Rapid Screening of Antioxidant Anthocyanins in Autochthonous Nero d'Avola Grape Clones by Pre-column DPPH Reaction Coupled to UHPLC-UV/Vis-IT-TOF: a Strategy to Combine Chemical data and Genetic Diversity

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Abstract

Grape is a rich source of bioactive compounds; among them anthocyanins are associated to many healthy properties, possessing a good antioxidant activity. In this work, we developed a fast and simple screening of antioxidant anthocyanins in six Sicilian Nero d'Avola autochthonous grape clones. The method was based on the pre-column reaction with 2,2′-diphenyl-1-picrylhydrazyl radical followed by the rapid separation by ultra high-pressure liquid chromatography coupled with ultraviolet and tandem mass spectrometry detection. Peak areas of antioxidant anthocyanins significantly reduced or even disappeared. The entire method took only 45 min per sample, showing good retention time and peak area repeatability with maximum CV% values ≤0.86 and 6.84, respectively. Samples rich in delphinidin derivatives showed lowest IC_{50} values, since those compounds possess the highest scavenging ability. The developed setup was less complex than online approaches and faster with respect to conventional high-performance liquid chromatography methods, taking advantage of ultra high-performance conditions, coupled for the first time with pre-column 2,2′-diphenyl-1-picrylhydrazyl assay. The proposed strategy is a valid tool for a rapid screening of antioxidant anthocyanins in grape samples, useful to correlate the genetic diversity with the production of secondary metabolites as well to assess their activity in nutraceutical products rich in anthocyanins.

Keywords: Anthocyanins, Antioxidant, DPPH-UHPLC, Grape, Nero d'Avola, Pre-column

Introduction

Several studies evidence the direct relationship between the social and dietary habits and the possible onset of chronic diseases, such as cardiovascular and neurodegenerative pathologies, as well as cancer (Espín et al. 2007). To improve and preserve the health status, many observations promote the regular intake of vegetables as well as fruit and juices; the benefits deriving from the consumption of these foods are related to the content of bioactive compounds, especially polyphenols. In this regard, the so-called French paradox has been linked to the consumption of wine (Fauconneau et al. 1997), which is the main product of grapevine, since it has proved to be a rich source of polyphenols. Grapevine (*Vitis vinifera* L.) is one of the earliest domesticated fruit and is a highly valuable crop, especially for the derived products wines and spirits. Despite that several genotypes have been selected during its millennial cultivation, in modern times extensive diffusion of only a few international varieties has

drastically reduced genetic diversity. In Italy, grapevines are extensively cultivated, with about 800,000 ha of vineyards; however, their distribution in the Country is unequal, with Sicily representing one of the most significant wine regions (Carimi et al. 2010). The long history of cultivation has led to a high number of autochthonous grapevine varieties, which represents an important source of genetic diversity. Accordingly, Sicilian varieties are characterized by high variability (Carimi et al. 2010). Within the group of Sicilian red grapevine, there are important cultivars. "Nero d'Avola" is currently the most important and diffused red berry cultivar in Sicily, with 15 % of the vineyard surface, and this ancient variety is utilized for the production of wines exported worldwide, among which are several other Denominazione di Origine Controllata (DOC) wines. Grape contains four main classes of polyphenols: flavonols, flavan-3-ols, their polymeric forms (tannins), and anthocyanins. Anthocyanins are natural pigments responsible for fruit and flower colors, including red grapes. In grapevines, they are localized in the skins and appear mainly during ripening, and their relative content can be influenced by many factors, including water availability and temperature. During harvest, the content of these pigments can reach values as high as 1000 mg/kg (Boulton et al. 1996); their presence is usually important for many processes and contributes to the sensory and quality properties of the final product. For this reason, each variety has a typical and characteristic anthocyanin profile that differs from the others, and contributes to their taxonomic identification (Picariello et al. 2014). The interest in these molecules is not merely restricted to a taxonomic classification; in fact, as evidenced in some studies, they are responsible for many healthy properties, with particular focus on cardiovascular diseases (Tenore et al. 2013; Burns et al. 2000), and possess an antioxidant activity close to ascorbic acid (Choi et al. 2010). Several in vitro methods have been developed for the evaluation of the antioxidant potential of natural as well as synthetic compounds, such as reactions with 2,2′-diphenyl-1-picrylhydrazyl (DPPH) or with 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Besides being a cheap and easy method, with respect to ABTS that needs to be incubated with potassium persulfate for 12 h, DPPH can be used immediately after preparation (Mishra et al. 2012). Although this method is useful for simple samples, it is not capable of giving information regarding the contribution of a single analyte in a complex matrix often resulting in under/overestimation. Besides, extensive and time-consuming purification steps are necessary to isolate pure compounds and further investigate their activity. In this regard, the coupling of DPPH assay together with a separation technique, such as high-performance liquid chromatography (HPLC), is an efficient tool to elucidate the activity of every single peak in a complex sample, by simply calculating the changes in the UV detector response after the reaction with the radical. Moreover, the further hyphenation with tandem mass spectrometry (MS/MS) ensures accurate identification of every possible antioxidant analyte in the sample. Thus, the comparison with pure standards could be also helpful to verify possible synergistic effects that occur in phytochemical complexes. In this work, a rapid antioxidant activity screening of anthocyanins in six Sicilian Nero d'Avola grape clones, together with their quali-quantitative profile, was carried out by DPPH ultra high-pressure liquid chromatography (UHPLC) coupled to UV/Vis-MS/MS detection. The method was based on the pre-column reaction with DPPH radical followed by fast LC separation and peak identification with ion trap-time-of-flight (IT-TOF) mass spectrometry. The changes in UV chromatogram peak areas, between the reference sample and the DPPH-spiked samples, express the antioxidant activity of the investigated compounds. The entire workflow, comprising reaction, injection, separation, and re-equilibration, took only 45 min per sample. Fourteen anthocyanins were identified and quantified, some present in traces, comprising monoglucosides as well as coumaroyl and acetyl derivatives, and their different antioxidant potential in relation with their abundance and chemical structure was highlighted. The proposed analytical strategy represents a fast and low-cost approach for the screening of anthocyanin profiles in grape, with respect to off-line standard in vitro antioxidant assays, and could be employed to highlight the connection between genetic diversity and expression of secondary metabolites as well as for nutraceutical purposes.

Materials and Methods

Clone Selection

Several surveys were carried out since 2006 in the main grapevine production areas of Sicily, where old vineyards are still cultivated. A total of 42 vineyards were surveyed. The oldest vineyards (more than 80 years) were located in Syracuse, while all the other vineyards were about 20–30 years old. A total of 62 plants comprising the varieties "Perricone", "Catarratto nero", "Nero d'Avola", "Nocera", and "Nerello Mascalese" were chosen for analysis of berry and bunch traits and subsequently sampled for DNA extraction and microsatellite analyses; three different plants were analyzed for each vineyard. The traits were chosen from the list of descriptors of the International Organisation of Vine and Wine (OIV). In order to obtain homogeneous growth conditions of plants for further chemical analysis, the genotypes that showed marked differences based on OIV descriptors were introduced in an ex situ collection of the experimental field of the National Research Council of Italy (CNR) Institute of Biosciences and Bioresources (IBBR) located in Collesano district, Italy (37° 59′ 19.9″ N, 13° 54′ 55.8″ E, 80 m above sea level) (Table S1). The climate is relatively dry, with a mean annual rainfall of 525 mm. The selected genotypes were grafted onto 1103 Paulsen (*V. berlandieri* x *V. rupestris*), spaced 3.2 m between rows × 1.2 m on the row, resulting in 2604 vines/ha. N, P, and K fertilization was similar for all genotypes. A block design with three replicates per treatment was used, and border effects were eliminated by guard rows and four guard vines at the edge of each block. Six clones of Nero d'Avola variety, which is the most important variety in Sicily, were initially selected for this study, for the development of the analytical method.

Reagents and Standards

Ultra pure water (H_2O) was obtained by a Milli-Q Direct 8 system (Millipore, Milan, Italy), acetonitrile (ACN), LC-MS grade, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Milan, Italy). For the DPPH-UHPLC, a Kinetex™ C18 100 mm × 2.1-mm (L. × I.D.), 1.7-μm column was employed (Phenomenex®, Castel Maggiore Bologna, Italy). Malvidin-3-*O*-glucoside standard was purchased from Extrasynthese (Lion, France). Unless stated otherwise, all other reagents were purchased from Sigma-Aldrich.

Sample Extraction

Extraction was carried out by a modified procedure described by Kammerer et al. (2004). Freezed grapes were defrosted at room temperature before use, and the berries were manually separated into skins and pulps using a knife. Nero d'Avola grape skins (7 g) were extracted with 40 mL of methanol/0.1 % HCl *v/v* for overnight stirring in the dark. The extracts were centrifuged (15 min, 4000×*g*), filtered on 0.45-μm filters (Phenex-RC, Phenomenex®), and injected without any other treatment.

Instrumentation

HPLC analyses were performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A

photo diode array detector (equipped with a 2.5-μL detector flow cell volume), a CTO-20A column oven, and a SIL-30 AC autosampler. The system was coupled online to an LCMS-IT-TOF mass spectrometer through an ESI source (Shimadzu, Kyoto, Japan). LC-MS data elaboration was performed by the LCMS Solution® software (Version 3.50.346, Shimadzu).

UHPLC-UV/Vis-ESI-IT-TOF Conditions

The optimal mobile phase consisted of 0.1 % TFA in water *v/v* (A) and ACN plus 0.1 % TFA *v/v* (B). Analysis was performed in gradient elution as follows: 0–10 min, 5–30 % B; 10–11 min, 30–95 % B; 11–11.50 min, isocratic at 95 % B; 11.50–11.60 min, 95–0 % B; and 11.60–15.00 min, 5 % B. Flow rate was 0.5 mL/min. Column oven temperature was set to 55 °C. Injection volume was 2 μL of extract. The following diode array detector parameters were applied: sampling rate, 10 Hz; detector time constant, 0.160 s; and cell temperature, 40 °C. Data acquisition was set in the range 190–800 nm, and chromatograms were monitored at 520 nm, at the maximum absorbance of the compounds of interest. MS detection was operated in positive ionization mode, with the following parameters: detector voltage, 1.55 kV; interface voltage, +4.5 kV; curve desolvation line (CDL) temperature, 250 °C; block heater temperature, 250 °C; and nebulizing and drying gas flow (N_2), 1.5 and 10 L/min. Full-scan MS data were acquired in the range of 150–1000 m/z, ion accumulation time, 35 ms; and IT repeat, 3. MS/MS experiments were conducted in data-dependent acquisition; precursor ions were acquired in the range 100–1000 m/z; peak width, 3 Da; ion accumulation time, 50 ms; CID energy, 55 %; repeat, 1; and execution trigger base peak intensity (BPC) at 95 % stop level. The instrument was tuned daily by a standard solution of sodium trifluoroacetate providing a mass accuracy level <10 ppm.

Total Antioxidant Capacity of Nero d'Avola Grape Clones

For the determination of antioxidant capacity of grape clones, several extract solutions were diluted in the appropriate ratio with methanol, and for each sample a calibration curve was built. The samples were added to DPPH solution (7.5 mM) in a 1:1 ratio, and the mixture was briefly sonicated then left to react for 30 min in the dark at room temperature. The determination of total antioxidant activity was carried out by UHPLC instead of spectrophotometric assay. The sample was filtered through 0.45 μm and injected in the LC system, running isocratic with mobile phases: (A) 0.1 % TFA in water *v/v*, and (B) ACN plus 0.1 % TFA in a ratio of 80/20. The blank control was prepared by diluting the DPPH solution with methanol in a 1:1 ratio. IC_{50} values, the concentration of grape extract scavenging 50 % DPPH, were calculated through linear regression analysis, by interpolation, and ascorbic acid was employed for positive control. The radical scavenging activity of the extracts was calculated with the following formula:

$$\text{Radical scavenging } (\%) = \frac{PA_{control} - PA_{spiked}}{PA_{control}} \times 100\% \quad (1)$$

where ($PA_{control}$) is referred to the DPPH peak area solution diluted with methanol, whereas (PA_{spiked}) is referred to the DPPH solution mixed with the grape extract.

DPPH-UHPLC-UV/Vis Method

DPPH-UHPLC analyses were carried out as reported previously (Tang et al. 2008). Two hundred microliters of Nero d'Avola extract and 200 μl of DPPH stock solution (at the final concentration of 7.5 mM) in a 1:1 ratio were briefly mixed and allowed to react for 30 min in the dark at room temperature. After filtration with a 0.45-μm filter, 2 μL of sample solution was injected for UHPLC

analysis with the conditions reported above. Two hundred microliters of methanol was added to the grape extract as control. The change in anthocyanin peak areas ($PA_{control}$) between control and DPPH-spiked (PA_{spiked}) sample was used to evaluate the antioxidant anthocyanins again according to Eq. 1.

Qualitative and Quantitative Analysis

Since standards for all compounds were not available, malvidin-3-*O*-glucoside was selected as external standard for the quantification. Stock solutions (1 mg/mL) were prepared in methanol; the calibration curves were obtained in a concentration range of 0.1– 0.0005 mg/mL with seven concentration levels, and triplicate injections of each level were run. Peak areas were plotted against corresponding concentrations. The amount of the compounds in the sample was expressed as milligrams per kilogram of malvidin-3-*O*-glucoside equivalents (ME) of grape skin, linear regression was used to generate calibration curve, and R^2 value was 0.995. Molecular formulas were calculated by the Formula Predictor software (Shimadzu), using the following parameters maximum deviation from mass accuracy: 10 ppm, MS/MS fragmentation data, nitrogen rule. The data obtained were compared with online free databases such as ChemSpider (http://www.chemspider.com), SciFinder Scholar (https://scifinder.cas.org), and Phenol-Explorer (www.phenol-explorer.eu).

Results and Discussion

Optimization of UHPLC Separation

Figure 1 shows the chromatograms extracted at 520 nm for an eastern Sicily Nero d'Avola grape variety. Separation parameters such as mobile phase composition, column oven temperature, and gradient were selected after several experiments, in order to obtain the best balance between resolution and analysis time. Since the optimal pH for the separation of anthocyanins ranges from 1 to 2 (García-Beneytez et al. 2003), the best compromise was obtained with TFA in concentration of 0.1 % *v/v*, instead of using high percentage of formic acid. Furthermore, high temperatures, together with the employment of a 1.7-μm core shell particle column, increased the kinetics of interconversion between flavylium cationic and carbinol pseudobasic forms, leading to sharper peaks and reduced analysis times (de Villiers et al. 2011); thus, the entire separation, comprising column re-equilibraton, was accomplished in 15 min, a considerable reduction in comparison with methods based on conventional particles, which are usually longer than 40 min (Fraige et al. 2014; Pati et al. 2006). Repeatability was established by triplicate injections of sample and solutions at low, medium, and high concentration levels of the analytical curve at the same day, with the same chromatographic conditions and analyst, showing good retention time and peak area repeatability with maximum CV% values ≤ 0.86 and 6.84, respectively, whereas inter-day repeatability values were 0.98 and 6.75 %, respectively. Recovery was evaluated by spiking the sample matrix with malvidin-3-O-glucoside at three concentration levels (12.5, 25, and 50 μg/mL); recovery values in the range of 81–105 % were obtained. Limits of detection (LODs) and quantification (LOQs) were calculated by the ratio between the standard deviation (SD) and analytical curve slope multiplied by 3 and 10, respectively. Results are reported in supporting Table S2.

DAD-ESI-IT-TOF Profile of Nero d'Avola Clones

The anthocyanin profile of Nero d'Avola grape is characterized by a marked prevalence of malvidin-3-O-glucoside and its acetylated and p-coumaroyl derivatives as confirmed by previous investigations (Casavecchia et al. 2007), with a content of malvidin-3-O-glucoside ranging from 1244 to 383 mg/kg (Table 1). Di-glucosides were not detected both in UV/Vis and MS traces, as also reported by other authors (Mazzuca et al. 2005), whereas acetylated and p-coumaroyl forms were also present, with the last being more abundant. The identification of compounds was carried out on the basis of diode array spectra as well as accurate MS molecular ions and MS/MS fragmentation patterns. The employment of a hybrid mass spectrometer, capable of high mass accuracy in both MS and MS^n stages, is particularly useful for accurate profiling of this class of compounds (Barnes et al. 2009). Results are shown in Table 2 in order of peak elution. Peaks 1, 2, 3, 4, and 5 (t_r 3.42, 4.11, 4.54, 5.14, 5.44) showed all a similar fragmentation pattern as a result of the cleavage of an hexose [M^+-162]. This information, together with the elution order, which is usually typical on C18 columns and depends from the number of hydroxyl groups, and their degree of methoxylation as reported previously (Valls et al. 2009), led to their identification as 3-O-glucoside forms of delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively. Compounds 6, 7, and 9 (t_r 6.67, 7.32, 7.85) were characterized by the loss of an hexose together with a difference of 42 amu [M^+-162-42] due to the presence of an acetyl group. Based on previous observations (Mazzuca et al. 2005), these compounds were identified as 3-O-(6″-acetyl)-glucoside derivatives of petunidin, peonidin, and malvidin, respectively. Among these compounds, peak 8 (t_r 7.40) showed a fragment at [M^+-308] probably originated from the cleavage of a p-coumaryl-glucose moiety; based on the elution order, this was recognized as delphinidin 3-O-(6″-p-coumaryl)-glucoside. Similar MS^2 spectra characterized peaks 10, 12, 13, and 14 (t_r 7.91, 8.21, 8.86, 8.97); thus, they were finally tentatively identified as 3-O-(6-p-coumaryl) glucoside derivatives of cyanidin, petunidin, peonidin, and malvidin, respectively. Lastly, peak 11 (t_r 8.05) showed two fragment ions, [M^+-324] and [M^+-162], revealing the possible presence of a caffeoyl moiety, thus leading to the identification as malvidin 3-O-(6-p-caffeoyl-glucoside). The presence of cis isomers for coumaryl derivatives, usually eluting before the trans forms, was slightly visible only for peaks 13 and 14, but those signals were very low and not clearly detectable even in MS trace (data not shown).

Table 1 Quantitative data and scavenging activity of grape extracts 1-6
Full size table
Table 2 ESI-IT-TOF anthocyanin profile of Nero d'Avola clones
Full size table

DPPH-UHPLC Method Optimization

The colorimetric DPPH assay is based on the reaction of this radical with an antioxidant compound. The DPPH radical possesses a strong absorbance at 517 nm, and, as a result of the reaction, this absorbance decreases. Although this method is widely used, it is not capable of giving detailed information about the contributions of single analytes in a matrix. In this regard, as shown by Tang et al. (2008), the coupling with a separation technique is useful to appreciate the contributions of every analyte in the matrix. In this case, after the reaction of the antioxidant compound with the DPPH radical, the conjugated system is destroyed, and thus, the relative peak area in the UV/Vis chromatogram will decrease or even disappear in relation to their antioxidant activity. No other peak was observed at 520 nm, probably due to a change in the absorbance of the molecular structures derived from the breaking of the conjugated system. A crucial aspect in this study was the ratio between the concentration of DPPH and the extract, together with the reaction time. When using an excess of DPPH, the differences in the antioxidant activity could not be measured, since every peak just disappeared in the UV/Vis trace, whereas, with an inadequate concentration of DPPH, no differences were noticed. After several observations, we found that the best conditions were obtained with 7.5 mM of DPPH. Regarding the reaction time, we observed, by injecting at different time

intervals, that the optimal reaction time was of 30 min; after this point, no further changes in peak areas were observed, which was confirmed by a stable yellow color of the solution.

Evaluation of Antioxidant Capacity

The coupling of a separation technique with antioxidant assays is particularly useful when dealing with natural matrices. Several approaches have been employed in the field, which can be divided mainly in post-column and pre-column methods (Zhang et al. 2012). In post-column methods, the radical solution is pumped and mixed with the sample eluting after the column outlet in a reaction coil; the matrix can be further detected by UV or MS to appreciate the reduction of peak areas (Karaçelik et al. 2015). Although those methods are fully online and do not need extensive sample preparation, since the reaction takes place in the reactor, they need hardware adjustments, such as an additional pumping system. Moreover, band broadening can occur in the reaction coil leading to coelutions and thus misleading peak area calculations. Furthermore, external sources of band broadening are detrimental for the employment of UHPLC conditions. In this regard, the pre-column approach, although needing manual sample preparation and off-line reaction, does not need any hardware adjustment and can take full advantage of UHPLC conditions, such as faster analysis times and higher peak capacities. In this regard, in comparison with both pre- (Tang et al. 2008) and post-column (Nuengchamnong and Ingkaninan 2009) DPPH-HPLC techniques based on conventional fully porous particles, the employment of a sub-2 μm core-shell particle column allowed to reduce significantly the analysis time, improving peak shape and resolution. The UV/Vis chromatogram relative to the separation of both untreated and DPPH-spiked sample is depicted in Fig. 2. As can be clearly observed, several peaks were significantly reduced, while only few completely disappeared. In particular, among all compounds listed in Table 1, how delphinidin derivatives possess a strong scavenging activity can be appreciated. This aspect is further confirmed from the graphic (Fig. 3), in which the contribution of every single analyte with respect to the total percent of DPPH scavenging activity is reported. In fact, grape extracts possessing lowest IC_{50} values (Table S3) are characterized by higher amounts of delphinidin derivatives, as also reported for other matrices containing anthocyanins (Sun et al. 2012). These data are in good agreement with previous observations; those compounds possessing more hydroxyl groups on the B ring (Fig. 4) show more efficiency toward the DPPH radical (Kähkönen and Heinonen 2003; Sun et al. 2012). Interestingly, also peonidin 3-O-(6″-acetyl)-glucoside showed a relevant scavenging ability; it is known that the methoxy group in the 3′ position improves the activity, but further studies are necessary to understand the role of the acetyl moiety. It is noteworthy that the fluctuation of some DPPH scavenging activity among samples can be related to the presence of the small amount of other polyphenols, not detected at 520 nm, whose synergistic effect could influence, negatively or positively, the antioxidant activity; this aspect has been reported previously especially in berry fruits (Sun et al. 2012). Among investigated grapes, samples 2 and 5, belonging respectively to Western and Eastern Sicily, showed higher scavenging activity. Despite showing the same qualitative profile, significant variation in the anthocyanin quantitative content was observed across samples, as can be also seen from values reported in Table 1 and from overlapped chromatogram in Fig. S1. This aspect is mainly related to the genetic diversity among clones of the same cultivar. Similar results were recently reported for Malbec grape clones (Muñoz et al. 2014), which observed high variations across clones of the same grape variety. In this regard, although not many samples were employed in this study, the developed analytical method could be useful to correlate the expression of healthy relevant secondary metabolites in grape samples possessing genetic diversity that reflect the historical and geographical background of the region. Further work will be focused on a large-scale investigation which will be coupled to both genetic and statistical analyses.

Conclusions

Clonal selection, in which a plant within a wineyard that showed desirable traits is selected for propagation, is the most worldwide common method to improve the performance of wine grapevine cultivars (Carimi et al. 2011). However, in practice, it is very difficult to identify improved clones for polyphenolic compound production because they are responsive to environmental conditions that influence their content. Among grape polyphenols, anthocyanins are interesting molecules, which possess good antioxidant activity. In this work, we developed a fast and easy method for the screening of antioxidant anthocyanins in six Nero d'Avola clones. The method possesses several advantages in comparison with both pre-column and post-column antioxidant assays, such as shorter analysis time and no further hardware adjustment. Our results show that grape clones rich in delphinidin derivatives possess the lower IC_{50} values, since these molecules exhibit the highest scavenging ability. The proposed strategy can be the starting point to correlate chemical and genetic data and is well suited to select elite clones for winemaking as well as for nutraceuticals and functional foods.

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Ethics declarations

# Table 1 Quantitative data and scavenging activity of grape extracts 1-6

| | | Western Sicily clones | | | | | | Eastern Sicily clones | | | | | |
| | | Sample 1 F5P17 | | Sample 2 F9P17 | | Sample 3 F9P20 | | Sample 4 F14P39 | | Sample 5 F14P42 | | Sample | |
Peak	Compounds	mg/kg	% Inhibition DPPH	mg/kg	% Inhibition DPPH	mg/kg	% Inhibition DPPH	mg/kg	% Inhibition DPPH	mg/kg	% Inhibition DPPH	mg/kg	
1	Delphinidin 3-O-glucoside	112.52 ± 1.80	99.85 ± 0.04	324.22 ± 0.47	99.94 ± 0.02	54.93 ± 0.07	99.67 ± 0.14	151.81 ± 0.25	97.24 ± 0.06	173.98 ± 0.21	95.88 ± 0.12	$76.75 \pm$	
2	Cyanidin 3-O-glucoside	16.12 ± 0.36	43.85 ± 2.84	30.56 ± 0.16	68.57 ± 1.38	24.69 ± 0.01	60.81 ± 0.41	12.35 ± 0.01	19.71 ± 2.17	11.78 ± 0.07	25.75 ± 2.04	$9.28 \pm$	
3	Petunidin 3-O-glucoside	138.93 ± 0.85	69.69 ± 2.85	326.26 ± 0.31	94.49 ± 0.11	71.14 ± 0.06	74.42 ± 0.34	180.14 ± 0.12	49.66 ± 0.52	200.78 ± 0.20	52.98 ± 0.41	101.68	
4	Peonidin 3-O-glucoside	87.01 ± 0.37	30.67 ± 2.03	111.54 ± 0.34	42.70 ± 1.12	109.44 ± 0.08	41.84 ± 0.13	65.03 ± 0.09	17.33 ± 2.55	63.70 ± 0.04	18.98 ± 2.95	$49.38 \pm$	
5	Malvidin 3-O-glucoside	638.09 ± 1.47	37.50 ± 1.06	1244.04 ± 1.17	85.17 ± 0.08	383.89 ± 0.05	52.04 ± 0.11	861.37 ± 0.30	25.74 ± 0.66	955.40 ± 0.59	24.95 ± 0.60	513.39	
6	Petunidin 3-O-(6″-acetyl)-glucoside	18.78 ± 0.04	62.03 ± 0.73	29.51 ± 0.03	90.73 ± 0.01	9.98 ± 0.01	69.70 ± 0.14	20.09 ± 0.11	41.11 ± 1.84	20.38 ± 0.08	47.00 ± 1.13	$14.65 \pm$	
7	Peonidin 3-O-(6″-acetyl)-glucoside	13.01 ± 0.13	100.00 ± 0.01	12.37 ± 0.19	100.00 ± 0.01	16.32 ± 0.03	100.00 ± 0.01	10.24 ± 0.16	100.00 ± 0.01	8.10 ± 0.22	100.00 ± 0.01	$10.40 \pm$	
8	Delphinidin 3-O-(6″-p-coumaroyl)-glucoside	37.55 ± 0.14	74.30 ± 0.97	66.18 ± 0.51	83.20 ± 0.89	26.08 ± 0.02	56.06 ± 0.31	49.55 ± 0.29	80.96 ± 1.12	56.94 ± 0.11	86.34 ± 0.34	$32.05 \pm$	

| | | Western Sicily clones | | | | | | Eastern Sicily clones | | | | | | Sampl... |
| | | Sample 1 F5P17 | | Sample 2 F9P17 | | Sample 3 F9P20 | | Sample 4 F14P39 | | Sample 5 F14P42 | | | |
Peak	Compounds	mg/kg	% DPPH Inhibition	mg/kg	% DPPH Inhibition	mg/kg	% DPPH Inhibition	mg/kg	% DPPH Inhibition	mg/kg	% DPPH Inhibition	mg/kg
9	Malvidin 3-O-(6″-acetyl)-glucoside	127.58 ± 0.27	31.93 ± 0.98	171.10 ± 0.45	76.11 ± 0.52	78.96 ± 0.23	47.93 ± 1.13	135.31 ± 0.07	20.47 ± 0.33	138.50 ± 0.18	18.51 ± 0.61	104.24
10	Cyanidin 3-O (6″-p-coumaroyl)-glucoside	4.01 ± 0.001	100.00 ± 0.01	3.42 ± 0.03	62.46 ± 2.46	5.34 ± 0.03	81.07 ± 0.69	3.90 ± 0.02	53.02 ± 1.21	<LOQ	57.97 ± 2.49	4.07 ± (
11	Malvidin 3-O-(6″-p-caffeoyl)-glucoside	30.01 ± 0.05	90.20 ± 0.26	38.48 ± 0.07	80.41 ± 0.12	31.44 ± 0.01	75.92 ± 0.19	46.37 ± 0.03	47.88 ± 0.22	30.41 ± 0.15	51.42 ± 2.17	37.69 ±
12	Petunidin 3-O (6″-p-coumaroyl)-glucoside	37.30 ± 0.07	53.36 ± 0.99	67.90 ± 0.05	84.25 ± 0.18	25.74 ± 0.04	57.13 ± 0.26	50.42 ± 0.04	42.89 ± 0.56	58.87 ± 0.28	47.64 ± 1.57	31.24 ±
13	Peonidin 3-O (6″-p-coumaroyl)-glucoside	21.05 ± 0.06	20.73 ± 0.23	23.93 ± 0.12	7.86 ± 2.07	33.10 ± 0.03	34.83 ± 1.51	16.09 ± 0.23	10.05 ± 0.96	19.27 ± 0.35	14.29 ± 2.47	14.92 ±
14	Malvidin 3-O (6″-p-coumaroyl)-glucoside	221.76 ± 0.30	33.99 ± 0.09	404.03 ± 0.28	78.18 ± 0.33	172.66 ± 0.21	48.92 ± 0.86	304.32 ± 0.27	24.88 ± 0.86	354.79 ± 0.45	23.99 ± 0.98	195.15

**Table 2 ESI-IT-TOF anthocyanin profile of Nero d'Avola clones**

| Peak	Molecular formula	Compounds	$[M]^+$ theoric	$[M]^+$observed	MS/MS	Error ppm
1	$C_{21}H_{20}O_{12}$	Delphinidin 3-O-glucoside	465.1028	465.1037	303.0498	1.94
2	$C_{21}H_{20}O_{11}$	Cyanidin 3-O-glucoside	449.1070	449.1093	287.0550	5.57
3	$C_{22}H_{22}O_{12}$	Petunidin 3-O-glucoside	479.1184	479.1202	317.0646	3.76
4	$C_{22}H_{22}O_{11}$	Peonidin 3-O-glucoside	463.1235	463.1258	301.0703	4.97
5	$C_{23}H_{24}O_{12}$	Malvidin 3-O-glucoside	493.1351	493.1363	331.0803	2.43
6	$C_{24}H_{24}O_{13}$	Petunidin 3-O-(6″-acetyl)-glucoside	521.1218	521.1238	317.1542	3.75
7	$C_{24}H_{24}O_{13}$	Peonidin 3-O-(6″-acetyl)-glucoside	505.1344	505.1365	301.1047	4.07
8	$C_{30}H_{26}O_{14}$	Delphinidin 3-O-(6″-p-coumaroyl)-glucoside	611.1318	611.1337	465.8745/303.0542	3.15
9	$C_{24}H_{24}O_{13}$	Malvidin 3-O-(6″-acetyl)-glucoside	535.1483	535.1493	331.2574	1.96
10	$C_{30}H_{26}O_{13}$	Cyanidin 3-O (6″-p-coumaroyl)-glucoside	595.1445	595.1462	287.6209	2.78
11	$C_{32}H_{30}O_{15}$	Malvidin 3-O-(6″-p-caffeoyl)-glucoside	655.1918	655.1943	493.2901/331.2571	3.78
12	$C_{31}H_{28}O_{14}$	Petunidin 3-O (6″-p-coumaroyl)-glucoside	625.1572	625.1587	317.1546	2.44
13	$C_{31}H_{28}O_{13}$	Peonidin 3-O (6″-p-coumaroyl)-glucoside	609.1616	609.1658	301.1051	6.89
14	$C_{32}H_{30}O_{14}$	Malvidin 3-O (6″-p-coumaroyl)-glucoside	639.1698	639.1747	331.2580	7.67

Fig. 1
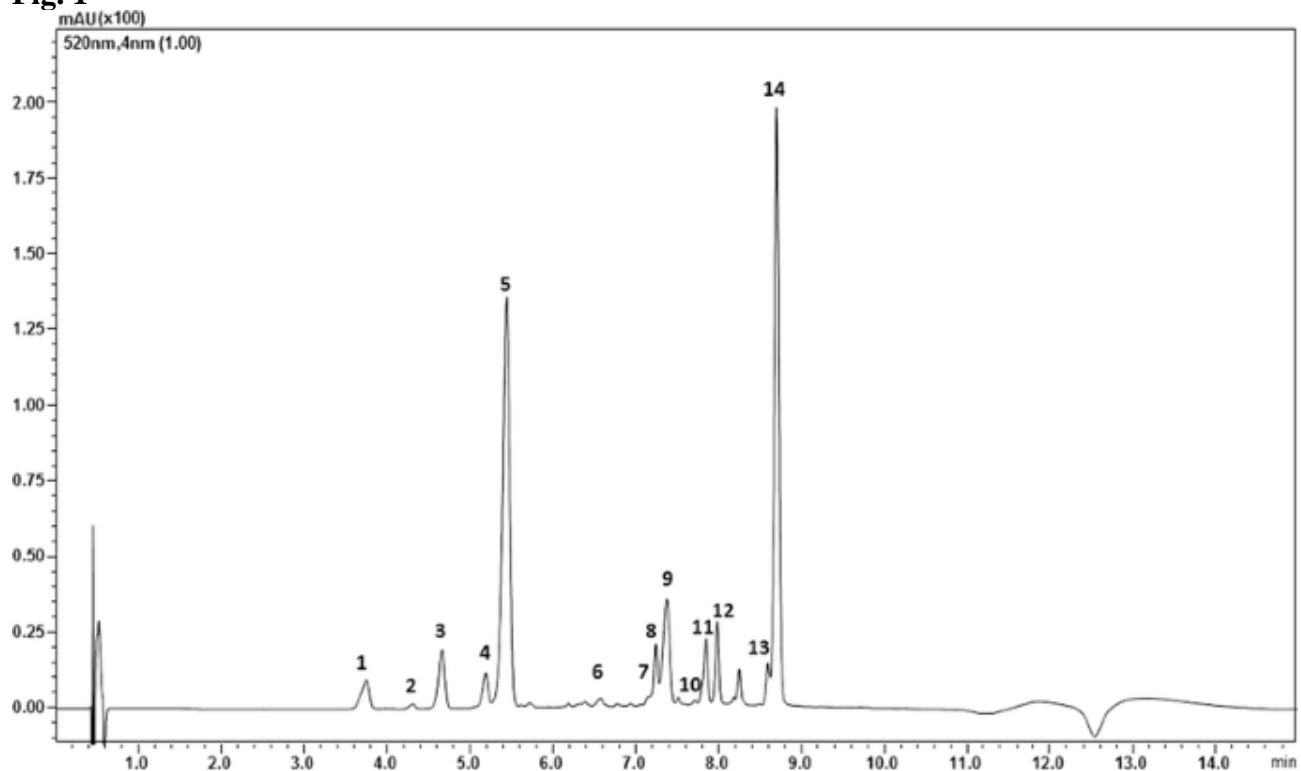
UV/Vis-UHPLC (520 nm) chromatogram of a Nero d'Avola grape extract from Eastern Sicily. Flow rate 0.5 mL/min, column oven 55 °C, injection volume 2 μL, column Kinetex™ C18 100 mm × 2.1 mm (L × I.D), 1.7 μm. For peak assignment, please see Table 2
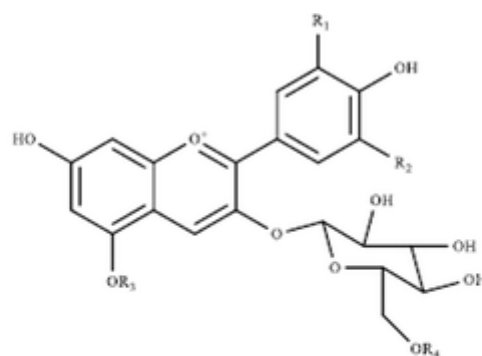
Fig. 2
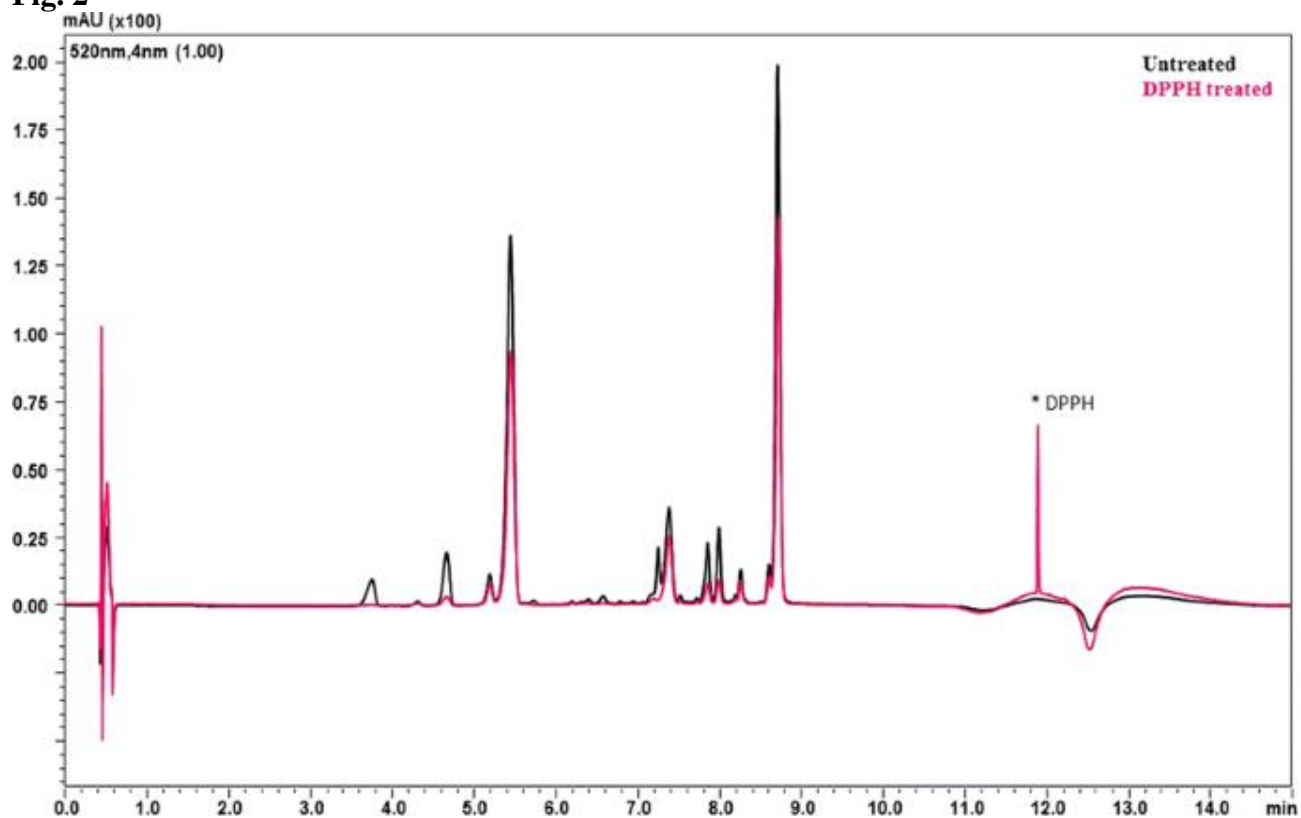
UV/Vis-UHPLC (520 nm) chromatograms of a Nero d'Avola grape extract before (*black line*) and after (*red line*) reaction with DPPH radical

Fig. 3
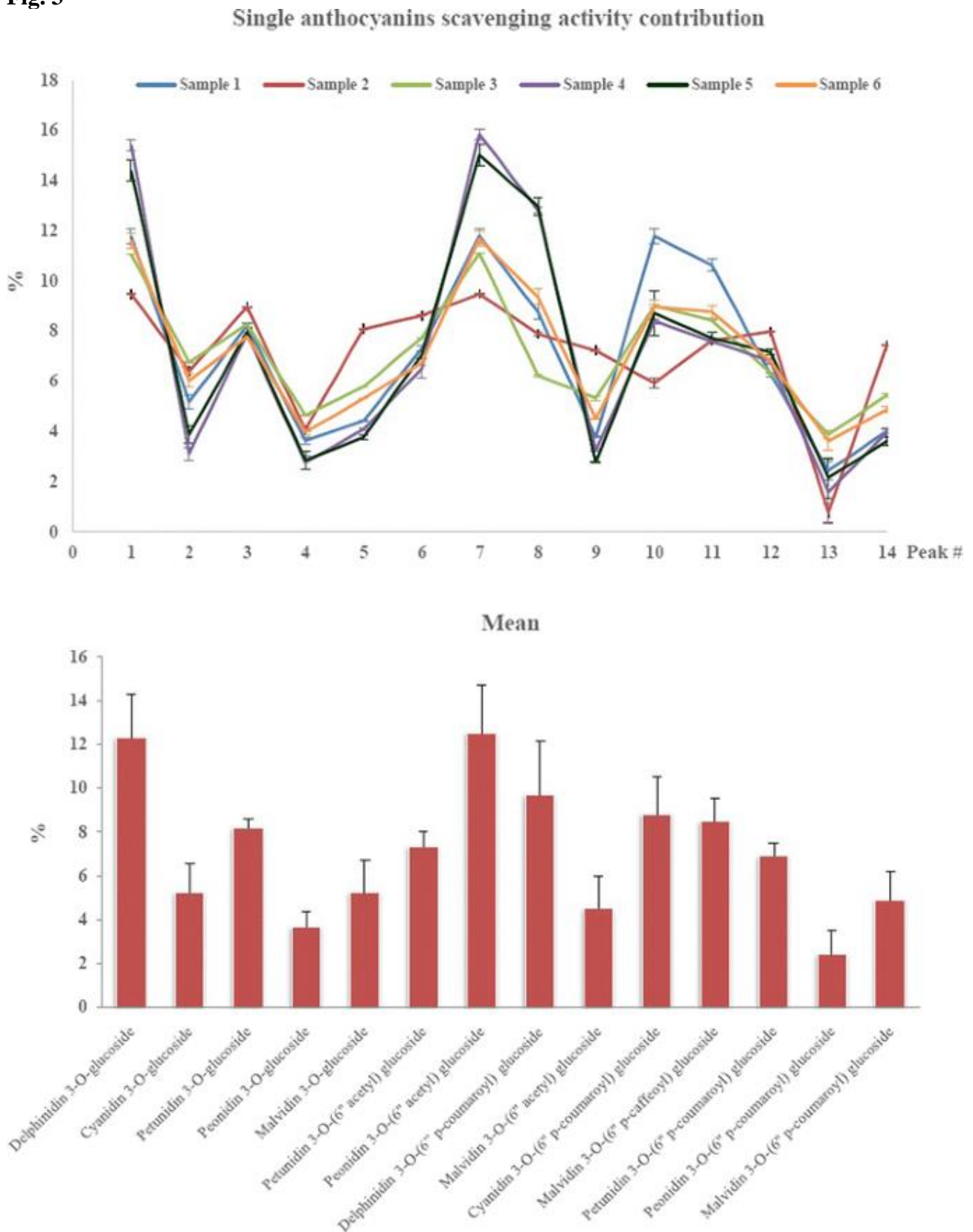
Contribution of single anthocyanins versus the total percent of DPPH scavenging activity and mean value

Fig. 4

Peak	Compounds	R_1	R_2	R_3	R_4
1	Delphinidin 3-*O*-glucoside	OH	OH	H	H
2	Cyanidin 3-*O*-glucoside	OH	H	H	H
3	Petunidin 3-*O*-glucoside	OCH₃	OH	H	H
4	Peonidin 3-*O*-glucoside	OCH₃	H	H	H
5	Malvidin 3-*O*-glucoside	OCH₃	OCH₃	H	H
6	Petunidin 3-*O*-(6" acetyl) glucoside	OCH₃	OH	H	acetyl
7	Peonidin 3-*O*-(6" acetyl) glucoside	OCH₃	H	H	acetyl
8	Delphinidin 3-*O*-(6" p-coumaroyl) glucoside	OH	OH	H	*p*-coumaryl
9	Malvidin 3-*O*-(6" acetyl) glucoside	OCH₃	OCH₃	H	acetyl
10	Cyanidin 3-*O* -(6" p-coumaroyl) glucoside	OH	H	H	*p*-coumaryl
11	Malvidin 3-*O*-(6" p-caffeoyl) glucoside	OCH₃	OCH₃	H	*p*-caffeoyl
12	Petunidin 3-*O*-(6" p-coumaroyl) glucoside	OCH₃	OH	H	*p*-coumaryl
13	Peonidin 3-*O*-(6" p-coumaroyl) glucoside	OCH₃	H	H	*p*-coumaryl
14	Malvidin 3-*O*-(6" p-coumaroyl) glucoside	OCH₃	OCH₃	H	*p*-coumaryl

Nero d'Avola grape anthocyanin structures