niche product.

KEYWORDS: *long-storage tomato, polyphenols, traceability*

INTRODUCTION

The exploitation of biodiversity may be useful in supporting food security and human nutrition and contributes to a general sustainable development. In this context new genetic material to increase yields or produce stress-resistant varieties can be achieved by using wild/traditional products. The horticultural production in southern Italy plays an important role in the Mediterranean agriculture for the relevant number of vegetables, representing a precious source of biodiversity. Among these productions, the local landraces of "long-storage" tomato, so-called for the textural properties of fruits that allow an extended shelf life, provide a niche product combining a good taste with excellent nutritional properties.[1−3] Furthermore, due to the high drought tolerance of the plant, traditionally cultivated under no water supply, long-storage tomato may represent an interesting genetic source in breeding programs for water stress resistance in both fresh-market and processing tomatoes.

Tomato (*Solanum lycopersicum* L. syn. *Lycopersicon esculentum* Mill.) is a good source of natural antioxidants including ascorbic acid, carotenoids, and a large number of phenolic compounds, thus playing an important role in human nutrition in the prevention of cancer and cardiovascular diseases.[4−10] The content of these constituents greatly depends on environmental and agronomic factors, such as cultivation area, variety, stage of ripening at harvest, and fertilization; for instance, tomato fruits grown in Mediterranean areas have been found to be richer in some phenols than those produced in northern Europe.[11] Several studies have examined the effects of genetic and environmental variability on defensive characters of plants, such as some secondary compounds or some physical structures of the plant and leaf, and these studies have been primarily focused on annual species that are easily manipulated. In particular, the abundance of phenolics in tomato fruits depends on both genetics and environmental conditions.[12,13] Within phenols, rutin (quercetin 3-*O*-rutinoside) was the first flavonoid identified in tomato fruits, back in the 1930s,[14] followed by naringenin and quercitrin (quercetin 3-*O*-rhamnoside). In a recent review, Slimestad and Verheul[15] gave a detailed overview of an unexpected number of flavonoids and phenolics in tomato fruits, including quercetin, kaempferol, eriodictyol, chalconaringenin, naringenin, and their variously glycosylated derivatives. Organic acids such as benzoic, hydroxybenzoic, and protocatechuic are also present.[15,16] Cinnamic acids (caffeic, ferulic, and coumaric) are reported as the main phenolic compounds, besides flavonoids, occurring in tomatoes, along with their derivatives with sugars (mainly glucose) and quinic acid (such as chlorogenic, 5-caffeoylquinic acid).[15,17] Phenols have recently gained attention as effective chemotaxonomic markers. Indeed, the use of phenolic compounds as chemotaxonomic markers in plant studies has evidenced even small differences within a wide number of wild and cultivated species, such as tea[18] and garlic.[19] Polyphenols have been also used for classification purposes, including the determination of the origin area, as in the case of thyme.[20] Analysis of phenolic profiles also contributed to demonstrate the biunivocal relationship between environmental conditions

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Table 1. Tomato Genotypes Examined and Some Fruit Morphological Characteristics

genotype	code	provenance	fruit shape	fruit apex[a]	fruit size[b]	fruit length, L (cm)	fruit width, W (cm)	L/W
Pizzutello di Sciacca	PS	Sciacca (Agrigento)	elongate	++	1	3.20	2.53	1.26
Pizzottello di Montallegro	PM	Montallegro (Agrigento)	round	+	4	3.40	3.43	0.99
Locale di Custonaci	C	Custonaci (Trapani)	round	++	4	3.20	3.30	0.97
Giallo Basicò	G	Basicò (Messina)	round	+	4	3.00	3.01	1.00
Locale di Salina 2	S2	Salina (Eolian Islands)	round	++	3	3.10	3.20	0.97
Locale di Filicudi	F	Filicudi (Eolian Islands)	round	−	2	2.90	3.00	0.97
Locale di Salina 6	S6	Salina (Eolian Islands)	round	−	2	2.53	2.73	0.93
Locale di Pollara	P	Salina (Eolian Islands)	round	−	1	2.70	2.80	0.96
Ruccaloru	R	San Pierniceto (Messina)	elongate	−	2	3.23	2.47	1.31
Principe Borghese	PB	SAIS selection (Cesena)	elongate	+	3	3.37	2.97	1.13
cv. Brigade (control)	B	ASGROW	elongate	−	5	5.10	4.21	1.21

[a]++, normal apex; +, small apex; −, no apex. [b]1, very small (<12 g); 2, small (12.1−15 g); 3, medium (15.1−18 g), 4, large (18.1−21); 5, very large (>21 g).

and ecophysiological response in plants.[21,22] Recently, Vallverdú-Queralt et al.[23] and Slimestad et al.[24] evaluated the differences in total and individual phenolic content in different local tomato varieties, showing the qualitative and quantitative study of phenol distribution in tomato fruits can be used as a differentiating tool among ecotypes. The present study aimed at assessing the phenolic profile in 10 landraces of long-storage tomato and identifying chemotaxonomic markers useful for the traceability of the product.

MATERIALS AND METHODS

Chemicals. All solvents used in this study were high-purity spectroscopic grade solvents by Carlo Erba (Milano, Italy). Pure cynarin (1,3-dicaffeoylquinic acid) was provided by Extrasynthese (Lyon, France); chlorogenic acid, rutin, quercetin, and naringenin were provided by Fluka (Sigma-Aldrich s.r.l., Milano, Italy).

Plant Material and Experimental Design. Nine landraces of long-storage tomato, belonging to the germplasm collection of the CNR-ISAFoM of Catania (Italy), were investigated in this study. The landraces were recovered throughout Sicily, from the western to the eastern part of the island (provinces of Agrigento, Trapani, and Messina) up to the Eolian islands (Salina and Filicudi). The commercial tomato 'Principe Borghese' (SAIS Sementi S.p.a., Cesena, Italy) was included in the study, being the sole long-storage tomato of which seeds are commercially available to farmers. The commercial hybrid 'Brigade' (Asgrow Italia Vegetable Seeds, Lodi, Italy) of processing tomato was also included in the experiments as control (Table 1).

All genotypes were open field cultivated in Sicily, southern Italy, on Catania plain (10 m asl, 37° 25′ N latitude, 15° 30′ E longitude), on a vertic xerochrepts soil (USDA Soil Taxonomy, 1999). The soil characteristics were as follows: clay, 28.3%; sand, 49.3%; loam, 22.4%; organic matter, 1.4%; pH, 8.6; total N, 1.0‰; available P_2O_5, 5 ppm; exchangeable K_2O, 245 ppm.

A randomized complete block experimental design with three replicates was used. Plants were transplanted at four-leaf stage on April 23, in plots of 24 m^2 (6 × 4 m) with a plant density of 3.3 plants m^{-2}. Before transplanting 75, 100, and 100 kg ha^{-1} of N (as ammonium sulfate), P (as mineral perphosphate), and K (as potassium sulfate) respectively, were distributed. A month after transplanting, a further 75 kg ha^{-1} of N (as ammonium nitrate) was supplied as top dressing.

A fixed total volume of approximately 40 mm of water was applied, split in two applications. After that, irrigation was interrupted. For the control only, two water regimes were applied: unirrigated (irrigation up to plant establishment, for a total amount of 40 mm) and fully irrigated (long season irrigation with 100% restoration of water evapotranspired, for a total amount of 233 mm).

Throughout the crop-growing season, air temperature, rainfall, and class A pan evaporation were daily recorded using a data logger (CR10, Campbell Scientific, Logan UT) located approximately 50 m from the experimental field. Meteorological data were those of a typically semiarid Mediterranean environment. During the crop-growing season, minimum temperatures ranged between 17.7 (April) and 20.9 °C (July) and maximum temperatures between 18.9 (April) and 32.6 (July). A total of 40 mm was recorded during the crop-growing season.

The crop was hand harvested when ripe fruit rate reached about 95% (mid July). At harvest, 10 fruits per replicate were randomly sampled, and the following biometric measurements were carried out: fruit fresh weight (g), fruit length and width (cm), which correspond to the polar and equatorial diameters, respectively. Finally, the fruit length/fruit width ratio was calculated, to indicate fruit shape.

Sample Preparation. Ripe fruits of the first and second trusses were sampled (approximately 2 kg per plot) at harvest for laboratory analyses. Before analyses, the tomatoes were washed with running water to remove dirt and dried thoroughly with absorbent paper. Approximately 50 g was finely grounded with an electric blender to a homogeneous reddish puree. Aliquots (1 g) of these "puree" samples were put in 8 mL amber sample vials, and 2 mL of a hydroalcoholic solution (80% methanol in water) was added. Samples were then maintained at room temperature (20 °C) overnight, in the dark and under vigorous shaking (350 rpm). The resulting heterogeneous mixtures, still containing solid tomato residues, were then filtered with PTFE filters (15 mm diameter, 0.45 μm pore size, Chemtek Analytica), thus obtaining 1.4−1.7 mL of clear yellowish solutions, which were further split into two aliquots and sent to qualitative and quantitative analyses. When required, the above-mentioned analytical samples were stored for short periods (1 week at the most) at −20 °C under nitrogen atmosphere.

HPLC/DAD/ESI-MS Qualitative Analyses. Variable aliquots (0.6−0.9 mL) of the above-mentioned hydroalcoholic solutions were transferred into standard laboratory vials and brought to dryness in vacuo with a rotary evaporator (Heidolph Laborota 400). The resulting yellow residues were then redissolved in 250 μL of the original 80% MeOH solution and submitted to qualitative analyses. Tomato polar extracts were analyzed by HPLC/DAD/ESI-MS using a Waters instrument (Waters Italia S.p.A., Milano, Italy) consisting of a 1525 binary HPLC pump, a PDA 996 photodiode array detector (DAD), and a Micromass ZQ Mass Analyzer equipped with an ESI Z-spray source. DAD analyses were carried out in the range between 600 and 190 nm, setting the detector at 280 nm for flavanones (naringenin and its derivatives), at 330 nm for mono- and dicinnamoylquinic acids, at 350 nm for glycosylated flavonoids, and at 370 nm for quercetin (aglycone).

Total ion current (TIC) chromatograms were acquired in negative mode, using a cone voltage of −30 V in the mass range between m/z 80 and 1200 units. The other parameters used for the acquisition of the TICs were the following: capillary voltage, 2.75 kV; source temperature, 150 °C; desolvation temperature, 280 °C; gas flow (L/h), 400 (desolvation) and 210 (cone). Chromatographic runs were performed using a reverse-phase column (Inertsil ODS-3, 100 × 3.0

mm, 3 μm particle size, Alltech, Italy) equipped with a guard column (Inertsil ODS 7.5 × 4.6 mm, 5 μm particle size, Alltech, Italy); polyphenols were eluted with the following gradient of B (formic acid, 1% solution in acetonitrile) in A (1% solution of formic acid in water): t = 0 min, B = 5%; t = 10 min, B = 10%; t = 15 min, B = 20%; t = 25 min, B = 35%, then at t = 35 min and B = 60%, for a total 45 min run time. The solvent flow rate was 0.7 mL min^{-1}, the temperature was kept at 20 °C with a column oven (Hitachi L-2300, VWR International, Milano, Italy), and the injector volume selected was 20 μL. Data from the LC-MS apparatus were analyzed and processed through Mass Lynx v. 4.0 standard software (Waters).

HPLC/DAD Quantitative Analyses. Small aliquots (0.8 mL) of the hydroalcoholic solutions described above were put in 2 mL amber conic vials and submitted to HPLC/UV−vis/DAD high-throughput analyses. Quantitative analyses were carried out on a Dionex instrument equipped with a P580 binary high-pressure pump, a PDA-100 photodiode array detector, a TCC-100 thermostated column compartment, and an ASI-100 automated sample injector. Collected data were processed through a Chromeleon Chromatography Information Management System v. 6.70. Chromatographic analysis runs were run using the same conditions (solvents, elution program, column) described in the previous paragraph. Quantification was carried out at 280 nm for naringenin using a calibration curve established with its corresponding analytical standard (naringenin, correlation coefficient R^2 = 0.9997) and at 330 nm for mono- and dicinnamoylquinic acids, using chlorogenic acid (R^2 = 0.9999) and cynarin (R^2 = 0.9998), respectively. Due to the differences in maximum absorption wavelength values, it was necessary to establish two separate calibration curves for flavonols; quercetin (R^2 = 0.9999) was quantified at 370 nm, whereas 350 nm was used as reference wavelength for rutin (quercetin 3-*O*-rutinoside, R^2 = 0.9998). All analyses were carried out in triplicate.

Data Analysis. The relationship between the different traits was quantified using principal component analysis (PCA). Principal components with eigenvalues >1.0 were selected. To distinguish high components, weights with values of 0.6 as their absolute value were arbitrarily adopted. Dendrograms were constructed based on Euclidean distances, and the furthest neighbor method was applied with the statistical package StatistiXL1.5 (StatistiXL Ltd.).

RESULTS AND DISCUSSION

Main Morphological Characteristics of Fruits. The fruit characteristics of genotypes assessed are reported in Table 1. Long-storage tomato genotypes widely differed in fruit size, ranging from 10.9 g ('Pizzutello di Sciacca') to 20.7 g ('Giallo Basicò'). Most of the types were round (L/W ratio from 0.93 to 1.00), and six of them showed a fruit apex. Neither fruit weight nor fruit apex was affected by genotype provenance, because genotypes originating from proximate areas (e.g., 'Locale di Salina 2' and 'Locale di Salina 6') differed for both characteristics.

Analysis of LC/UV-vis-DAD/ESI-MS Data. Besides carotenoids, polyphenols are the most abundant class of secondary metabolites present in tomato, for a total of more than 100 different compounds.[15] Rutin, naringenin, chalconaringenin, narirutin, and quercetin are the main flavonoids found in tomato fruits, followed by cinnamic acids and their derivatives with sugars and/or quinic acid.[15,17] A deep analysis of the mass spectra in the TIC chromatogram, together with the single ion extraction method, contributed to distinguishing one class of derivatives from the other and to identifying the molecules of interest. The LC-MS analysis of the different long-storage tomatoes showed the presence of chlorogenic acid (5-caffeoylquinic acid, t_R = 15.6) in all samples; other peaks ubiquitously detected were rutin (t_R = 18.2), quercetin (t_R = 26.9), and naringenin (t_R = 27.8) (Table 2). The UV−vis data of the unidentified signals present in the chromatograms suggested they may belong to the chemical class of the cinnamic acid derivatives; the extraction from the corresponding TIC chromatograms of the diagnostic ion at m/z 191 units (deprotonated quinic acid molecule) confirmed their tentative identification as cinnamoylquinic acid derivatives. Further studies on the data deriving from the MS allowed the identification of 12 different molecules belonging to this class (Table 2). Peak assignments were made through the analysis of pseudomolecular ions and main fragments; MS data were in agreement with those reported in the literature.[17,25,26] Unfortunately, it was impossible to distinguish among the different isomers within the same subclass (Table 2). As previously mentioned, isomers of mono-, di-, and tricaffeoylquinic acids, as well as coumaroylquinic and feruloylquinic acids, are already reported in the literature to occur in tomato fruits;[15,17] nevertheless, it is worth mentioning that the presence of some of these long-storage tomato metabolites (such as compounds **10**−**14**) is here reported for the first time.

Table 2. Peak List and Diagnostics of Long-Storage Tomato Extract Chemotaxonomic Markers

peak	t_R[a] (min)	compound identification	MW	MS: ESI$^-$ data, m/z[b]
1	15.6	5-caffeoylquinic (chlorogenic) acid[c]	354	353* (M − 1), 191
2	16.9	feruloylquinic acid 1[d]	368	367 (M − 1), 191*
3	17.3	feruloylquinic acid 2[d]	368	367 (M − 1), 191*
4	18.2	rutin (quercetin 3-*O*-rutinoside)[c]	610	609* (M − 1), 300 (M − glc)
5	19.0	coumaroylquinic acid 1[d]	338	337 (M − 1), 191*
6	19.4	coumaroylquinic acid 2[d]	338	337 (M − 1), 191*
7	19.8	dicaffeoylquinic acid 1[d]	516	515* (M − 1), 353, 191
8	20.6	dicaffeoylquinic acid 2[d]	516	515* (M − 1), 353, 191
9	21.1	dicaffeoylquinic acid 3[d]	516	515 (M − 1), 353, 191*
10	21.6	caffeoyl-feruloylquinic acid 1[d]	530	529 (M − 1), 353*, 191
11	22.1	caffeoyl-feruloylquinic acid 2[d]	530	529 (M − 1), 367, 353*, 191
12	22.8	caffeoyl-feruloylquinic acid 3[d]	530	529 (M − 1), 353, 191*
13	23.7	diferuloylquinic acid 1[d]	544	543 (M-1), 191*
14	24.6	diferuloylquinic acid 2[d]	544	543 (M − 1), 191*
15	26.9	quercetin[c]	302	301* (M − 1)
16	27.8	naringenin[c]	272	271 (M − 1)

[a]Average value of 3 × 36 = 108 analytical measurements. [b]Base peaks marked with an asterisk. [c]Co-injection with pure analytical standards. [d]Correct isomer not identified.

Quantitative Data Analysis. Analyses of 10 different ecotypes of long-storage tomato plus a processing tomato cultivar, based on the marker identification, revealed a great genetic variation in total phenolic content (from 0.21 mg g^{-1} for 'Pizzutello di Sciacca' to 0.09 mg g^{-1} for 'Locale di Salina 6'), with values that were up to 10-fold higher than that of the control 'Brigade' (Figure 1). This phenomenon may be considered as a result of the environmental pressure, which has exerted a natural selection toward long-storage tomatoes higher in phenol biosynthesis, this crop being traditionally cultivated under no water supply. Indeed, the defense function of these metabolites has been widely demonstrated against biotic and abiotic stimuli such as UV-B radiation, attacks by pathogens, and drought,[22,27−29] and it is also known that plant resistance to various stresses is associated with antioxidant

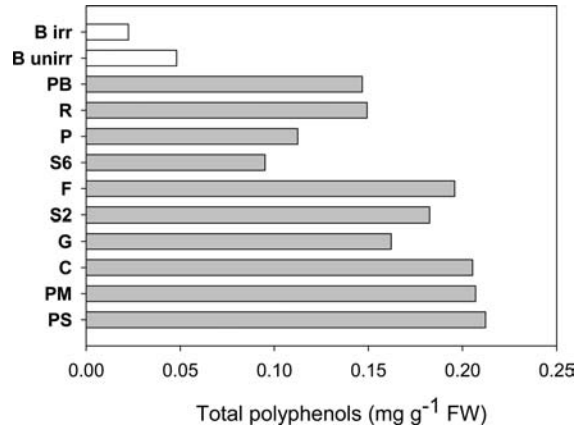

Figure 1. Total polyphenol content in tomato genotypes described in the text (capital letters on the left refer to Table 1; irr, well irrigated; unirr, unirrigated). White bars correspond to processing tomato control 'Brigade'.

capacity and that increased levels of antioxidants may prevent stress damage.[29,30]

From a chemotaxonomical point of view, an interesting result is the variation of each marker along the analytical batch. Figure 2 reports the sequential chromatographic profile of five selected long-storage tomatoes and the control. When peaks 1 (chlorogenic acid), 4 (rutin), and 15 and 16 (quercetin and naringenin, respectively), the variations of which are rather negligible, are excluded, all components change considerably with genotype. In particular, a high variance was observed between 18 and 25 min; this interval is the most interesting portion of chromatograms; thus, it could be considered as a sort of "fingerprint" area. During this time interval, a series of peaks elute (peaks 5−14), which differentiate the various extracts assessed. The corresponding markers belong to the family of cinnamoylquinic acid derivatives.

These data were analyzed by means of PCA. The first two components (PC1 and PC2) provide satisfactory information, because they explain 76.4% of the total variance and were used to score plot (Figure 3). The first component accounted for 53.8% of total variation, had larger positive coefficients (>0.6)

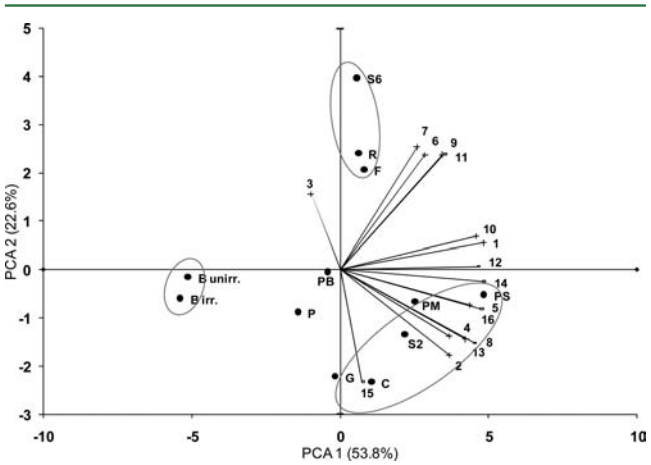

Figure 3. Scatter plot grouping of tomatoes based on PC1 and PC2 of the principal component analysis (see Table 1 for the sample list and Table 2 for the numbering of chemical markers; irr, well irrigated; unirr, unirrigated).

for all traits except for compound **3**, and showed relatively small negative coefficients for compounds **7**, **9**, and **15**. These last three are mainly represented by the second component, responsible for 22.6% of the variability. Examination of the score plot suggests a clustering into three distinct groups; 'Pizzutello di Sciacca', 'Pizzottello di Montallegro', 'Locale di Custonaci', 'Giallo Basicò', and 'Locale di Salina 2' clustered on the right side of the ordination along PC1, characterized by high contents of **2**, **4**, **5**, **8**, **13**, **15**, and **16**. 'Brigade' was characterized by low contents of these constituents. 'Locale di Salina 6', 'Ruccaloru, and 'Locale di Filicudi' clustered together on the upper quarter of the ordination along PC2 and were characterized by a lower content of **15** and higher levels of **6**, **7**, **9**, and **11**. In Figure 3, the vector length of a trait measures the magnitude of its effect (positive or negative). All of the studied

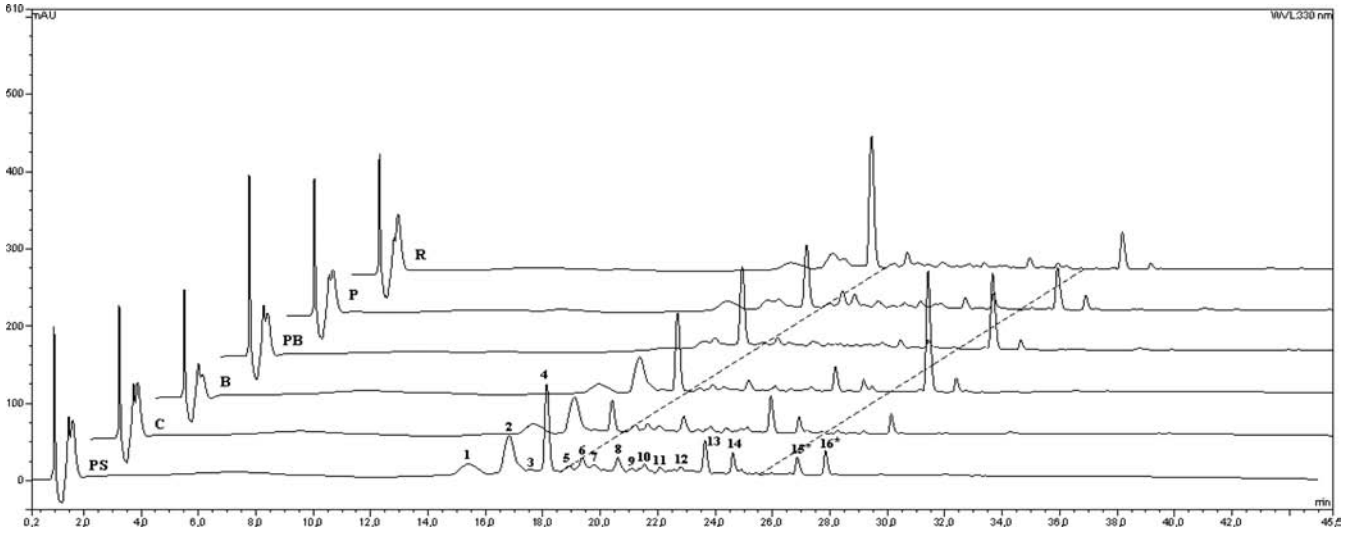

Figure 2. HPLC chromatograms, visualized at 330 nm, of selected samples from the long-storage tomatoes as described in the text. The chemotaxonomic markers are numbered 1−16. Capital letters on each row on the left refer to Table 1, whereas peak numbers refer to Table 2. The area covering 18−25 min (containing high-variance peaks 5−14) is the fingerprint area. Peaks marked with an asterisk are visualized through their residual absorptions at the selected wavelength.

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traits, when feruloylquinic acid 2 (**3**) was excluded, had relatively long vectors, suggesting that they could have relatively large weight on the selection of representative traits.

The dendrogram based on the cluster analysis of biochemical data (Figure 4) shows a clear distinction in two main groups.

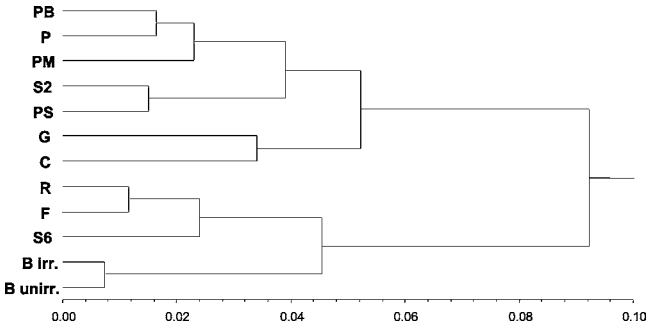

Figure 4. Dendrogram of 12 tomatoes based on 16 chemotaxonomical markers (capital letters on the left refer to the list of Table 1; irr, well irrigated; unirr, unirrigated).

The first one includes the landraces originating from the Sicilian mainland, except 'Principe Borghese' and 'Locale di Salina 2'. The second one consists of the remaining three landraces originating from the Eolian islands and 'Brigade' (control). The two crops of 'Brigade' (well irrigated and unirrigated) are placed in the same cluster (distance = 0.0007), suggesting that these secondary metabolites in tomato fruits may be more genetics-dependent than environment-dependent (i.e., soil−water availability).

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