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Polyphenol metabolomics of twenty Italian red grape varieties

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Abstract. "Suspect screening analysis"method to study grape metabolomics, was performed. This method is a middle-way "targeted" and "untargeted"approach aiming at identifying the largest number of metabolites in grape samples. A new database of putative grape and wine metabolites (*GrapeMetabolomics*), which currently contains around 1,100 compounds, was constructed by CREA at Conegliano. By performing high-resolution mass spectrometry analysis of the grape extract in both positive and negative ionization mode, averaging 320-450 putative compounds are identified. Most of them are grape polyphenols, such as anthocyanins, flavonols and stilbene derivatives. By performing PCA and Cluster Analysis the composition in anthocyanins and flavonols of 20 Italian red grape varieties, was studied.

Introduction

Anthocyanins are polyphenolic compounds found in the grape skin that are responsible for the red color of the grapes and wine. These secondary metabolites play a key role both in terms of sensory profile and in terms of the health value, with antioxidant, antimicrobial, anticancer effects and protection of the cardiovascular system (De Pascual-Teresa et al., 2008).

The anthocyanins may also be used as natural dyes in food industry and as useful compounds for the pharmaceutical and nutraceutical industries.

V. vinifera flavonols are an interesting class of polyphenols both studied for their copigmentation with anthocyanins in red wines (Boulton, 2001) and for food and health implications (Manach al., 2004). Anthocyanins and flavonols are also widely studied for variety characterization (Mattivi et al., 2006).

In this work, a *suspect screening analysis* method developed for the grape metabolomics (Flamini et al., 2013) was used to study anthocyanins and flavonols of twenty Italian native red grape varieties. The relationship between variety and polyphenolic composition was studied by multivariate statistical analysis (PCA and Cluster Analysis).

Materials and methods

Red grape varieties Aglianico, Barbera, Cannonau, Cesanese d'Affile, Corvina, Enantio, Grignolino, Lambrusco Grasparossa, Montepulciano, Nebbiolo, Negroamaro, Nero d'Avola, Primitivo, Raboso Piave, Refosco dal Peduncolo Rosso, Rossese, Sagrantino, Sangiovese, Terrano, and Uva di Troia, were studied.

For each variety about 100 grape berries were collected at physiological maturity from the CREA-VIT grapevine germplasm collection (Susegana, TV, harvest 2013). Twenty berries were weighed. Following seed removal, the sample was homogenized using liquid nitrogen and the resulting powder immediately extracted with methanol ratio 2:1 (v/w) under stirring for 20 min. After the addition of 200 μL of 4′,5,7-trihydroxy flavanone (500 mg/L) as internal standard, the sample was centrifuged at 4000 g/min (10 °C) for 20 min. The supernatant was filtered on 0.22 μm filter and subjected to LC/MS analysis. Each sample was replicated twice. The analyses were performed in both negative and positive ionization mode by using a ultra-performance Agilent 1290 Infinity liquid chromatography system coupled to a high resolution time of flight mass spectrometer AgilentQ TOF 6540 (40,000 nominal resolution). The gradient chromatographic separation was performed on a reversed-phase column Zorbax (RRHD SB-C18 150 × 3 mm, 1.8 μm) and mobile phase composed of A) aqueous 0.1% v/v formic acid, B) acetonitrile containing 0.1% v/v formic acid, at a flow rate 0.4 mL/min Sample injection 10 μL. Settings of QTOF mass spectrometer: nitrogen sheath gas flow 10 L/min at 400 °C; nitrogen drying gas flow 8 L/min at 350 °C; nebulizer pressure 60 psi; cone voltage 1 kV (positive), 0 kV (negative); capillary voltage 3.5 kV. The signals were recorded in the m/z range 100-1700. Statistical analysis was performed using the software PAST 3.13 (Hammer et al., 2001).

Results and discussion

The utilization of database GrapeMetabolomics allowed the identification of 16 anthocyanins and 15 flavonols in each V. vinifera sample. This database of metabolites

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Table 1. Anthocyanins identified in *V. vinifera* grape extracts.

Anthocyanin	RT (min)	Formula	M^+ (*m/z*)
Delphinidin-3-*O*-glucoside	12.03	$C_{21}H_{21}O_{12}$	465.1028
Cyanidin-3-*O*-monoglucoside	12.57	$C_{21}H_{21}O_{11}$	449.1078
Petunidin-3-*O*-monoglucoside	12.74	$C_{22}H_{23}O_{12}$	479.1184
Peonidin-3-*O*-monoglucoside	13.12	$C_{22}H_{23}O_{11}$	463.1240
Malvidin-3-*O*-monoglucoside	13.27	$C_{23}H_{25}O_{12}$	493.1341
Delphinidin-3-*O*-(6-*O*-acetyl)monoglucoside	13.59	$C_{23}H_{23}O_{13}$	507.1133
Cyanidin-3-*O*-(6-*O*-acetyl)monoglucoside	14.06	$C_{23}H_{23}O_{12}$	491.1184
Petunidin-3-*O*-(6-*O*-acetyl)monoglucoside	14.14	$C_{24}H_{25}O_{13}$	521.1290
Malvidin-3-*O*-(6-*O*-acetyl)monoglucoside	14.64	$C_{25}H_{27}O_{13}$	535.1446
Peonidin-3-*O*-(6-*O*-acetyl)monoglucoside	14.65	$C_{24}H_{25}O_{12}$	505.1341
Delphinidin-3-(6-*O*-*p*-coumaroyl)monoglucoside	14.82	$C_{30}H_{27}O_{14}$	611.1395
Malvidin-3-(6-*O*-caffeoyl)monoglucoside	15.14	$C_{31}H_{31}O_{15}$	655.1663
Cyanidin-3-(6-*O*-*p*-coumaroyl)monoglucoside	15.24	$C_{30}H_{27}O_{13}$	595.1446
Petunidin-3-(6-*O*-*p*-coumaroyl)monoglucoside	15.30	$C_{31}H_{29}O_{14}$	625.1552
Malvidin-3-(6-*O*-*p*-coumaroyl)monoglucoside	15.77	$C_{32}H_{31}O_{14}$	639.1708
Peonidin-3-(6-*O*-*p*-coumaroyl)monoglucoside	15.78	$C_{31}H_{29}O_{13}$	609.1603

contains approximately 1,100 compounds and was expressly constructed to study grape metabolomics (Flamini et al., 2015). Anthocyanins identified in positive ion mode were delphinidin, cyanidin, petunidin, peonidin, and malvidin in monoglucoside, acetylmonoglucoside and p-coumaroyl-monoglucoside forms. Malvidin caffeoyl-monoglucoside was also identified. Compounds are reported in Table 1 with their M+ m/z signal.

Flavonols were detected in negative ion mode, the compounds identified are reported in Table 2 with their $[M\text{-}H]^-$ m/z signal. Among them also B-ring trisubstituted glycosides were identified (i.e. myricetin, larycitrin and syringetin), which are typical of red grape varieties.

Due to the lack of standards available, semi-quantitative analysis was performed by normalization of the signal area with that of the internal standard. This method allowed the comparison among the different varieties. Multivariate analysis (PCA and cluster analysis) to study the effect of variety on these secondary metabolites, was performed, and results are shown in the Fig. 1 and Fig. 2.

As may be seen from the figures, was possible classify the samples into five groups on the basis of their polyphenolic profile: A) Rossese, B) Aglianico, Cannonau,

Table 2. Flavonols identified in *V. vinifera* grape extracts.

Flavonols	RT (min)	Formula	$[M\text{-}H]^-$ (*m/z*)
Myricetin-3-*O*-glucuronide	14.26	$C_{21}H_{18}O_{14}$	493.0624
Myricetin-3-*O*-glucoside	14.31	$C_{21}H_{20}O_{13}$	479.0831
Dihydroquercetin-3-*O*-hexoside	14.40	$C_{21}H_{22}O_{12}$	465.1038
Rutin	14.68	$C_{27}H_{30}O_{16}$	609.1461
Quercetin-3-*O*-galactoside	15.00	$C_{21}H_{20}O_{12}$	463.0882
Quercetin-3-*O*-glucuronide	15.02	$C_{21}H_{18}O_{13}$	477.0675
Larycitrin-3-*O*-hexoside	15.06	$C_{22}H_{22}O_{13}$	493.0988
Quercetin-3-*O*-glucoside	15.06	$C_{21}H_{20}O_{12}$	463.0882
Dihydroquercetin-3-*O*-rhamnoside	15.34	$C_{21}H_{2}2O_{11}$	449.1089
Kaempferol-3-*O*-galactoside	15.47	$C_{21}H_{20}O_{11}$	447.0933
Kaempferol-3-O-glucoside	15.65	$C_{21}H_{20}O_{11}$	447.0933
Kaempferol-3-*O*-glucuronide	15.66	$C_{21}H_{18}O_{12}$	461.0725
Syringetin-3-*O*-glucoside	15.73	$C_{23}H_{24}O_{13}$	507.1144
Isorhamnetin-3-*O*-hexoside	15.78	$C_{22}H_{22}O_{12}$	477.1038
Dihydrokaempferol-3-*O*-ramnoside	16.08	$C_{21}H_{22}O_{10}$	433.1140

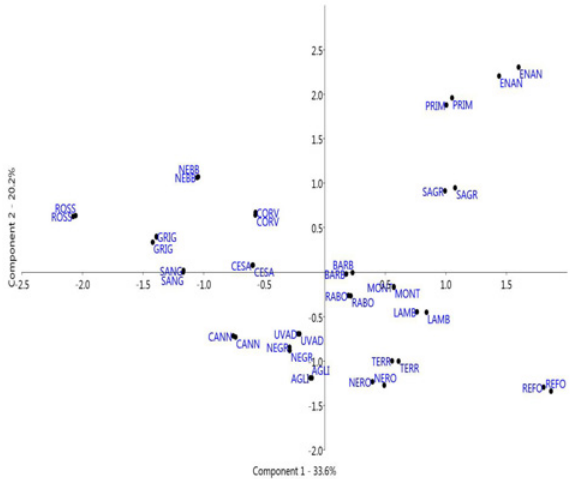

Figure 1. PCA analysis performed by using anthocyanin and flavonol signals measured in the extracts of the 20 red grape varieties studied.

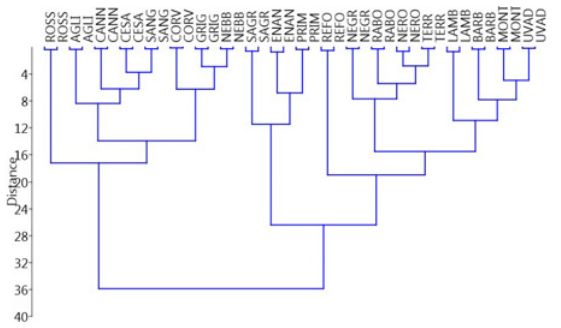

Figure 2. Hierarchical clustering analysis (Ward's method, Euclidean similarity index) performed by using anthocyanin and flavonol signals measured in the extracts of the 20 red grape varieties studied.

Cesanese d'Affile, Sangiovese, Corvina, Grignolino, Nebbiolo, C) Sagrantino, Enantio, Primitivo, D) Refosco dal peduncolo rosso, E) Negroamaro, Raboso Piave, Nero d'Avola, Terrano, Lambrusco Grasparossa, Barbera, Montepulciano, Uva di Troia.

Conclusions

Anthocyanins and flavonols are secondary metabolites useful for grape chemotaxonomy because of their dependence on genetic factors. The metabolomic approach was associated with the statistical analysis of qualitative and semi quantitative data. This approach proved to be useful tool for the varietal characterization of the vine. Specifically, it was possible to clearly divide the twenty Italian native varieties into five groups according to their polyphenolic profile. This result may be supplemented and confirmed by the analysis of different vintages.

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