SUPPLEMENTARY MATERIAL

Nero d'Avola and Perricone cultivars: determination of polyphenols, flavonoids and anthocyanins in grapes and wines

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ABSTRACT

In 2011 vintage, the evolution of monomer and total anthocyanins, as well as of total flavonoids and polyphenols of grapes and wine of Nero d'Avola and Perricone, varieties cultivated in Sicily, were studied. Anthocyanin profiles are commonly used for grapevine cultivar identification because it is currently accepted that this trait is closely related to their genetic characteristics. The concentration of Nero d'Avola and Perricone anthocyanins was determined by HPLC-DAD.

Keywords: Nero D'Avola, Perricone, polyphenols, flavonoids, anthocyanins profile, HPLC-DAD.

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EXPERIMENTAL

Samples and Extraction

The study was conducted in 2011 vintage, in the periods indicated in Tab.1S.

The samplings were carried out, in triplicate, during the different stages of grapes ripening in order to analyze the content of the substances under investigation.

Also after the harvest and wine making, the samples of wine, obtained from Perricone and Nero d'Avola grapes, were collected for the analysis.

The skins of 50 grapes, for each rate, were weighed and put into 50 ml of tartaric buffer at pH 3.2 obtained dissolving 5 g of $C_4H_6O_6$, 22.2 ml of 1N NaOH, 2 g of $Na_2S_2O_5$, 125 ml of 95% ethanol and brought to 1 L with water). The samples were incubated for 3 hours in order to extract the phenol compounds and then stored at $-20^{\circ}C$ until analysis. The SO_2 used in the extraction solvent ensures polyphenol oxidase (PPO) inactivation, this enzyme is responsible for the phenol oxidations that causes a rapid browning in the in must and wine.

The samples were then homogenized for 5 minutes and centrifuged at 4000 rpm for 10 minutes, the extracts were collected in a 100 ml volumetric flask and brought to volume with tartaric buffer.

Both wine samples were made by skin fermentation with stem-contac method. After harvesting at optimal maturity ($22^{\circ}Brix$), vinification took place in small concrete vats that would allow more contact between the skins and the juice. To further encourage this mixing of the skins and the juice the wine was pumped-over.

As reported in literature the type of winemaking technology affect the levels of phenolic compounds of wine The stem-contact and the period of contact increased both total and polymeric phenol levels (Sun et al. 2001).

After bottling, were stored at room temperature, protected from light and heat, and analyzed in triplicate.

Total Phenolos Determinations

All spectrophotometer measurement were carried out by using a UV–Vis spectrophotometer (Thermo, MA, USA).

Total phenolic contents in the extract were evaluated using the Folin–Ciocalteu assay (Ivanova et al. 2010; Slinkard & Singleton 1977).

Briefly an aliquot (1 mL) of the samples, previously extracted and eluted in a Sep-Pak C18 Cartridge (300 mg sorbent per cartridge) (Waters, MA, USA), previously activated, with 2 ml of MeOH, was added to a 10 mL volumetric flask, containing 5 mL of distilled water. Then, 1 mL of Folin-Ciocalteu reagent, a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMO_{12}O_{40}$), was added and the contents mixed. After 3 min, 1.5 mL Na_2CO_3 solution of concentration 5 g/L was added and made up to a total volume of 10 mL distilled water. After 16 min incubation of the samples at $50^{\circ}C$ (water bath) in sealed flasks, samples were cooled and read their absorbencies at 750 nm. The total polyphenol content (PT) of the grapes (Singleton et al.1965), and wines (Di Stefano et al 1989; Di Stefano & Cravero 1991), was calculated using the following equations:

$$\begin{aligned} PT \text{ (mg/100 grapes)} &= E_{750nm} \times 173,3 \times (V_T/1000)/v \\ PT \text{ (mg/ grapes Kg)} &= E_{750nm} \times 173,3 \times (V_T/P)/v \\ PT \text{ (mg/L)} &= E_{1cm,750nm} \times 173,3/V \end{aligned}$$

E_{750nm} = (absorbance measured at 750)

173,3 = (coefficient of (+) - Catechin molar absorptivity)

V_T = (buffer volume in which the skin of 100 grapes was placed)

v = (extracted volume passed on the cartridge and incubated)

P = (100 grapes weight)

V = (wine volume)

Total Anthocyanins and Flavonoids determinations

Determination of total anthocyanins and flavonoids was performed by the method proposed by Di Stefano et al. (1989). For total skin anthocyanins and flavonoids analysis grape skin extract samples were concentrated and purified with Sep-Pak C18 Cartridge (300 mg sorbent per cartridge), previously activated with methanol to remove the sugar and the SO₂ content. The matrix tested was eluted with 1 ml of MeOH in a graduated flask, brought to volume with ethanol chloride (70 ml of C₂H₅OH, 30 ml of H₂O, 1 ml of 37% HCl) and analyzed by a UV-visible spectrophotometer, in a range from 230 nm to 700 nm (Di Stefano et al. 1989). Absorbance at 540 for cultivars in which B-ring tri substituted anthocyanins prevailed and absorbance at 536 nm for cultivars with high percentage of B-ring di substituted anthocyanins were recorded, and E₂₈₀ was calculated

For the determination of total anthocyanins (AT) and total flavonoids (FT) concentration, the following equations were used:

$$\text{Total anthocyanins mg L}^{-1} = 16.17 \times E_{540} \times d$$

$$\text{Total flavonoids mg L}^{-1} = 82.4 \times E_{280} \times d$$

MW/e = 16.17 for malvidin-3-glucoside in ethanol-HCl was calculated from e = 33,700 for malvidin-3-glucoside in methanol-HCl (Wulf & Nagel, 1978). The ratio of (+)-catechin concentration/E'₂₈₀ determined on a 10-mg L⁻¹ solution of (+)-catechin was 82.4. "d" represent the dilution coefficient of the extracts. E₂₈₀ was the length (in absorbance units) of the segment joining the peak at 280 nm of the spectrum of the skin extracts diluted in ethanol-HCl, with the intersection point between the perpendicular drawn from the 280-nm peak to the x axis and the tangent to the spectrum in the UV region (Corona et al. 2015).

Anthocyanin Profiles of Grape and Wine by HPLC-DAD

The anthocyanin pattern was determined by a HPLC method. Many authors have studied the phenolic compounds in grapes and wines using HPLC as the most suitable analytical technique (McMurrough and McDowell 1978; Porter et al. 1986; Wulf and Nagel 1978).

Extraction from grape skins was performed as reported in Squadrito (2007). For HPLC analysis, total extracts were acidified with 1 M H₃PO₄ (4.5 ml extract + 0.5 ml 1 M H₃PO₄), centrifuged (10,000 rpm) filtered through a 0.45 µm size nylon membrane filter, and placed in a 1.5 ml vial in an automatic sampler for injection.

Wine anthocyanins were pre-concentrated on Sep-Pak C18 (400 mg sorbent per cartridge) (Waters, MA, USA). A volume of wine, that could be sufficient to obtain an anthocyanin sample of 100-200 mg/l for HPLC analysis, was diluted with 5 × 10⁻³ M H₂SO₄ to an ethanol content of 3-4 % and passed through the C₁₈ cartridge, previously activated with 2 ml of methanol and 3 ml of 5 × 10⁻³ M H₂SO₄. After hydrophilic substances removal by washing with 2 ml of 5 × 10⁻³ M H₂SO₄ and cartridge dehydration, anthocyanins were recovered with methanol, collected in a 1.5 ml vial from the first colored drop (about 0.5 ml of CH₄O), diluted with an equal volume of solvent A (10 % HCOOH / 90 % H₂O v/v) and placed in the automatic sampler for HPLC injection (Squadrito et al. 2010).

For HPLC analysis an Agilent Technologies (Agilent, CA, USA) Series 1100 liquid chromatography equipped with a quaternary pump equipped with a microdegasser, thermostat autosampler, thermostat column compartment and DAD detector was used. The chromatographic conditions are those reported by Squadrito (2007).

SAMPLES	SAMPLING DATE
PERRICONE GRAPES	August 4
	August 11
	August 24
	September 28
PERRICONE WINE	December 4
NERO D'AVOLA GRAPES	August 4
	August 11
	August 24
	September 28
NERO D'AVOLA WINE	December 4

Tab.S1 –Sampling data of wine and grape samples examined

Fig. S1 – HPLC chromatogram of Perricone grapes

Fig. S2 – HPLC chromatogram of Nero d'Avola grapes

Fig.S3 – HPLC chromatogram of Nero d'Avola wine

Fig. S4 – HPLC chromatogram of Perricone wine

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