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Phenolic profile and antioxidant activity of different grape (*Vitis vinifera* L.) varieties

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Abstract. In the last years, the interest in non-alcoholic grape products has considerably increased. Table grapes are largely produced in the Mediterranean area and their consumption has raised worldwide. Beside the positive pattern of nutrients, table grapes could provide benefits on human health. Among the health-promoting substances contained in table grapes, flavonoids (mainly anthocyanins, flavan-3-ols and flavonols) seem to be the most interesting. The aim of this study was the characterization of the phenolic pattern of sixteen grape varieties, and the evaluation of the associated antioxidant activity. The methods were: 1) Folin-Cocalteau's assay for the quantification of total polyphenol content; 2) DPPH (1,1,-diphenil-2—picrylhydrazyl) assay and 3) ORAC (Oxygen Radical Absorbance Capacity) spectrophotometric assays for the assessment of radical scavenging activity; 4) High Performance Liquid Chromatography (HPLC) method, coupled with electrospray ionization mass spectrometer (ESI-MS) and DAD detector was developed in order to obtain the phenolic pattern of grape samples. Data obtained in this study underline that some table grape varieties can show interesting phenolic pattern independently from the presence or not of seeds. This observation suggests that selected varieties of seedless grapes could represent an interesting source of healthy compounds, satisfying consumers' preferences and reducing concerns versus alcoholic beverages.

1. Introduction

Grape (*Vitis vinifera* L.) is one of the world's largest fruit crop with apple, watermelon and banana [1]. In 2014, the worldwide production was approximately 75 million tons, of which 41% was produced in Europe, 29% in Asia and 21% in the Americas. About 45% of grape production is not pressed, while the other 55% is mainly used for wine production [1]. Since the "French paradox" [2], wine has been extensively investigated for the health promoting effects (in particular for cardiovascular diseases), which are associated with its phenolic compounds. Nevertheless, the wine market has recently shown a decreasing trend due to the frequent misuse/abuse of alcoholic beverages also in young people; this social problem was faced in December 2009 by WHO with the paper: "Strategies to reduce the harmful use of alcohol: draft global strategy" [3]. This market situation has stimulated a considerable interest in non-fermented products, especially table grapes, as a potential alternative source of phenolic compounds. In Europe, production of table grapes remains concentrated in Mediterranean-type areas: Italy, Spain, Portugal and Greece are among the most important producers [1]. In addition, three countries produce over 50% of table grapes: People's Republic of China (9.2 million tons), India (2.1 tons) and Turkey (2.1 million tons, mainly used for raisin production) [1].

Compared to wine grapes, table grapes usually have larger berries and firmer pulp. These characteristics make table grapes less prone to be damaged during shipping, since they don't wilt and crush easily. On the other hand, consumers generally prefer seedless varieties with medium sized berries, that are crunchy and characterized by a thin skin and sweet tasting. From a nutritional point of view, despite the higher caloric content compared to other fruits (65 kcal/100 g vs. 51 kcal/100 g of apple and 36 kcal/100 g of orange), grapes are characterized by a good nutritional profile: in fact, they are a good source of potassium (11%), manganese (3%) and vitamins B6 (3%), B1 (6%), and C (3%) [1].

In addition, grapes contain a wide number of health-promoting compounds, like polyphenols: flavonoids, and flavanols, flavonols and anthocyanins are the most representative in red varieties. In more detail, grape skins and leaves contain anthocyanins and flavonols, while pulp and seeds contain mainly proanthocyanidins and non-flavonoid compounds. Polyphenols show a broad spectrum of health promoting properties, including antioxidant activity. Oxidative stress is closely related to pathophysiological processes, such as metabolic syndrome and neurodegenerative disorders [4]; on this process antioxydant can interact with and decrease the risk factors for chronic degenerative diseases. Antioxidant activity is mainly associated with the chemical structure of phenolic

compounds, which contain cyclic aromatic molecules with one or more hydroxylic groups bound to the aromatic rings that facilitate their ability to scavenge free radicals due to resonance stabilization of the captured electron [5]. Due to the lacking characterization of their phenolic compounds, the aim of this study was to investigate the phenolic patterns of different table grape varieties with correlation to their antioxidant activity.

2. Materials and methods

The assessment of the total phenolic content and antioxidant activity was performed by applying the most frequently used assays: 1) Folin-Ciocalteu's assay for total polyphenol content quantification; 2) DPPH (1,1-diphenyl-2-picrylhydrazyl) and ORAC (Ogen Radical Absorbance Capacity) spectrophotometric assays for the assessment of radical scavenging activity. In parallel, a High Performance Liquid Chromatography (HPLC) method, coupled with electrospray ionization mass spectrometric (ESI-MS), was developed in order to obtain the phenolic pattern of grape samples. Simultaneously, a photodiode-array detector (DAD) was used. MS and MSn fragmentation data were employed for the structural characterization of phenolic compounds, whereas DAD detection provided their UV-Vis spectra.

2.1. Samples

Thirteen table grapes and three wine varieties were analysed in this study. Table grapes included six red and seven white varieties. Apart from Red Globe, table grapes included only seedless varieties. Grape varieties, their provenience and the abbreviations used in this paper are reported in Table 1.

About 100 g of berries from each variety were weighed.Then, about 50 g were homogenated and freeze-dried. All samples were maintained at −20 °C till the use. For the successive spectrophotometric and chromatographic analysis, 0.4 g of each homogenate grape sample were mixed with 3 mL of a methanol:water (1:1, v/v) mixture, sonicated for 15 minutes by using a ultrasonic bath; red and white were centrifuged for 15 minutes at 3000 and 8000 r.c.f. (relative centrifugal force), respectively. The supernatant was collected and filtered on a 0.45 µm filter (Millipore, Billerica, MA, USA). Grape residues were extracted again with 2 mL of methanol:water mixture and the two supernatants were combined and brought to volume (5 mL) with the extraction mixture described above.

2.2. Spectrophotometric assays

In order to evaluate the total polyphenol content (TPC) and the total antioxidant activity of grape samples, three spectrophotometric assays were applied.

2.2.1. Folin-Ciocalteu's assay

Total polyphenol content (TPC) was determined according to Singleton and Rossi [6].

Aliquots of 300 µL from different samples prepared as described in paragraph 2.1 were solubilized in 1 mL of a 50:50 water:methanol solution (v/v), mixed in test tubes with 1.5 mL of Folin–Ciocalteu's reagent diluted 10 times, and 1.2 mL of 7.5% (w/v) sodium carbonate. After 30 min, the absorbance was measured at 765 nm in a UV-visible spectrophotometer (Varian Cary 50 SCAN, Palo Alto, CA, USA). The polyphenol content in samples was calculated using a linear regression bases on increasing concentrations of standard (gallic acid). Results were expressed as equivalents of gallic acid in mg/g.

Table 1. Grape varieties included in the study, their origin and code used.

Variety	Cultivar	Origin	Code
Red table grapes	**Pasiga**	Conegliano Veneto, Italy	**PA**
	Red Flame	Conegliano Veneto, Italy	**RF**
	Red Globe	Beja, Portugal	**RG**
	Beauty Seedless	Conegliano Veneto, Italy	**BEs**
	King's Ruby Seedless	Conegliano Veneto, Italy	**KRs**
	Nerona	Conegliano Veneto, Italy	**NR**
Red wine grapes	**Nebbiolo**	Asti, Italy	**NB**
	Barbera	Asti, Italy	**BR**
	Albarossa	Asti, Italy	**AL**
White table grapes	**King's Husainy**	Conegliano Veneto, Italy	**KH**
	Sugraone B.	Conegliano Veneto, Italy	**SC**
	Exalta	Conegliano Veneto, Italy	**EX**
	Sultanina	Conegliano Veneto, Italy	**SL**
	Canner Seedless	Conegliano Veneto, Italy	**CAs**
	Centennial Seedless	Conegliano Veneto, Italy	**CEs**
	Sugraone	Beja, Portugal	**SG**

2.2.2. Antioxidant activity by DPPH assay

The antioxidant activity (AOA) of grape samples was measured spectrophotometrically, as a measure of radical scavenging activity, using 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) [7, 8].

Samples were prepared as described in 2.1. Aliquots of 1 mL of DPPH (Sigma Aldrich, Germany) in methanol (5 mg/100 mL) were mixed with 0.5 mL of each sample suitably diluted. The absorbance was measured after 30 minutes at 517 nm. Results were expressed as equivalents of trolox (mg/g).

2.2.3. Antioxidant activity by ORAC assay

The assay was applied according to the method proposed by Ou et al. and modified by Davalos et al. [9, 10]. The procedure was carried out using a Victor X3 plate reader (Perkin Elmer, USA) equipped with a fluorescence detector set at excitation and emission wavelengths of 484 and 528, respectively. Analyses were conducted in a

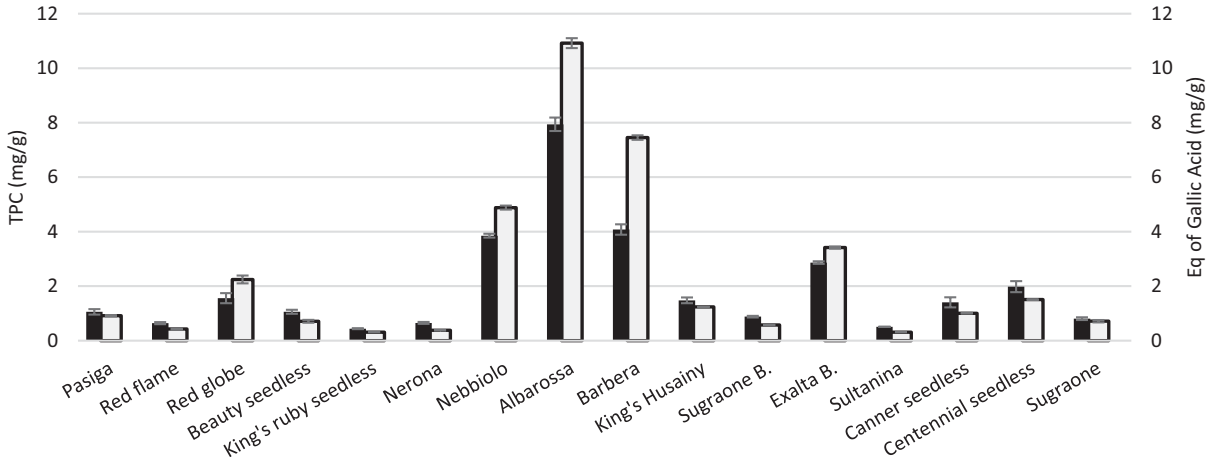

Figure 1. Total polyphenol content (black bars) and antioxidant activity measured by DPPH assay (grey bars) expressed as gallic acid (mg/g) and Trolox equivalents (mg/g), respectively.

phosphate buffer (pH 7.4, 75 mM). Peroxyl radicals were generated using AAPH (12 mM) and fluorescein (70 nM) was used as substrate. Trolox (6.25–50 μM) or samples (400 μg/mL) were added and, after shaking, readings were taken every minute for 120 min at 37 °C. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC was calculated by subtracting the AUC of the blank. The final ORAC values were determined by linear regression of trolox concentrations and are expressed as μM trolox/mL.

2.3. Analysis by HPLC-DAD-MS

A HPLC method combined with electrospray ionization mass spectrometric (ESI-MS) and with Diode Array Detector (DAD) has been set up. The analytic platform was composed by a Surveyor LC system, which was connected to a LCQ Advantage mass spectrometer through a Finnigan IonMax electrospray ionization (ESI) source assembled with a high flow stainless steel emitter (Thermo Fisher Scientific, Rodano, MI, Italy). Full instrument control and data analysis were provided by Xcalibur software (version 2.0.7, Thermo Fisher Scientific, Rodano, MI, Italy). The chromatographic column was a Synergi 4u MAX-RP 80A (250 × 2.0 mm 4 μm) (Phenomenex, Torrance, CA, USA).

The analysis was performed using a linear gradient elution at a flow rate of 0.3 mL/min, where: A) water:acetonitrile:formic acid 96.99:3:0.01 (v/v/v); and B) acetonitrile:water:formic acid 50:49.99:0.01 (v/v/v).

The mass spectrometer operated in electrospray for negative ions (ESI-) using nitrogen as a sheath gas. Chromatograms were recorded at 200–800 nm. Different classes of phenolic compounds (anthocyanins, flavan-3-ols, proanthocyanidins, flavonols, stilbenes and organic acids) were identified by their UV spectra recorded with a DAD detector, by their MS spectra and their corresponding MSn fragments.

3. Results and discussion

3.1. Spectrophotometric assays

Figure 1 shows the total polyphenol content and the antioxidant activity of samples measured by DPPH assay.

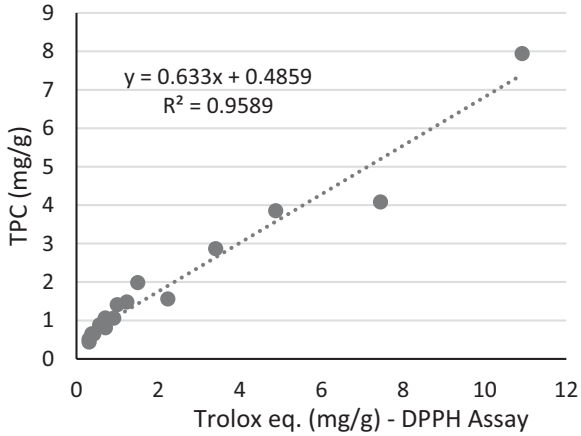

Figure 2. Correlation between TPC (mg/g gallic acid equivalents) and antioxidant activity measured by DPPH assay (mg/g Trolox equivalents).

According to data from the literature, total phenolic content (TPC) measured in grape samples included in this study ranged between 0.44 ± 0.02 and 7.94 ± 0.19 mg gallic acid equivalents/g grape sample [11, 12]. TPC of red wine varieties was higher than that of red table grapes: Albarossa presented the highest phenol content (7.94 ± 0.19 mg/g) followed by Barbera and Nebbiolo (4.08 ± 0.25 and 3.85 ± 0.07 mg/g, respectively). Surprisingly, Exalta (a white variety) showed the highest total polyphenol content (2.86 ± 0.04 mg/g) considering both white and red table grapes (0.44 ± 0.02 and 1.6 ± 0.18 mg/g, respectively). The lowest contents were found in the red table grape King's Ruby (0.44 ± 0.019 mg/g) and in the white Sultanina (0.51 ± 0.003 mg/g).

Among table grapes, the red Nerona showed the highest antioxidant activity (4.88 ± 0.18 mg/g), followed by the white grape Exalta (3.41 ± 0.04 mg/g), Centennial seedless and King's Hussainy (1.51 ± 0.01 mg/g and 1.24 ± 0.01 mg/g, respectively). The lowest activities were found in the white table grape variety Sultanina (0.32 ± 0.001 mg/g) and in the red table grape King's Ruby (0.31 ± 0.002 mg/g).

When the TPC was correlated with DPPH assay (antioxidant activity), a good linear coefficient was

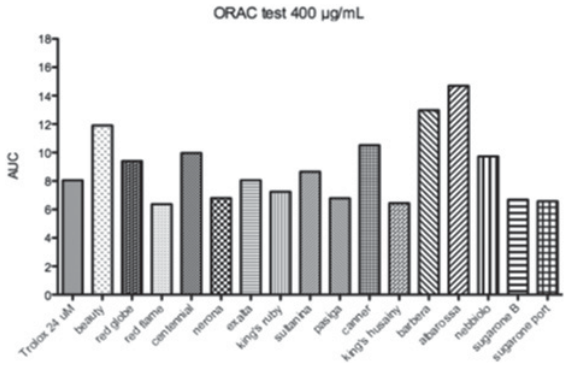

Figure 3. Antioxidant activity of grape samples measured by ORAC assay.

obtained ($R^2 = 0.96$), indicating a high correlation between TPC and antioxidant activity (Fig. 2).

According to the data obtained by ORAC test (Fig. 3), the red grape Beauty Seedless and the white grape Canner Seedless showed the highest antioxidant activity when assayed at 400 µg/mL (0.45 µmol/g and 0.39 µmol/g Trolox equivalents, respectively), followed by Centennial (0.38 µmol/g trolox equivalents), Sultanina (0.32 µmol/g trolox equivalents) and Exalta (0.30 trolox equivalents).

The differences observed between ORAC and DPPH test could be due to some interferences in the development of the colorimetric reaction. In fact, many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH due to steric inaccessibility [5].

3.2. HPLC-DAD-MS analysis

HPLC coupled to mass spectrometry was used to obtain a first preliminary evaluation of grape phenolic pattern. The UV spectral data, fragmentation patterns and chromatographic behaviours were evaluated by comparing the results with the information found in the scientific literature or with on-line databases (e.g. Phenol-Explorer, Metabolome and Massbank). The HPLC-DAD and MS/MS spectra of four of the main phenolic compounds (epicatechin, procyanidin B2, malvidin-3-glucoside, quercetin) are illustrated in Fig. 4.

LC-MS technique allowed the identification of a high number of compounds: n. 10 flavonols, n. 3 organic acids and stilbenes, n. 25 anthocyanins and n. 24 flavan-3-ols.

3.2.1. Anthocyanins

A total of 25 anthocyanin compounds were identified by LC-MS: 3-O-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin and their corresponding conjugates, including acetyl, coumaroyl and caffeoyl esters. Interestingly, pelargonidin and its derivatives were not detected, although their presence was reported in wine grapes by other authors [10]. This disagreement could be due to the fact that, in the present study, only few wine varieties were included. Figure 5 shows the distribution of the identified anthocyanin compounds among the samples analysed.

Malvidin was the anthocyanidin with the highest number of derivatives (mainly glicosides and cumaroyl-derivates), while peonidin-glucoside and malvidin-glucoside were the anthocyanins detected in the highest number of samples, being present in 100% and 89% of grapes, respectively. Malvidin-coumaroyl-hexoside, petunidin-glucoside and peonidin-coumaroyl-hexoside were detected in 78% of samples, while caffeoyl, acetyl hexosides of petunidin and peonidin were the less frequently observed (11% of samples). Interestingly, the first two compounds (petunidin caffeoyl and acetyl hexosides) presented a specific presence in wine varieties, since they were not identified in table grapes. A similar pattern was observed for coumaroyl and acetyl derivatives of cyanidin and peonidin, respectively.

It is known that the anthocyanin pattern influences not only the grape colour, but also the grape antioxidant potential. For example, the presence of malvidin and peonidin is considered positive, since their chemical structure does not contain hydroxyl groups in ortho-position (like other anthocyanidins), resulting in a higher resistance to oxidation and great stability [13]. Glycosylation and acetylation of anthocyanidins further increase their antioxidant activity [13]. Considering that glycosylated and acetylated forms of malvidin and peonidin were generally widely distributed and relatively abundant in some table grapes, there is a great probability that these varieties could be a good source of antioxidant compounds. The importance of anthocyanin concentration in berries is closely correlated to their positive implications in human health; their antioxidant potential is associated with the reduction of some cardiovascular risk factors, such as reduction of oxidized LDL levels and increased serum antioxidant capacity [14]. Anti-inflammatory effects of grape anthocyanins were also investigated, demonstrating the reduction of TNF-α-induced inflammation in humans through modulation of endothelial monocyte chemoattractant protein-1 [15].

3.2.2. Flavan-3-ols

Figure 6 shows the distribution of the n.16 flavan-3-ols identified in grape samples under study. The distribution of flavan-3-ols shows that the dimers and trimers were generally more frequent than the monomers: proanthocyanidin dimers were detected in all samples analyzed, while 93% of the varieties contained the trimers. Epicatechin gallate was identified in only 12.5% of samples (only wine varieties Albarossa and table grape Red Globe, data not shown). Among the other monomers, catechin and epicatechin were the most frequently detected, being found in 62.5% and 50% of grapes, respectively. Dimers and trimers of proanthocyanidins were equally distributed among red and white varieties but, unfortunately, most of these compounds were only tentatively identified. The only certain identification regarded the procyanidin B2 (detected in 56.25% of samples: 37.5% red and 25% white grapes), since the relative commercial standard was available. Interestingly, proanthocyanid dimers and trimers and their derivatives, mostly present in seeds, were abundant also in seedless white grape varieties (e.g. King's Husainy and Exalta, data not shown). A possible explanation of this result is the presence in these varieties of little seeds, probably due to the not total successful process of seedless grapes production, the so-called "stenospermocarpic process". As a consequence, the high antioxidant activity of some

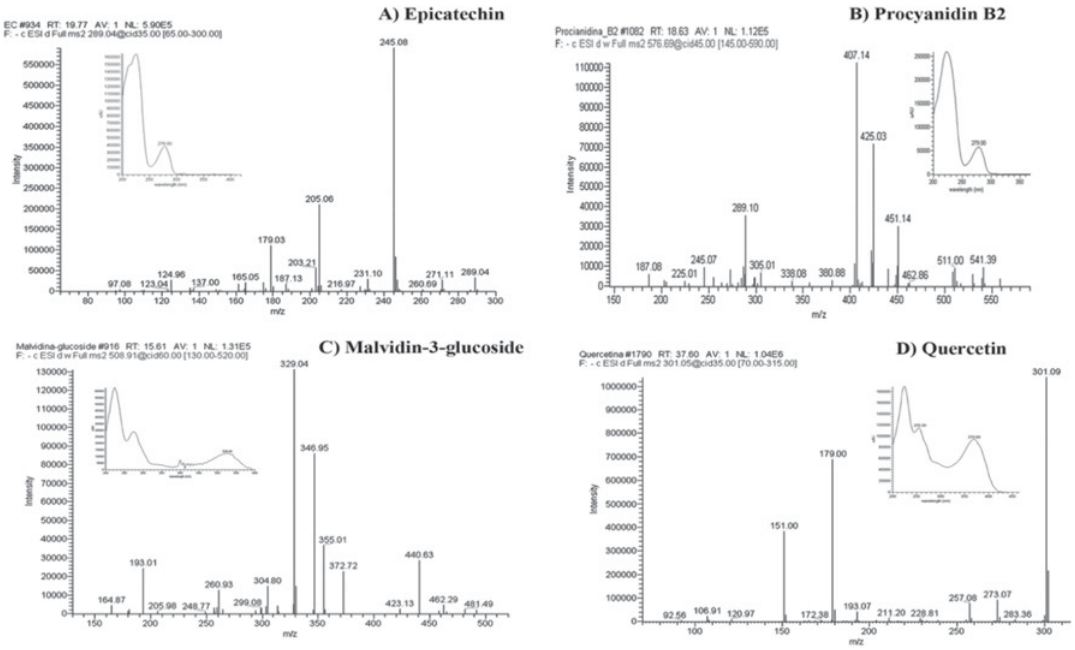

Figure 4. HPLC-DAD and MS/MS spectra of the four most representative phenolic compounds: A) Epicatechin; B) Procyanidin B2; C) Malvidin-3-glucoside; D) Quercetin.

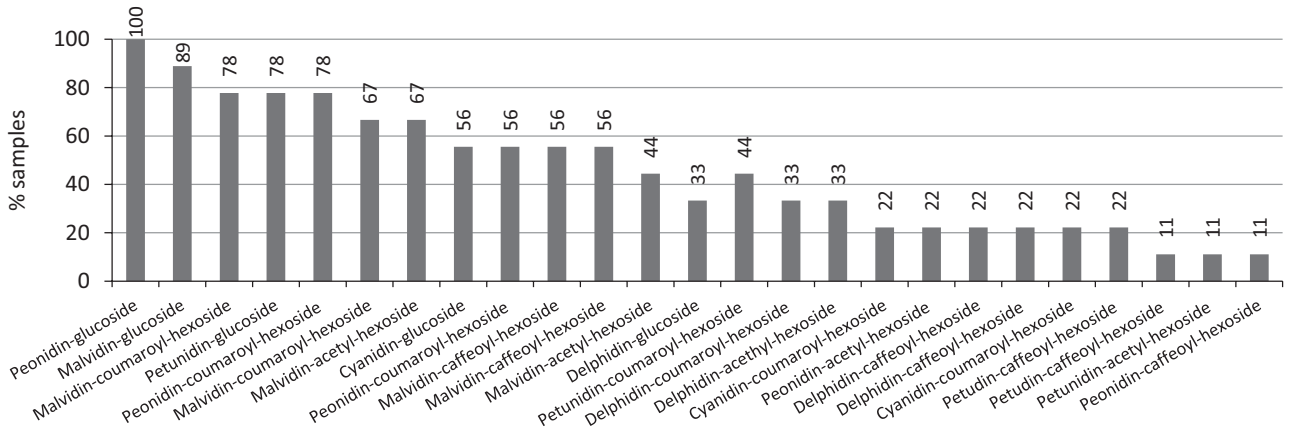

Figure 5. Distribution of anthocyanins in red grape samples similar pattern was observed for coumaroyl and acetyl identified in grape samples under study.

varieties measured in spectrophotometric assays could be due to the presence of proanthocyanidins coming from the partially developed seeds. A suitable intake of flavan-3-ols with the diet is important for their activities as free radicals scavenger and chelator of transition metals, which are among the mechanisms responsible for their positive effects in human health. The most important evidences for the human health-promoting effects of flavan-3-ols are associated with the reduction of risks factors for cardiovascular disease though several mechanisms. In particular, proanthocyanidins and flavan-3-ols monomers are capable to modulate plasma cholesterol levels, inhibit the LDL oxidation, activate endothelial nitric oxide synthase and prevent platelet adhesion and aggregation [16–18]. Taking into consideration the consumers' preferences for seedless varieties, the presence of little seeds could provide additional positive phenol compounds without interfering with the organoleptic characteristics.

3.2.3. Flavonols

The LC-MS analysis allowed the identication of n. 18 phenol compounds belonging to flavonols. The distribution of these flavonoids among grape varieties is illustrated in Fig. 7.

Quercetin derivatives were the most abundant flavonols detected in the samples analyzed: the glucoside and glucuronide compounds were present in all varieties, followed by the galactoside and rutinoside, identified in 87.5% and 62.5% of grapes, respectively.

Other flavonols were widely distributed in grape varieties: trihydroxyflavone-riboside and isorhamnetin-hexoside were detected in 15 and 14 samples, representing the 93.5% and 87.5% of grapes, respectively. Suprisingly, kaempferol derivatives were rarely detected compared to other *Vitis vinifera* grape varieties reported in the literature [19]; in fact, the rutinoside was detected in only 2 samples.

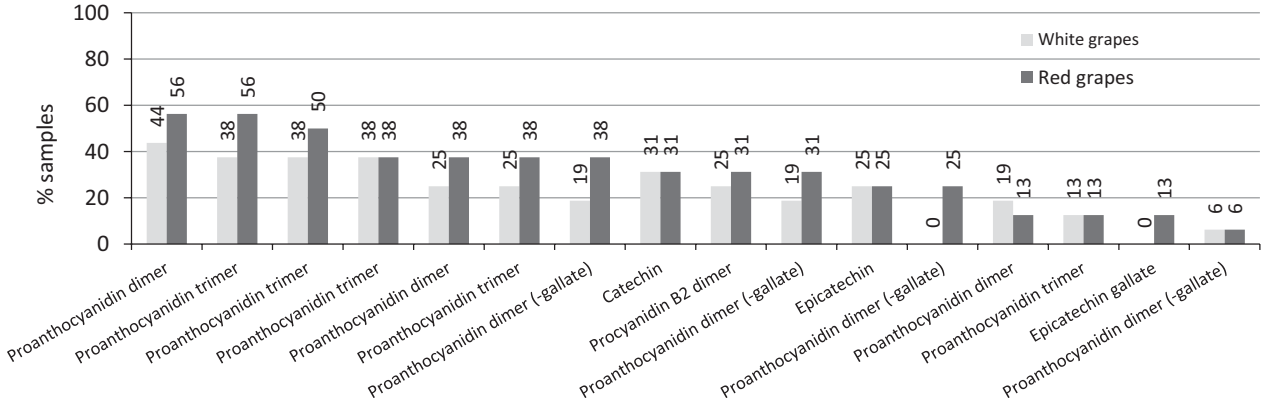

Figure 6. Distribution of flavan-3-ols in grape samples.

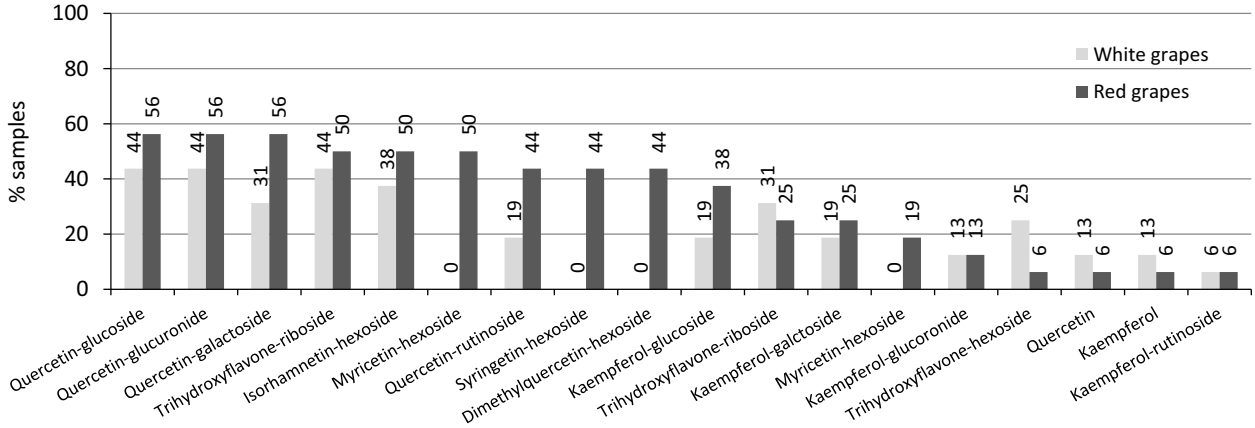

Figure 7. Distribution of flavonols in grape samples.

As the other classes of flavonoids reported above, three minor compounds were identified only in red varieties (table grapes) as syringetin-hexoside and dimethyl-quercetin-hexoside, indicating, that they could be suggested as useful biomarkers for the screening of red table grapes. These data need further confirm.

Apart from one sample (white Sultanina, not shown), all white varieties contained several flavonols; interestingly, the presence of quercetin aglycone, undetectable in most samples, was observed in two white varieties (Canner seedless and Centennial seedless). Some authors reported that the presence of free form of quercetin could be due to hydrolysis [19], while others stated that flavonol aglycones are generally present only in red varieties [1]. The observed great variability of results can be attributed to several factors; among them, the grape variety and the degree of grape ripening. As regards the potential beneficial effects, the high content in flavonols, and in particular of quercetin derivatives, is considered important for its strong antioxidant properties; as known they are involved in the reduction of risk factors for several chronic diseases [20, 21]. Quercetin, but also kaempferol and myricetin, have shown a possiblel antithrombotic effects because they are capable to scavenge free radicals, improving endothelial function. Other studies report that these flavonoids inhibit the activity of cyclooxygenase and lipoxygenase pathways, which are responsible for anti-inflammatory properties [11].

4. Conclusion

After the WHO documents on the possible harmful consequence of the misuse of alcoholic beverages, the interest in studying table grapes and their non-fermented derivatives has increased progressively. Other international organizations have also stimulated the research in this field. In particular, the OIV (International Organization of Wine and Vine) in its strategic plan (2015–2019), underlines the importance in identifying and stimulating research axes, on the investigation of the healthy aspects and nutritional potential of all non-alcoholic vine-derived products. This study characterized the chemical profile and the antioxidant activity of different varieties of *Vitis vinifera* L. (grape) samples; thirteen of them were table grapes. Considering the table grapes, several phenolic compounds were detected in seed-containing varieties (e.g. wine grapes and red table grape Red Globe), but also in some seedless white grapes (e.g. Exalta and Centennial), where there was the presence of undeveloped seeds. Antioxidant properties, measured in parallel, showed a correlated pattern of activity. Different classes of flavonoids were preliminary identified by LC-MS. Malvidin-3-glucoside and peonidin-3-glucoside were the most abundant anthocyanins in red grape samples. Proanthocyanidins were also identified by MSn fragmentation; among them, dimers and trimers of flavan-3-ols were the most frequently detected and

abundant in both red and white grapes. With regards to flavonols, quercetin and kaempferol derivatives were widely distributed in all the varieties analysed. These results showed that table grapes and, consequently, their unfermented derivatives (e.g. juice), could have healthy properties thanks to their content in phenolic compounds. They could be a good source of antioxidant compounds for people who, for different reasons, do not drink wine. To date, several beneficial effects have been demonstrated in humans for the classes of polyphenols identified in the grape varieties investigated in this study, including the reduction of risk factors for chronic illness and in particular, for cardiovascular diseases. This awarness strongly stimulates further efforts in this area.

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