PAPER

ANTHOCYANIN PROFILE AND ANTIOXIDANT ACTIVITY OF FRESHLY SQUEEZED POMEGRANATE (*PUNICA GRANATUM* L.) JUICES OF SICILIAN AND SPANISH PROVENANCES

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ABSTRACT

Pomegranate (*Punica granatum* L., *Punicaceae*) fruit is traditionally consumed in several countries, especially in Middle East, and has gained increasing popularity all over the world due to its assumed health benefits. Juices derived from the arils of the seeds were shown to be rich in anthocyanin glucosides, typically composed of cyanidin, delphinidin, and pelargonidin. The aim of the present study was the characterization of diverse Sicilian and Spanish pomegranate accessions regarding their anthocyanin and total polyphenol contents using the Folin-Ciocalteu reagent. The anthocyanin profiles were determined by HPLC-DAD and LC-MS, and color parameters were characterized using the CIELAB coordinates. Antioxidant activities were measured using a fluorimetric assay, and expressed as ORAC values. The anthocyanin and polyphenol contents were correlated with their antioxidant activities. Results obtained were correlated and evaluated for the identification of the most suitable accessions to be selected for cultivation, juice processing, and further breeding.

Keywords: pomegranate, juice, ORAC, anthocyanins, antioxidant, polyphenols, cultivars

1. INTRODUCTION

Pomegranate is a wide-spread fruit crop in the entire Mediterranean area. The fruit is highly appreciated due to the sweet taste of its juice made from the edible part of the fruit, the arils. The species native to Iran and the Himalayas in northern India has a deeply rooted history dating back to ancient times. As reported by MELGAREJO *et al.* (2012), the world production is probably exceeding 3,000,000 mt, and over 80% thereof is originating from Iran, India and China (HOLLAND and BAR YAAKOV, 2008). The growing interest in pomegranate cultivation stems from a number of advantages ranging from agronomic performance in marginal environments to the broad variety of products obtained therefrom, and the health properties of the juice traditionally used for the treatment of diabetes, osteoporosis, cataracts, cardiovascular and neoplastic diseases (ASGARY *et al.*, 2014; KUMAGAI *et al.*, 2015; MALIK and MUKHTAR, 2006; VIUDA-MARTOS *et al.*, 2010). Many research dealt with the anthocyanins profile and the antioxidant activity of pomegranate juices originating from Iran, Israel, Morocco, Spain, Turkey, USA including their benefits for human health (LEGUA *et al.*, 2012; MOUSAVI DERAZMAHALLEH *et al.* 2013; QU *et al.*, 2011; RINALDI *et al.*, 2013; SCHWARTZ *et al.*, 2009); however, little is known about the different characteristics of the varieties despite recent findings indicating a broad genetic and geographic diversity (CRISTOFORI *et al.*, 2011; FERRARA *et al.*, 2011; FISCHER *et al.*, 2011a). Sicilian pomegranate germplasm has been characterized so far (BARONE *et al.*, 2001; DOMINA *et al.*, 2007), and a new promising accession named "Primosole" has been described (LA MALFA *et al.*, 2009). Different authors also considered the germplasm of other Italian regions. Recently, CALANI *et al.* (2013) evaluated the phenolic profiles of some ancient Italian accession.
In this paper, the phenolic contents of 12 Sicilian accessions should be studied comparing them with four Spanish varieties for the first time. In order to shed light on the diversity of anthocyanin profiles and antioxidant activities and other basic quality traits such as total soluble solids, total acidity, pH, and ascorbic acid contents of 16 accessions originating from Sicily (Italy) and Spain should be determined. This study is aimed to identify high-yielding varieties with potential health benefits suitable for growing in marginal areas.

2. MATERIALS AND METHODS

2.1. Sample preparation

Sixteen accessions, 12 belonging to the Sicilian germplasm, and four originating from Spain, (Table 1) were grown applying standard horticultural practices in the experimental farm of the Catania University (Italy) located near the eastern coast of Sicily (lat. 37°24'37'' N; long. 15°03'16'' E); 20 kg of fruits from four different trees of each accession were harvested at marketing ripeness stage (i.e. October 10 for Violetto and Valenciana, November 10 for PG-CT5, PG-CT6 and Valenti, October 25 for the others) transported to the laboratory, and stored at 4°C for 24 h. Fruits were sanitized with chlorinated tap water; seeds (arils) were isolated using a commercial pomegranate aril separator (Pomeke, Netanya, Israel), and juice was freshly squeezed with a juice extractor (Termozeta, model 405003, Milan, Italy). Juices obtained were analyzed for their pH, total acidities (TA), soluble solids (TSS), maturity index (MI), total polyphenol contents, anthocyanin contents, ellagic acid, tannin contents, and antioxidant activities. All analyses were performed in triplicate, and the data collected were treated statistically.

Table 1: Composition of juices made from the arils of 16 pomegranate accessions.

Accession	pH	TA (g/100 ml of citric acid)	TSS (°brix)	M Index
Dente di cavallo acc.1	4.00±0.02^{abc}	0.18±0.01^{c}	15.49±0.89^{e}	86.04±1.95^{f}
Dente di cavallo acc.2	3.88±0.01^{de}	0.25±0.01^{a}	14.88±0.39^{g}	59.55±0.94^{h}
Noto	4.04±0.03^{a}	0.15±0.00^{d}	14.88±0.68^{g}	99.22±2.45^{e}
PG-CT1	4.03±0.04^{ab}	0.25±0.02^{a}	16.09±0.72^{c}	64.36±1.12^{h}
PG-CT5	3.90±0.03^{de}	0.14±0.00^{de}	17.59±0.95^{a}	125.62±3.11^{a}
PG-CT6	3.85±0.01^{e}	0.14±0.00^{de}	15.49±0.69^{e}	110.64±3.43^{bc}
PG-SR1	3.96±0.02^{abcd}	0.20±0.01^{b}	14.58±0.56^{h}	70.92±2.25^{g}
PG-SR3	3.95±0.01^{bcd}	0.14±0.00^{de}	14.58±0.56^{h}	104.16±3.21^{de}
Primosole	3.67±0.02^{g}	0.15±0.01^{d}	15.78±0.74^{d}	105.27±3.74^{cde}
Rosolini	3.89±0.03^{de}	0.14±0.01^{de}	15.49±0.81^{e}	110.64±2.96^{bc}
Valenti	3.94±0.02^{cd}	0.14±0.01^{de}	15.49±0.79^{e}	110.64±2.58^{bc}
Violetto	3.96±0.01^{bcd}	0.18±0.01^{c}	13.36±0.52^{i}	74.24±1.96^{g}
Mollar de Elche	3.84±0.02^{ef}	0.14±0.01^{de}	17.59±0.89^{a}	125.64±4.79^{a}
Piñon tierno	3.53±0.03^{h}	0.13±0.00^{e}	15.18±0.67^{f}	116.80±3.41^{b}
Piñonenca	3.75±0.01^{fg}	0.21±0.01^{b}	16.69±0.93^{b}	80.82±1.85^{f}
Valenciana	3.71±0.02^{g}	0.14±0.01^{de}	14.88±0.74^{g}	106.29±3.63^{cd}

Values in the same column not followed by the same letter are significantly different ($p< 0.01$). pH value, TA total acidity expressed as citric acid, TSS total soluble solids, Mindex maturity index TSS/TA.

2.2. Chemicals

All reagents and solvents were of analytical or HPLC grade. Trolox [(+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid], ABTS [2,20-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] and Folin-Ciocalteu reagent were supplied by Sigma (St. Louis, MO, USA). Cyanidin 3-glucoside (CY3), delphinidin 3-glucoside (DP3) pelargonidin 3-glucoside (PL3), cyanidin 3-rutinoside (CY3R), gallic acid and ellagic acid were from Extrasynthèse (Genay, France). Delphinidin-3-rutinoside was from Apin Chemicals (Oxfordshire, UK). All other reagents and solvents were from Carlo Erba (Milan, Italy).

2.3. Determination of ascorbic acid

The ascorbic acid concentration was determined according to RAPISARDA and INTELISANO (1996) using an HPLC (Shimadzu, Japan) equipped with two pumps (LC-10A), a control system (SCL-10A), an injector (Rheodyne with 20 uL loop), a photodiode detector (SPD-M10A), a C18 Alltima ODS Hypersil column 250 mm x 4.6 mm I.D. (Milan, Italy), and a similarly packed pre-column. The elution was performed using a buffer consisting of KH_2PO_4/H_3PO_4 at pH 2.3, at a flow rate of 1 mL/min, and detection wavelength was set at 260 nm.

2.4. Determination of anthocyanins and ellagic acid

Total anthocyanins were determined spectrophotometrically as described by RAPISARDA *et al.* (2000). An aliquot of juice (2 mL) was diluted up to 25 mL with a pH 1 solution (125 mL of 0.2 M KCl and 375 mL of 0.2 M HCl). A second aliquot (2 mL) was diluted to 25 mL with a pH 4.5 buffered solution (400 mL of 1 M sodium acetate, 240 mL of 1 M HCl, and 360 mL of H_2O). Absorbance of the solutions was measured at 510 nm. Anthocyanin concentrations were calculated by Equation (1):

$$C_{mg/L} = \left(Abs_{pH1} - Abs_{pH4.5}\right) * 484.82 * 1000/24825 * DF \qquad (1)$$

where the term in parentheses is the difference of absorbance at 510 nm measured at pH 1 and pH 4.5, 484.82 is the molecular mass of cyanidin-3-glucoside chloride, 24825 is its molar absorptivity (ε) at 510 nm in the pH 1 solution, and DF is the dilution factor. Anthocyanins and ellagic acid were also quantitated by HPLC-DAD, and assigned by HPLC-MS according to Fischer *et al.* (2011b). Anthocyanin analyses were conducted using a Merck Hitachi La-Chrom Elite HPLC system (Merck, Darmstadt, Germany) equipped with an L-2200 auto sampler, an L-2130 pump, a jetstream column oven, and a L-2450 diode array detector. The separation was carried out on an analytical Phenomenex (Torrance, CA, USA) C 18 Synergi 4 μm Hydro-RP 80 Å pore size (150 x 3.0 mm) column fitted with a Phenomenex security guard column (4 x 3.0 mm) operated at 30°C. The acquisition range of the diode array detector was set at 200-600 nm. The mobile phase consisted of 5% (v/v) formic acid in water (eluent A), and of water, formic acid and methanol (10/10/80, v/v/v; eluent B). The rate flow was 0.4 mL/min, and the gradient program was optimized as follows: 10-14% B (5 min), 14-23% B (11 min), 23-35% B (5 min), 35-40% B (14 min), 40-100% B (3 min), 100% B isocratic (3 min), 100-10% B (3 min), 10% B isocratic (4 min). Total run time was 48 min, the injection volume of all samples was 10 μL, and monitoring was performed at 520 nm and 280 nm for ellagic acid.

In order to remove undesirable sugars, acids, amino acids, and proteins that could interfere with anthocyanin separation, solid-phase extraction (SPE) was conducted prior to instrumental analysis employing C18 ODS SPE cartridges (Sep-Pak Waters Milford, MA, USA). Aliquots of anthocyanin extracts (10 mL) were passed through SPE preconditioned cartridges with methanol (5 mL) and water (10 mL). Cartridges were washed with water (15 mL), and anthocyanins eluted drop wise with methanol containing formic acid 0.4% (10 mL). The methanolic extract was concentrated under reduced pressure using a Büchi rotavapor (Flavil, Switzerland) at 30°C, and the residue diluted with 5 mL of deionized water containing 7% (v/v) of formic acid. LC-MS analyses were carried out using an Agilent HPLC 1100 system (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1379A degasser, a model G1312A binary gradient pump, a model G1313A thermo-auto sampler, a model G1316A column oven, and a model G1315A diode array detection system. The HPLC system was connected in series with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an ESI source. The LC-MS program was the same used for LC-DAD. Data acquisition and processing were done using Esquire Control software. Positive ion mass spectra of the column eluate were in the range of m/z 50-1500 at a scan speed of 13000 m/z/s. Nitrogen was used both as drying gas at flow rates of 10.0 L/min and nebulizing gas at pressures of 50.0 psi. The nebulizer temperature was 365°C. Helium was used as collision gas at a pressure of 4 x 10^{-6} mbar.

2.5. Determination of total polyphenols and total tannins

The total polyphenol (TP) and tannin (TT) contents of the samples were estimated by a colorimetric assay based on the procedures described by Glasl (1983) with slight modifications. For the determination of total tannins, a sample size of 10 g was used. Briefly, the samples were dissolved in 250 mL water to obtain the mother liquor (ML). A 5 mL aliquot of ML was diluted with water to 25 mL, and 2 mL of this solution were transferred into a 25 mL vial containing 1 mL of Folin-Ciocalteau reagent and 10 mL of bi-distilled water, and subsequently made up to volume of 25 mL with a 10.0% sodium carbonate solution. After 15 minutes, the absorbance was read at 730 nm. Water was used as the blank. To determine the non-adsorbed polyphenols (NAP), 10 mL of ML was mixed with 100 mg of hide powder (Merck) and shaken for 90 min. A 2 mL aliquot of this solution was assayed for polyphenolics as above. The absorbance wavelength (730 nm) was previously selected by spectrophotometric scanning of samples of extract and gallic acid. The percentage of total phenolics and tannins were determined as follows (Equation 2):

$$TP\% = \frac{15625 * Abs}{1000 * m} \quad NAP\% = \frac{15625 * Abs}{1000 * m} \quad TT\% = TP - NAP \qquad (2)$$

where TP are the total polyphenolics (%); NAP the non-adsorbed phenolics (%); TT the total tannins (%); Abs the absorbance; and m the mass (g).

2.6. Determination of antioxidant activity

In addition to the Folin-Ciocalteu assay, which does not only measure the total content of phenolics but also that of the total reducing capacity of the samples, the ORAC assay was performed as described by CAO *et al.* (1993) with some modifications. The measurements were carried out on a Wallac 1420 Victor III 96 well plate reader (EG and Wallac, Turku, Finland) with a fluorescence filter (excitation 485 nm, emission 535 nm). Fluorescein (116 nM) was the target molecule for free radical attack by AAPH (153 mM) used as the peroxyl radical generator. The reaction was performed at 37°C, pH 7.0, and Trolox (1 μM) was taken as the control standard, while phosphate buffer was used as blank. All solutions were freshly prepared prior to analysis. All samples were diluted with phosphate buffer (1:25-100, v/v) prior to analysis, and results were expressed as micromoles (μMol) of Trolox equivalents per 100 mL of juice.

2.7. Color analysis

The CIELAB coordinates of pomegranate juice were determined without previous sample preparation by reading the L^* a^* b^*-values on a Cary 1E spectrophotometer (Varian, Mulgrave-Victoria, Australia), and estimating the color intensity by Cary Color Calculation software. Color index (C*index) was calculated according to equation (3):

$$(180\text{-Hue})/(L^*+\text{Chroma}) \qquad (3)$$

2.8. Statistical analysis

Experimental data were processed by statistical analyses using the Statistica software (StatSoft, Tulsa, OK, USA). The descriptive analysis was followed by an analysis of variance method applying the Tukey's HSD test. Principal component analysis (PCA) and cluster analysis (CA) were also performed.

3. RESULTS AND DISCUSSIONS

In this study, juices were obtained from the arils of four Spanish and 12 Italian pomegranate accessions whose pomological characteristics were previously described (LA MALFA *et al.*, 2009) illustrated by Fig 1. Their main chemical characteristics are compiled in Table 1. The lowest pH value was determined for "Piñon tierno" (pH 3.5), while "Noto" and "PG-CT1" were characterized by higher pH values (pH 4.0). "Dente di cavallo acc. 2" had the highest acidity (TA = 0.25 g/100mL), whereas the remaining cultivars (Piñon tierno", 0.14 "PG-SR3", "Rosolini", "PG-CT5", "Valenti") had considerably lower total acidities around 0.13-0.14. To provide information about the sensory quality of the fruits, the maturity index (MI) being the ratio of TSS and TA measured in the juices was also determined. The highest maturity index (MI) was found for "Mollar de Elche" (125.14), and the lowest in "Dente di cavallo acc.2" (60.73). All accessions were classified as sweet according to the classification of Martínez *et al.* (2006). The contents of the main bioactives are listed in Table 2. In accordance with Chace *et al.* (1981), all accessions are suitable both for fresh consumption and juice production.

Figure 1: Pomegranate accessions investigated: from top to bottom and from left to right: Valenciana, Mollar de Elche, Primosole, Piñonenca, Piñon tierno, Valenti, Violetto, Rosolini, Noto, Dente di cavallo acc. 1, PG-SR3, PG-SR1, PG-CT1, PG-CT5, Dente di Cavallo acc. 2, PG-CT6.

Ascorbic acid content; however, without considering the dehydroascorbic acid contents, ranged between 80.2 and 417.4 mg/L with statistical significance between all accessions. In particular, maximum values were found for the "Violetto", "Rosolini", "PG-CT1" and "PG-SR1" accessions that are well separated among others accessions. We detected very high values of ascorbic acid much higher than the values reported in the literature (FERRARA *et al.*, 2011; DROGOUDI *et al.*, 2005; TEHRANIFAR *et al.*, 2010).

Table 2: Contents of bioactive compounds and antioxidant potential of 16 pomegranate accessions.

	Ascorbic Acid (mg/L)	Anthocyanins (mg/L)	Tannins (mg/L)	Ellagic Acids (mg/L)	Total polyphenols (mg/L)	ORAC-value (μmol TE/100mL)
Dente di cavallo acc. 1	179.8±9.3^{de}	76.9±2.2^{ef}	3.3±0.2^{fgh}	37.3±1.7^{f}	948.6±42.4^{gh}	7272±369^{gh}
Dente di cavallo acc. 2	100.6±5.7^{hi}	154.1±6.1^{b}	4.2±0.1^{cde}	47.5±2.7^{d}	1186.9±58.3^{efg}	7269±362^{gh}
Noto	80.2±4.4^{i}	56.6±1.5^{g}	5.2±0.24^{b}	34.8±1.7^{f}	1338.8±65.2^{e}	7050±353^{h}
PG-CT1	358.8±14.5^{b}	104.3±9.8^{cd}	7.4±0.3^{a}	83.2±3.6^{b}	2118.7±101.8^{cd}	10540±536^{bc}
PG-CT5	124.5±6.9^{fghi}	160.2±8.0^{b}	4.4±0.2^{bcde}	33.4±1.9^{f}	2497.0±122.8^{b}	8408±410^{defg}
PG-CT6	106.6±5.1^{ghi}	156.3±8.5^{b}	4.8±0.1^{bc}	70.5±3.0^{c}	1100.9±55.0^{efgh}	8536±427^{def}
PG-SR1	417.4±20.2^{a}	97.5±5.2^{cd}	2.7±0.2^{h}	49.0±2.6^{d}	857.0±42.8^{h}	8317±280^{defg}
PG-SR3	139.4±7.7^{fg}	65.0±3.3^{fg}	3.6±0.2^{efg}	26.0±1.2^{g}	2252.8±112.6^{bc}	7721±368^{fgh}
Primosole	175.9±9.2^{de}	196.6±10.3^{a}	3.9±0.2^{defg}	41.7±2.0^{e}	3055.1±151.1^{a}	11835±585^{a}
Rosolini	252.0±11.5^{c}	168.1±4.1^{ab}	4.1±0.2^{cdef}	24.7±1.0^{g}	1011.2±60.2^{fgh}	8285±415^{defg}
Valenti	164.7±8.2^{ef}	92.9±7.4^{de}	4.7±0.2^{bcd}	19.0±0.9^{h}	1205.6±97.0^{efg}	8548±417^{def}
Violetto	209.0±10.2^{d}	53.0±2.0^{g}	7.1±0.1^{a}	41.8±2.1^{e}	1899.5±96.0^{d}	9400±469^{cd}
Mollar de Elche	101.5±5.3^{hi}	102.8±3.2^{cd}	4.6±0.2^{bcd}	36.5±1.4^{f}	1281.3±66.6^{ef}	9064±458de
Piñon tierno	115.9±6.5^{gh}	64.5±1.1^{fg}	2.7±0.1^{h}	16.1±0.4^{h}	1982.0±96.1^{cd}	11411±568^{ab}
Piñonenca	184.8±10.6^{de}	114.6± 4.9^{c}	3.7±0.2^{fgh}	104.3±4.6^{a}	3097.7±154.4^{a}	5593±269^{i}
Valenciana	118.2±6.0^{gh}	97.3±2.8^{cd}	3.2±0.1^{gh}	34.3±1.2^{f}	2398.1±119.3^{b}	8010±406^{efgh}

Values in the same column not followed by the same letter are significantly different ($p< 0.01$).

Total anthocyanins content as determined by spectrophotometry (Table 2) ranged between 53.0 and 196.6 mg/L with a mean value of 110.0, and a standard deviation of 44.3, thus revealing a high variability among the accessions; in particular "Rosolini" and "Primosole" showed the highest anthocyanins contents. This was in according with previous reports providing evidence of high variability in other accessions investigated (SCHWARTZ *et al.*, 2009; DAFNY-YALIN *et al.* 2010). Analysis of the anthocyanin profiles (**Table 3**) revealed statistically significant differences between the accessions. In particular, cyanidin 3-O-glucoside (CY3) was the predominant anthocyanin followed by cyanidin 3,5-O-diglucoside (CY3.5), and delphinidin 3,5-O-diglucoside (DP3.5). In six out of 16 accessions, among them two of the Spanish accessions, anthocyanins diglucoside (DI) contents exceeded those of the monoglucosides (MO), and seven out of total accessions were richer in monoglucosidic (MO) than diglucosidic anthocyanins (DI). In particular, the cumulated relative amounts of DI accounted for 23.7% and 78.3% of total anthocyanins,

with "Violetto" and "Piñonenca" showing the lowest and highest proportions, respectively (Table 3).

Table 3: Individual and total anthocyanin contents of 16 pomegranate accessions including the relative amount of mono (MO) - and diglycosidic (DI) anthocyanins of total anthocyanin contents.

Accession	Anthocyanins (mg/L)									
	CY3	PL3	CY3R	DP3.5	CY3.5	PL3.5	DP3	Total	% MO	% DI
Dente di cavallo acc. 1	19.2	4.0	0.4	4.9	17.8	2.3	2.1	50.7	50.5	49.5
Dente di cavallo acc. 2	40.5	4.8	0.8	23.5	41.3	2.6	14.2	127.7	47.2	52.8
Noto	15.8	2.0	0.4	3.8	5.8	0.8	1.8	30.4	65.9	34.1
PG-CT1	78.7	5.8	1.7	47.3	46.7	1.9	44.7	226.8	57.7	42.3
PG-CT5	44.9	5.3	0.8	43.8	55.3	3.4	23.1	176.6	41.9	58.1
PG-CT6	58.7	6.2	1.1	38.5	48.1	2.8	33.1	188.5	52.6	47.4
PG-SR1	53.6	6.1	0.8	18.0	41.1	2.5	12.7	134.8	54.3	45.7
PG-SR3	24.5	4.2	0.4	4.0	28.6	2.7	1.7	66.1	46.6	53.4
Primosole	52.7	5.6	0.9	59.1	57.4	3.2	33.6	212.5	43.7	56.3
Rosolini	49.5	8.1	0.7	4.1	32.4	2.7	3.6	101.1	61.2	38.8
Valenti	67.1	8.5	1.0	21.8	47.9	3.1	16.0	165.4	56.0	44.0
Violetto	21.1	2.2	0.5	0.0	9.0	0.7	7.4	40.9	76.3	23.7
Mollar de Elche	38.4	9.8	0.5	3.9	48.8	5.9	3.0	110.3	46.9	53.1
Piñon tierno	24.3	3.6	0.5	8.7	23.6	2.1	4.8	67.6	49.2	50.8
Piñonenca	12.6	4.6	0.2	17.2	52.1	7.8	4.0	98.5	21.7	78.3
Valenciana	19.7	4.5	0.4	5.7	19.1	2.7	2.7	54.8	49.7	50.3

CY3 cyanidin 3-O-glucoside; PL3 pelargonidin 3-O-glucoside; CY3R cyanidin 3-O-rutinoside; DP3.5 delphinidin 3,5 diglucoside; PL3.5 pelargonidin 3,5 diglucoside; DP3 delphinidin 3-O-glucoside; %MO per cent of monoglucoside anthocyanins; %DI per cent of diglucoside anthocyanins.

To the best of our knowledge, this approach has so far solely been applied by FISCHER *et al.* (2011a), and we found the same results for accessions of identical provenance, being in agreement with the data reported by FISCHER *et al.* (2011a), the Spanish accession showed the same proportion of anthocyanin diglucoside as "Pinõnenca" accounting for 77% of the total amount, and the glycosidic relations of the Italian cultivars IT-1 and IT-2 were quite similar to the ratios found for "Dente di cavallo acc1" and "Rosolini", both exhibiting 35% of diglucosidic anthocyanins. LEGUA *et al.* (2012) characterized the profiles of six Spanish cultivars, while we found a relative amount of anthocyanin diglucosides ranging between 36 and 51%. However, the latter group did not determine total phenolic contents, thus precluding the comparison of anthocyanin and total phenolic ratios. ZHAO *et al.* (2013) studied the anthocyanin profiles of three Chinese cultivars, reporting the predominance of monoglucosidic anthocyanins accounting for 92% of their total amount. BOROCHOV-NEORI *et al.* (2011) characterized four pomegranate accession for anthocyanins

composition showing a different anthocyanins glucosylation dependent on the harvest date and climate. GOMEZ-CARAVACA *et al.* (2013) found same results on 17 cultivars from different provenances. We evaluated the proportion of total anthocyanins to total phenolics, finding consistent differences to earlier published data. In particular, in our samples, anthocyanin share in total polyphenols ranged between 2.9 and 16.6%, being in agreement with the results published by GIL *et al.* (2000) for commercial juices and juices made from fresh arils, whereas according to FISCHER *et al.*, (2011a) the ratio of total anthocyanins to total phenolics was even higher (17-93%) in juices produced from the aril. In an investigation of 20 Iranian cultivars, TEHRANIFAR *et al.* (2010) found total anthocyanin ratios to total phenolics ranging between 0.88 and 3.29. Such differing traits may be used as a discriminant factor of diverse accessions. Fig. 2 shows the anthocyanin patters of juices made from the arils of "Primosole" pomegranate cultivar analyzed by HPLC–DAD. Identification and anthocyanins peak assignment were based on the comparison of their UV–Vis spectra and retention times (RT) with those of standards and references (FISCHER *et al.*, 2011b; WU and PRIOR, 2005), eight peaks were found, of which six were identified according to FISCHER *et al.* (2011b). In addition, we were able to tentatively identify a cyanidin-pentoside (CYPENT) and a cyanidin-rutinoside (CY3RUT) for the first time in Sicilian accessions (MS data not show). In particular, all accessions contained cyanidin pentoside (CYpent), while small amounts of cyanidin rutinoside were found in "Primosole", "PG-CT1", "PG-CT5", "PG-CT6", and "Mollar de Elche", respectively.

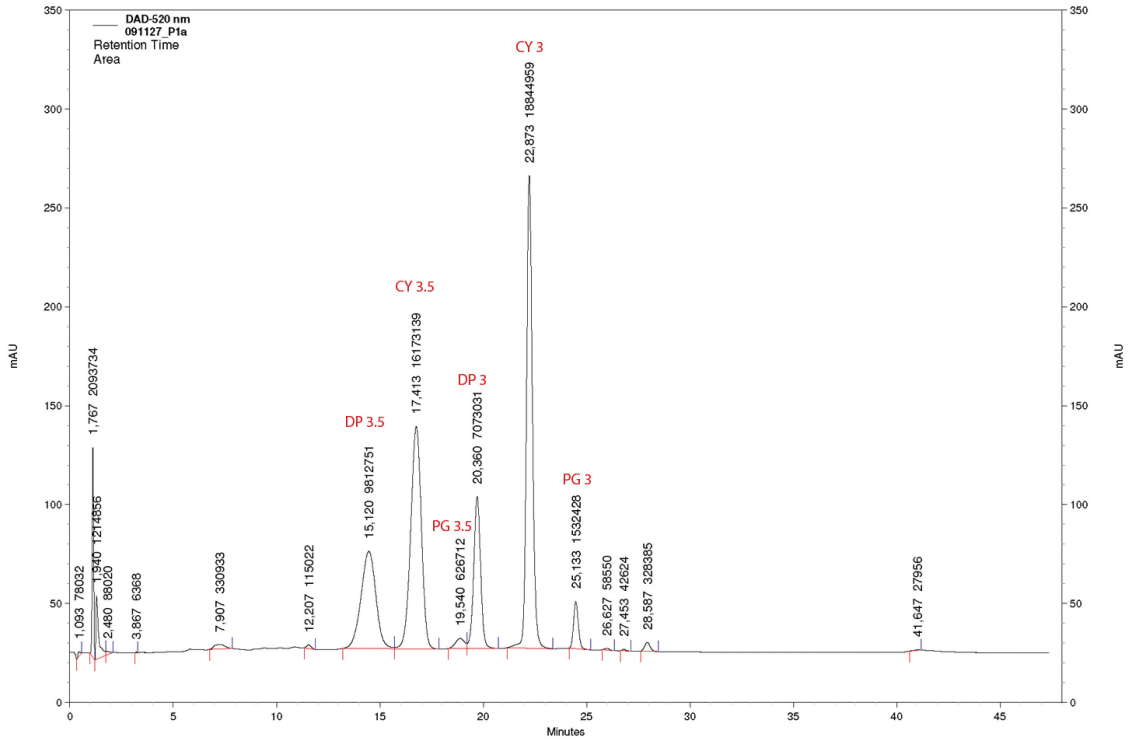

Figure 2: HPLC anthocyanin profile of a pomegranate juice of the "Primosole" accession.

Colorless phenolic compounds appear to be mostly responsible for the health-promoting properties of pomegranate fruits and juices obtained therefrom. Total polyphenol contents ranged between 948.6 and 3097.6 mg/L being in agreement with the literature data (GIL *et al.*, 2000; EL CAR *et al.*, 2011; OZGEN *et al.*, 2008). While "Primosole" accession showed the highest value, each of the accessions exhibited a high level of antioxidant activity ranging from 5,593 to 11,835 ORAC value compared to strawberry, plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear, and honeydew melon (WANG *et al.*, 1996; KALT *et al.*, 1999; WU *et al.*, 2004). Among the Sicilian accessions, "Primosole" had a slightly higher antioxidant activity (11,835 ORAC units) than "Piñon tierno" (11,411 ORAC units) exhibiting the maximum value among the Spanish accessions.
Hydrolyzable tannins comprising gallotannins and ellagitannins together with gallagyl-esters, were the predominant phenolic compounds in pomegranate fruits and juices prepared from the entire fruits, accounting for an average of 99 to 100 % of total phenolics in the pericarps, 87 % in pomegranate juices from entire fruits, and only 33 % in pomegranate juices made from isolated arils [42]. In the present study, tannin contents were rather uniform within the different accessions, with "Violetto" showing the highest value. However, in contrast to FISCHER *et al.* (2011a; 2011b; 2013) the method applied did not allow the distinction of gallotannins, ellagitannins and gallagyl esters, respectively. "Valenti" and "Piñon tierno" were characterized by low ellagic acid contents in contrast to "PG-CT1" and "Piñonenca" displaying the maximum values. Variability of ellagic acid contents was found to be high, which was confirmed by statistical analysis allowing to divide into 8 separate groups of the 16 accessions considered. Based on these findings, selection of high-yielding cultivars is recommended.
Anthocyanin amounts varied markedly which went along with visually noticeable differences in the red color of the juices. Therefore, monitoring of total color variations using the *CIE-L*a*b** color space, was instrumental. Higher a^* values were characteristic of a more reddish overall impression of the juices, whereas b^* values reflected blue ($-b^*$) to yellow ($+b^*$) tonalities of the juices.
Color analysis revealed significant differences among the cultivars as can be seen from the differing values of color parameters (Table 4) (SCHWARTZ *et al.*, 2009; FISCHER *et al.*, 2011a; DAFNY-YALIN *et al.*, 2010; FISCHER *et al.*, 2013).
In particular, it has been possible to distinguish deeply pigmented pomegranate juices by their high anthocyanin content which well corresponded with their high a^* values. This may allow rapid analysis and identification of adulterated commercial pomegranate juice because the pattern of anthocyanins is strictly related to the a^* value in accordance with DAFNY-YALIN *et al.* (2010) who found a significant correspondence ($p<0.05$) between a^* values and anthocyanin contents. Calculating the ΔE values, and taking "Primosole" as a reference, i.e. the index for the differences perceived by the human eye, differences between "Primosole", "PG-CT1", "Valenti", "PG-CT5" and "Valenciana" were insignificant; however, we found significant differences between "Primosole" and "Noto", Dente di Cavallo acc.1", "PG-SR3", "Violetto" among Italian accessions and significant differences between "Primosole" and "Piñon tierno". We have calculates the ΔE values taking "Piñonenca" as a reference of the Spanish accessions, and we did not find perceivable differences by the human eye.
From Table 5, a significant correlation ($P<0.05$) between ORAC values, anthocyanin contents and total polyphenol contents is obvious. The same holds true for the anthocyanin contents and the C^* values.
Our findings are in accordance with previously published data (LEGUA *et al.* 2012; TZULKER *et al.*, 2007; CUI *et al.*, 2013), thus confirming the high nutritional relevance of pomegranate juice. PCA analysis allowed identifying different clusters within the accessions. The total variability was explained by the 24 principal components, 9 of which

representing 95.6% of quality. Using Kaiser's rule to identify the principal components, only the eigenvalues greater than 1 have been considered (Table 6).

Table 4: Color characteristics of 16 pomegranate accessions.

	*L**	*a**	*b**	*C**	h°	Color index	ΔE
Dente di cavallo acc.1	92.7±2.5^{abc}	4.8±0.4^{g}	4.0±0.2^{d}	6.2±0.3^{g}	40.2±0.5^{c}	1.4±0.0^{gh}	9.8±0.1
Dente di cavallo acc.2	89.0±2.0^{f}	5.0±0.2^{g}	4.1±0.2^{cd}	6.5±0.4^{fg}	38.9±0.5^{c}	1.5±0.0^{de}	8.4±0.1
Noto	93.1±2.6^{a}	4.3±0.4^{h}	3.2±0.1^{f}	5.3±0.3^{h}	36.8±0.4^{d}	1.5±0.0^{ef}	10.5±0.1
PG-CT1	89.2±2.2^{fg}	11.6±0.6^{b}	4.5±0.2^{b}	12.4±0.7^{b}	21.3±0.3^{hi}	1.6±0.0^{ab}	2.3±0.0
PG-CT5	85.8±2.1^{i}	12.9±0.5^{a}	5.5±0.4^{a}	14.0±0.8^{a}	23.0±0.4^{h}	1.6±0.0^{a}	1.8±0.0
PG-CT6	91.6±2.1^{de}	6.0±0.3^{e}	3.5±0.1^{ef}	7.0±0.5^{de}	30.1±0.4^{f}	1.5±0.0^{c}	8.2±0.1
PG-SR1	91.8±2.3^{cde}	6.4±0.4^{d}	4.8±0.4^{b}	8.0±0.6^{c}	36.7±0.5^{d}	1.4±0.0^{fg}	7.9±0.1
PG-SR3	93.4±2.3^{a}	3.1±0.3^{i}	3.4±0.1^{f}	4.6±0.2^{i}	47.7±0.6^{ab}	1.3±0.0^{i}	11.7±0.1
Primosole	87.5±2.3^{h}	13.0±0.6^{a}	4.7±0.2^{b}	13.9±0.6^{a}	20.0±0.3^{i}	1.6±0.0^{a}	*
Rosolini	92.0±2.1^{bcd}	5.6±0.4^{f}	3.8±0.2^{de}	6.8±0.4^{ef}	34.4±0.5^{e}	1.5±0.0^{de}	8.7±0.0
Valenti	87.4±2.1^{h}	11.2±0.6^{c}	5.4±0.3^{a}	12.4±0.6^{b}	26.0±0.4^{g}	1.5±0.0^{bc}	2.0±0.0
Violetto	92.8±2.3^{ab}	4.3±0.3^{h}	4.7±0.3^{b}	6.3±0.5^{g}	47.6±0.5^{ab}	1.3±0.0^{i}	10.2±0.1
Mollar de Elche	91.0±2.4^{e}	5.9±0.3^{e}	4.0±0.4^{d}	7.2±0.3^{d}	34.0±04^{e}	1.5±0.0^{d}	7.9±0.0
Piñon tierno	93.2±2.5^{a}	2.8±0.1^{i}	3.2±0.2^{f}	4.3±0.2^{i}	49.1±0.5^{a}	1.3±0.0^{i}	11.8±0.2
Piñonenca	88.7±2.1^{g}	4.2±0.2^{h}	4.5±0.4^{bc}	6.2±0.4^{g}	46.5±0.6^{b}	1.4±0.0^{h}	8.9±0.1
Valenciana	88.7±2.2^{g}	13.0±0.6^{a}	4.7±0.4^{b}	13.9±0.7^{a}	20.0±0.3^{i}	1.6±0.0^{ab}	1.2±0.0

Values in the same column not followed by the same letter are significantly different ($p< 0.01$).
*Primosole value is the reference for ΔE calculation. ΔE indicates the values that have a just noticeable difference.

Table 5: Correlation matrix of characteristics of different pomegranate juices.

	MI	ASC	PT	TAN	ELL	ORAC	L*	CI	ANT
MI		0.1908	0.0904	-0.2902	-0.5223	0.2081	-0.1434	0.2392	-0.0463
ASC	n.s.		0.0275	-0.1068	-0.0540	0.0280	-0.1249	0.2793	-0.1420
PT	n.s.	*		0.0218	0.2999	0.1921	-0.4858	0.1278	0.1753
TAN	*	n.s.	n.s.		0.2869	0.2010	-0.0624	0.1529	0.2482
ELL	***	n.s.	*	*		-0.2606	-0.2026	0.1090	0.3998
ORAC	*	n.s.	*	*	*		-0.0820	0.1980	0.3538
L*	n.s.	n.s.	***	n.s.	*	n.s.		-0.7755	-0.6710
CI	*	*	n.s.	n.s.	n.s.	*	***		0.7221
ANT	n.s.	n.s.	*	*	**	**	***	***	

*P < 0.05, **P < 0.01, ***P < 0.001, n.s.: not significant.
MI maturity index; ASC ascorbic acid content; PT total polyphenols; TAN tannins content; ELL ellagic acid; ORAC ORAC value; L* lightness parameter; CI color index; ANT anthocyanins content.

Table 6: Eigenvalues and proportion of total variability among pomegranate accessions as explained by the first 24 principal components (PC).

PC	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative%
1	7.384571	30.76905	7.38457	30.7690
2	4.426785	18.44494	11.81136	49.2140
3	3.448635	14.36931	15.25999	63.5833
4	2.490409	10.37670	17.75040	73.9600
5	1.770037	7.37515	19.52044	81.3351
6	1.303753	5.43230	20.82419	86.7675
7	0.949781	3.95742	21.77397	90.7249
8	0.710033	2.95847	22.48400	93.6833
9	0.448733	1.86972	22.93274	95.5531
10	0.356859	1.48691	23.28959	97.0400
11	0.230059	0.95858	23.51965	97.9986
12	0.216912	0.90380	23.73656	98.9024
13	0.129161	0.53817	23.86573	99.4405
14	0.096041	0.40017	23.96177	99.8407
15	0.019683	0.08201	23.98145	99.9227
16	0.010061	0.04192	23.99151	99.9646
17	0.004536	0.01890	23.99605	99.9835
18	0.002681	0.01117	23.99873	99.9947
19	0.000949	0.00395	23.99968	99.9987
20	0.000139	0.00058	23.99982	99.9992
21	0.000095	0.00040	23.99991	99.9996
22	0.000062	0.00026	23.99998	99.9999
23	0.000020	0.00008	24.00000	100.0000
24	0.000005	0.00002	24.00000	100.0000

The projections of the observations on the first two principal component axes are shown in Fig. 3. The accessions are distributed on the factor plane. These two coordinates represent 49.2 % of the total variance. The major contribute on factor 1 is due to anthocyanin contents, whereas b* values contribute to the factor 2. "Violetto" has a positive contribution to factor 1, being well distributed along the factor 2 direction. On the other hand, both the "Valenciana" and "PG-CT5" have negative contributions on factor 1 and factor 2. "PG-CT1" positively contributes to factor 1, but opposite sign contributions on factor 2. Cultivars "Piñonenca" as well as "Dente di cavallo acc.2" and PG-SR1" were neither discriminated by the principal factor 1 x factor 2 not by any of the others. PCA is a good starting point to understand the component issues relevant for the discrimination of the different pomegranate accession samples. In fact, we found significant differences among the accessions, and this information may be useful for the authenticity control of pomegranate juices from different origins.

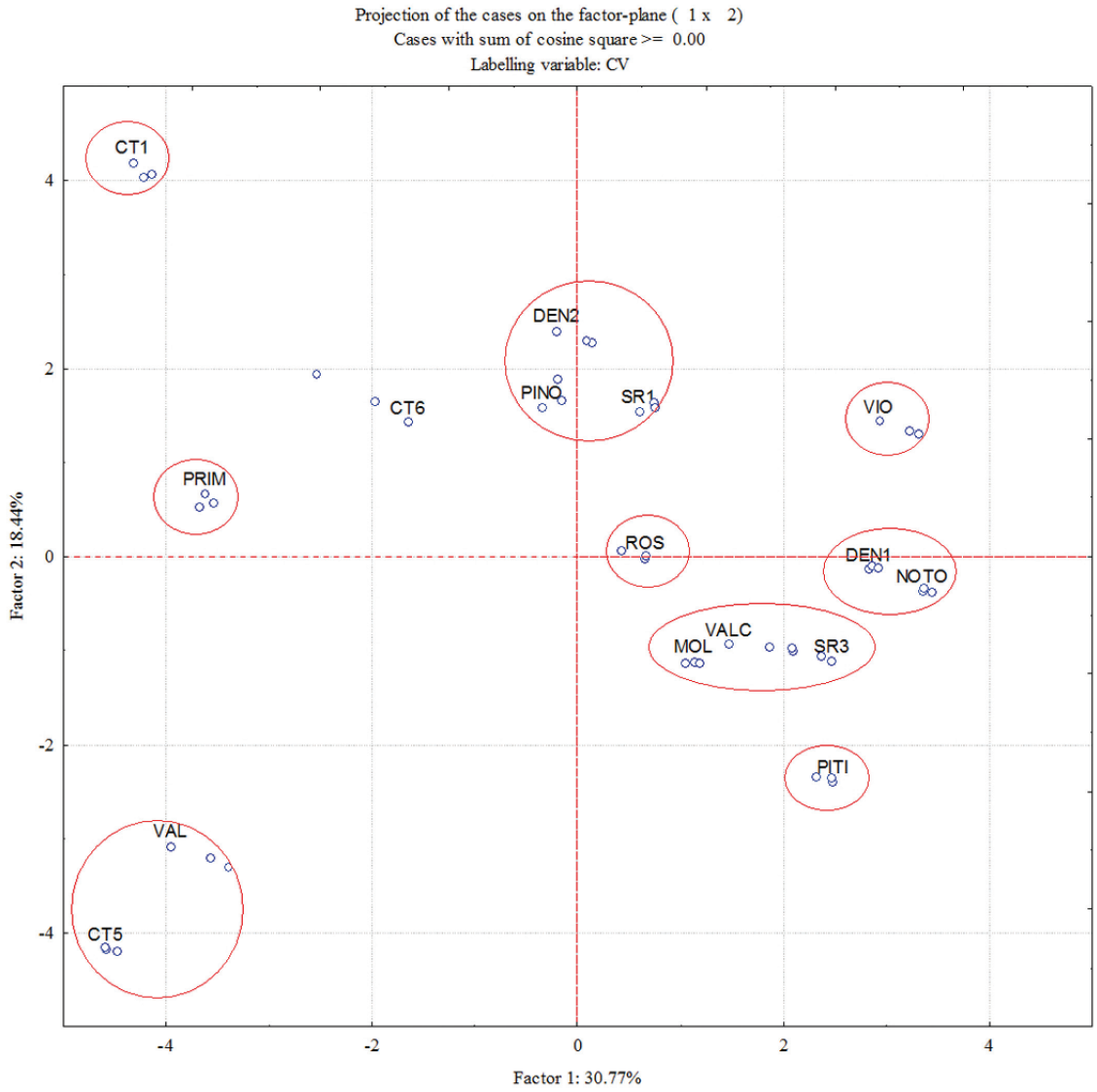

Figure 3: Projections of sample replicates of the five groups on the factor 1 vs. factor 2 plane.

Fig. 4 shows the hierarchical cluster analysis with the linkage method. We found seven main groups with the first main group made of "Dente di Cavallo acc.1" "Dente di cavallo acc. 2" and "Noto", the second one was made of "PG-CT5" "Valenti" "PG-SR1" and "Rosolini". High dissimilarity levels were found for accessions "Primosole", "Mollar del Elche", and "Violetto", being highly heterogeneous among the studied accessions.
Among all cultivars investigated, "Primosole" has interesting properties making this cultivar suitable for juice production and further breeding. In further investigations, anthocyanin pattern and contents in pomegranate of different provenances should be investigated in more detail.

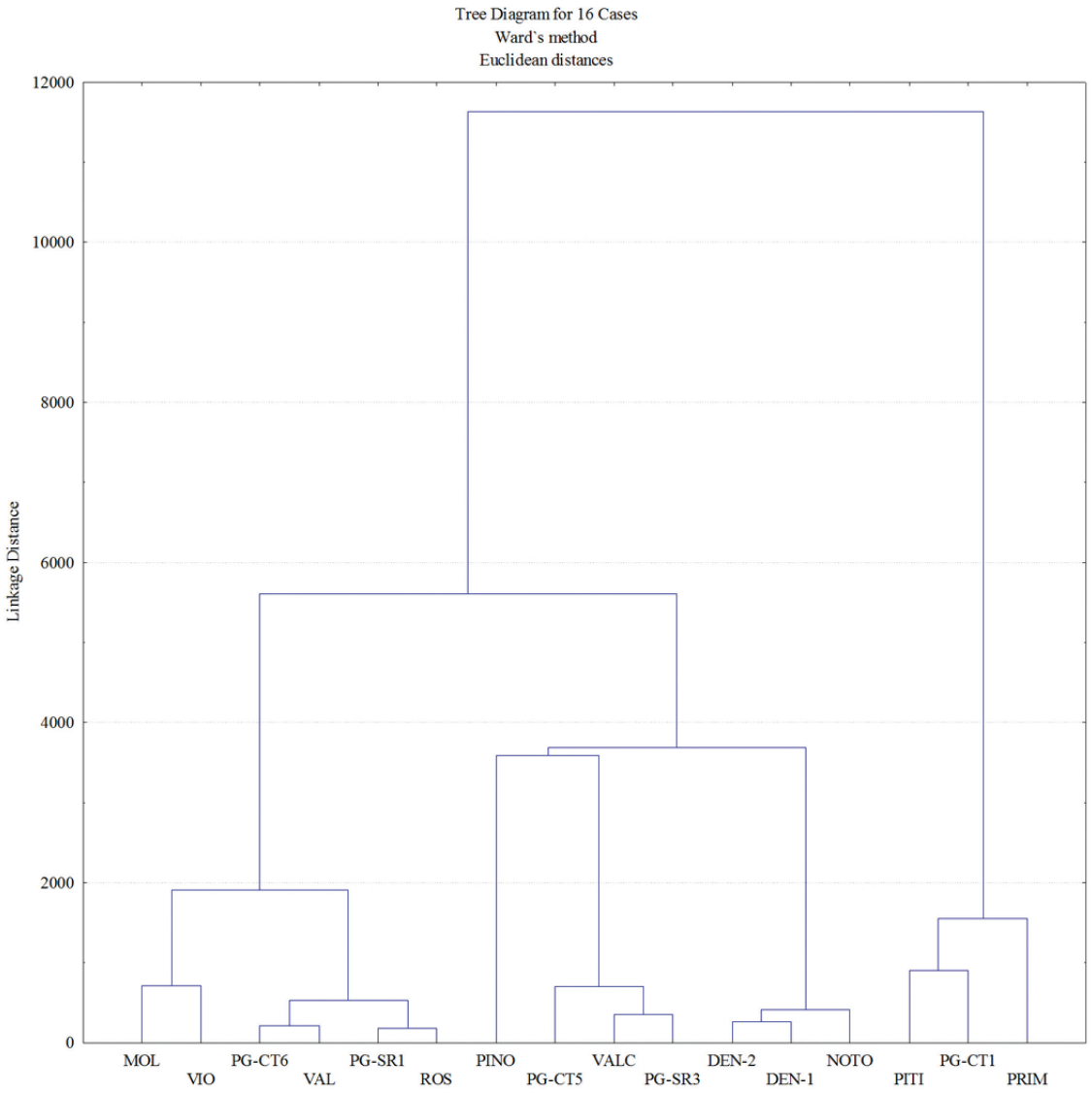

Figure 4: Cluster dendrogram of the pomegranate accessions under investigation.

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