ANTHOCYANIN PROFILE AND ANTIOXIDANT ACTIVITY OF UNFERMENTED GRAPE DERIVATIVES

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ABSTRACT

Since the first observations of the "French paradox", numerous studies have been developed to test the antioxidant and health-promoting effects of phenolic compounds present in grapes and wine. These products are also employed as ingredients of dietary supplements. The aim of this study was to correlate the antioxidant activity with the total polyphenol content (TPC) and the HPLC anthocyanin profiles of grape derivatives (fresh grape juices and dietary supplements). Anthocyanins were analyzed by HPLC using a gradient elution. The antioxidant activity was measured spectophotometrically at 517 nm after reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Total polyphenol content (TPC) was determined with the Folin-Ciocalteu colorimetric assay. Our data demonstrate that non fermented grape derivatives contain polyphenols in concentration that can justify their use as an alternative source for antioxidant compounds.

A partire dalle prime osservazioni del "paradosso francese", sono stati avviati numerosi studi per valutare l'attività antiossidante e gli effetti benefici dei composti fenolici presenti nell'uva e derivati. Tra questi vanno inclusi anche gli ingredienti di integratori alimentari. Scopo dello studio è stato quello di correlare l'attività antiossidante con il contenuto in polifenoli totali (TPC) e antocianine, in prodotti derivati dall'uva quali succhi d'uva e integratori alimentari. Le antocianine sono state analizzate tramite HPLC utilizzando un'eluizione in gradiente. L'attività antiossidante è stata misurata per via spettrofotometrica a 517 nm in seguito a reazione con il radicale libero 1,1-difenil-2-picrilidrazile. Il TPC è stato determinato mediante dosaggio colorimetrico di Folin-Ciocalteu. I risultati ottenuti mostrano come i prodotti non fermentati derivanti dall'uva contengano polifenoli, in concentrazioni tali da giustificarne l'utilizzo, quale fonte alternativa di composti antiossidanti.

INTRODUCTION

Since the first observations of the "French paradox" (Renaud and De Lorgeril, 1992), numerous studies have demonstrated the antioxidant and the health promoting effects of the

phenolic compounds present in grapes and wine. *Vitis vinifera* fruits contain several non-flavonoid compounds in the pulp, and flavonoid compounds in the skins, seeds and stems. Total concentrations of phenolic compounds were measured by some authors and were about 2178.8, 374.6, 23.8 and 351.6 mg/g GAE (Gallic Acid Equivalents) in seed, skin, pulp, and leaf, respectively (Pastrana-Bonilla et al., 2003). The reduction of coronary heart disease (CHD) mortality in moderate wine consumers suggested a possible protective effect of wine, but quantification of active compounds in plasma and urine after consumption of light to moderate amounts of wine suggested that plasma and urine concentrations reached values in the order of ppb (μg/L) or even less (Waterhouse, 2002). This amount is significantly lower (up to 10 or even 100-fold) than that required for a physiological role as shown by *in vitro* tests and animal studies. On the other hand, wine market shows a decreasing trend due to negative campaign associated with abuse of alcoholic beverages 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.* This market situation has led to a considerable interest in the evaluation of winery by-products as a potential source of phenolic compounds to be used as functional food ingredients (Shrikhande, 2000). Besides the grape pomace used to produce spirits, two other winery by-products are seeds and skins, discarded from white wine and juice production. The main anthocyanins identified in *Vitis vinifera* spp. are the 3-O-monoglucoside and the 3-O-acylated monoglucosides of the five main anthocyanins: delphinidin, cyaniding, petunidin, peonidin and malvidin (Mazza and Miniati, 1993). In *Vitis vinifera* spp., flavonols exist as the 3-O-glycosides of myricetin, quercetin, kaempherol and isorhamnetin. Glucose, galactose, and glucuronide acid are the main sugar units. Grape seeds, skin and stems are also an important source of proanthocyanidins (PROs), oligomers and polymers of (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate. Skin and stems also contain prodelphinidins, oligomers and polymers of (-)-epigallocatechin and trace amounts of (+)-gallocatechin and (-)-epigallocatechin gallate (Monagas et al., 2005). Grape pulp contains mainly non-flavonoid compounds, as stilbenes, hydroxycinnamic acids and benzoic acids and organic acids (tartaric, malic and citric acids). PROs and non-flavonoid compounds are considered responsible for the healthy effects associated with the consumption of grape juice, such as the improvement of the endothelial function, the increase of the serum antioxidant capacity, the protection of LDLs against oxidation, the decrease of native plasma protein oxidation and the reduction of platelet aggregation (Chou *et al*., 2001). Extracts from *Vitis vinifera* L. are commonly used to formulate dietary supplements, but although the dietary industry based on wine by-products is rapidly growing, practically no scientific research has been performed on the wide range of products currently available on the market. The aim of this study was the correlation of the antioxidant activity with the total polyphenol content (TPC) and the HPLC anthocyanin profiles in two types of grape derivatives: fresh grape juices and dietary supplements.

MATERIALS AND METHODS

Samples. The samples assayed in this study were: 3 fresh red grape juices; 3 fresh white grape juices; 2 red wines; 1 white wine; 1 food supplement containing grape skin extract; 2 food supplements containing grape seeds; 2 food supplements containing grape leave extract. Grapes juices were freshly prepared by corresponding grapes immediately before the analysis. To prepare fresh juices, about 100 g of berries from each type of grape were weighed, pressed and centrifuged at 5000 rpm for 40 minutes at 10 °C. The juice obtained (about 50 mL) was filtered by using a paper filter. In the case of food supplements, 0.5-3 g of each product were

added to 50 mL of 0.025 M KCl pH 1, vortexed, sonicated for 10 minutes and filtered on filter paper.

Total polyphenol content

Total polyphenol content (TPC) was determined according to the Folin-Ciocalteu method as reported by Singleton and Rossi (Singleton, Rossi, 1965). Aliquots of 300 μL from different samples were mixed in test tubes with: 1.5 mL of Folin-Ciocalteu's reagent (Sigma Aldrich, Germany) diluted 10 times, and 1.2 mL of 7.5% sodium carbonate (Sigma Aldrich, Germany). After 30 minutes, the absorbance was measured at 765 nm in a UV-visible spectrophotometer (Varian Cary 50 SCAN, Palo Alto, California, U.S.A.). Results were expressed as equivalents of gallic acid (GA) in mg/L for juices and wines, mg/g for supplements.

Antioxidant activity

The antioxidant activity (AOA) was determined spectrophotometrically as a measure of radical scavenging using 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) (Brand-Williams et al.; Leong, Shui, 2002). Aliquots of 1 mL of DPPH (Sigma Aldrich) solubilised in methanol (5 mg/100 mL) were mixed with 0.5 mL of each sample. The absorbance was measured after 30 minutes at 517 nm. The amount of sample necessary to inhibit by 50% the absorbance of DPPH represents the IC50. The concentration of antioxidants was calculated from the calibration curve considering the dilution factors used in the samples preparation.

Total Anthocyanin Content

Total anthocyanin content was determined according to the AOAC method. This method is based on the property of anthocyanin pigments to change colour at different pHs. The difference in absorbance at 520 nm is proportional to the pigment concentration. The absorbance of samples, opportunely diluted with pH 1.0 and pH 4.5 buffer, was measured both at 520 and 700 nm, using the last lecture to correct for haze. Results are expressed on as cyanidin-3-glucoside, according the following relationship:

Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L) = A x MW x DF x 1000/e x 1
Where:
$A = (A_{520nm} - A_{700nm})$ pH 1.0 – $(A_{520nm} - A_{700nm})$ pH 4.5;
MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu);
DF = dilution factor;
l = path length in cm;
e = 26 900 molar extinction coefficient,
L x mol–1 x cm–1, for cyd-3-glu;
1000 is the factor for conversion from g to mg.

High Performance Liquid Chromatography (HPLC)

Standard solutions of the anthocyanins delphinidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside (Extrasynthese, Lyon, France) were prepared by adding 1 mg of each analyte in 10 mL of distilled water. Analyses of anthocyanins in samples (prepared as reported in materials and methods) were performed using a Thermo Separation Products (TSP) system (San Jose, CA) equipped with a TSP UV 6000 LP photodiode array detector. Separations were achieved on a reverse phase Synergi 40, MAX-RP 80 A column, 25 cm long x 4.6 mm, 4 μm i.d. supplied by Phenomenex (Torrance,

CA, USA). The column temperature was maintained at 40°C. A flow rate of 0.8 mL/min and a run time of approximately 45 minutes were used. Solvent A was: water/ formic acid/ acetonitrile 87:10:3 (v/v/v); solvent B was: water/formic acid/ acetonitrile 40:10:50 (v/v/v). The gradient used was: 0-15 min from 94 to 70% A;15-30 min from 70 to 50% A; 30-35 min from 50 to 40% A; 35-41 min from 40 to 94% A. The elution profile was monitored at 254-600 nm. The peak areas were measured at 520 nm.

RESULTS AND DISCUSSION

Total polyphenols content, antioxidant activity and total anthocyanin content

The total polyphenol content (TPC), the antioxidant activity (AOA) and the total anthocyanin content (TAC) measured in different samples are listed in Tables 3-4.

Table 3 - Total polyphenol content (TPC), antioxidant activity (AOA) and total anthocyanin content (TAC) of fresh grape juices and wines (mean ± SD)

Samples analyzed	TPC (mg GA/L)	AOA (mg GA/L)	TAC (cyanidin-3-glucoside equivalents mg/L)
Red wine	3117.5 ± 40.7	717.3 ± 10.9	235.8 ± 0.5
White wine	223.6 ± 1.6	23.7 ± 0.5	N.D.
Fresh red grape juice 1	1441.8 ± 1.6	463.8 ± 0.5	207.4 ± 3.5
Fresh red grape juice 2	1217.7 ± 4.2	343.5 ± 3.1	126.0 ± 0.2
Fresh red grape juice 3	1570.5 ± 1.6	495.2 ± 2.4	45.3 ± 0.1
Fresh white grape juice 1	593.9 ± 4.8	273.7 ± 2.8	N.D.
Fresh white grape juice 2	1099.2 ± 9.6	456.5 ± 2.8	N.D.
Fresh white grape juice 3	212.4 ± 6.3	58.0 ± 5.5	N.D.

Table 4 - Total polyphenol content (TPC), antioxidant activity (AOA) and total anthocyanin content (TAC) of dietary supplements containing grape derivatives (mean ± SD)

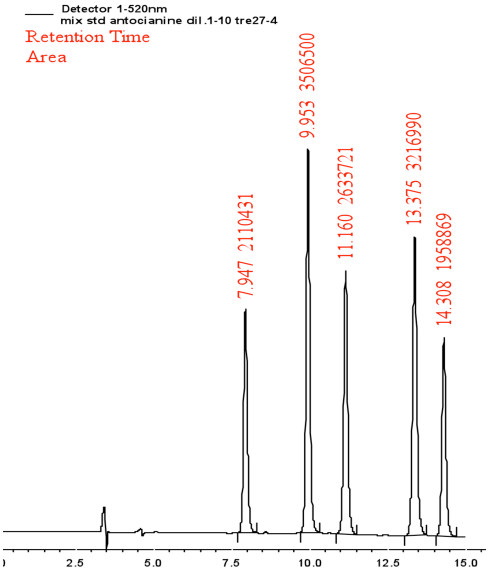
Samples analyzed	TPC (mg GA/g)	AOA (mg GA/g)	TAC (cyanidin-3-glucoside equivalents mg/g)
Food supplement containing grape skin	284.8 ± 6.7	2.25 ± 0.02	354.5 ± 12.3
Food supplement containing grape seeds	49.5 ± 2.2	12.0 ± 0.01	N.D.
Food supplement containing grape leaves 1	84.4 ± 5.2	6.3 ± 0.004	117.0 ± 4.2
Food supplement containing grape leaves 2	37.3 ± 1.5	0.19 ± 0.00004	23.0 ± 1.1

The data presented in Table 3 show that red wine sample has the highest polyphenol content (3117.5±40.7 mg GA/L), followed by the fresh red grape juice 3 (1570.5±1.6 mg GA/L) and

1 (1441.8 ± 1.6 mg GA/L). Unexpectedly, the white grape juice number 3 showed a very high TPC value when compared to red grape juices. A correlation between total polyphenol content and the corresponding antioxidant activity can be appreciated only in some samples. This could be due to the influence of the different flavonoid and non-flavonoid subgroups on the antioxidant capacity; for example, from data in Table 3 is possible to observe that anthocyanins contribute in different manner to the antioxidant activity. Fresh red juice shows a higher antixidant activity, but TAC value is the lowest among red derivatives. Furthemore, the chemical structure of phenolic compounds, particularly the number or position of OH and OCH_3 groups could affect antioxidant capacity (Finotti et al., 2003). The degree of polymerization and the ratio between monomeric and polymeric compounds can also influence the inhibition of radicals, that tends to increase with the order of polymerization. Finally, wine phenolic composition could be influenced by different factors: grape variety, vineyard location, agronomical approaches, climate, soil type, harvesting time, oenological process and ageing. In the case of red wine, TPC and AOA could be modulated by the phenolic composition of wood and by the condensation/polymerization reactions which take place in wine during maturation (Bartolomè et al., 2004). Considering the data shown in Table 4, higher TPC and TAC are present in the grape skin extract, followed by food supplement containing grape leaves 1. Both food supplements, containing grape leaves, showed a very low antioxidant activity, while a good antioxidant profile could be appreciated in supplement containing grape seed extract, confirming that other flavonoids, such as catechins and procyanidins, can contribute to the antioxidant activity. Significative differences between the two food supplements containing grape leaves (different brands) was observed.

High Performance Liquid Chromatography (HPLC)

Figure 1 shows the HPLC chromatogram of a mixture of standard solutions, containing 5 anthocyanins, with their relative retention times, peaks areas and concentrations. Figure 2 shows the chromatogram of a food supplement containing grape skin.



Name	Retention Time (min)	Concentration (µg/mL)	Area
Delfinidin-3-Glu	7.947	80	2110431
Cyanidin-3-Glu	9.953	100	3506500
Petunidin-3-Glu	11.160	100	3633721
Peonidin-3-Glu	13.375	100	1958869
Malvidin-3-Glu	14.308	100	1958869

Figure 1- HPLC chromatogram of a standard solution and retention times, concentrations, peak areas of anthocyanin present in standard solution

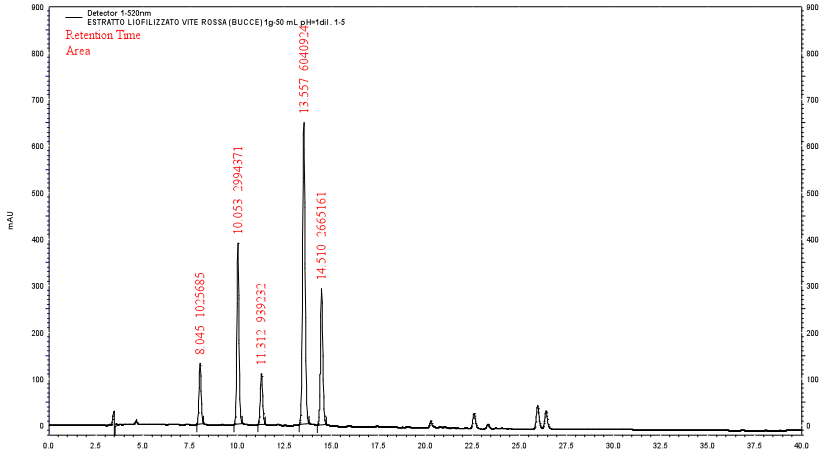


Figure 2 – HPLC chromatogram of a food upplement containing grape skin

Peak identification was made according to the retention times of authentic standards: the identification was then confirmed by comparing the UV spectra of peaks present in samples and those of the corresponding standard. Figures 3-4 show the anthocyanins concentration in fresh red grape juices, red wine and in food supplements.

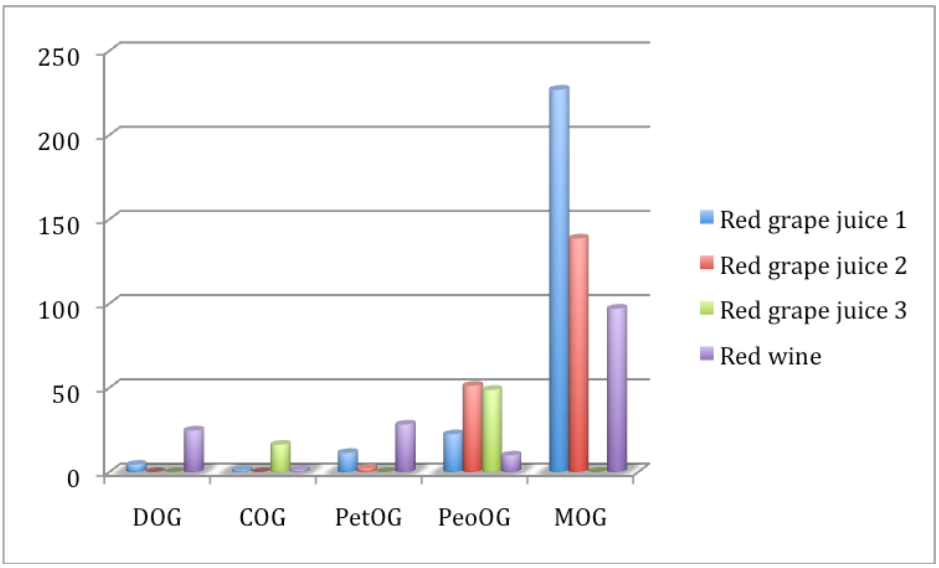


Figure 3 – Anthocyanins content in red grape juices and red wine

Legenda

DOG = Delphinidin-3-O-glucoside, COG = Cyandin-3-O-glucoside,
PetOG = Petunidin-3-O-glucoside, PeOG =Peonidin-3-O-glucoside
MOG = Malvidin-3-O-glucoside

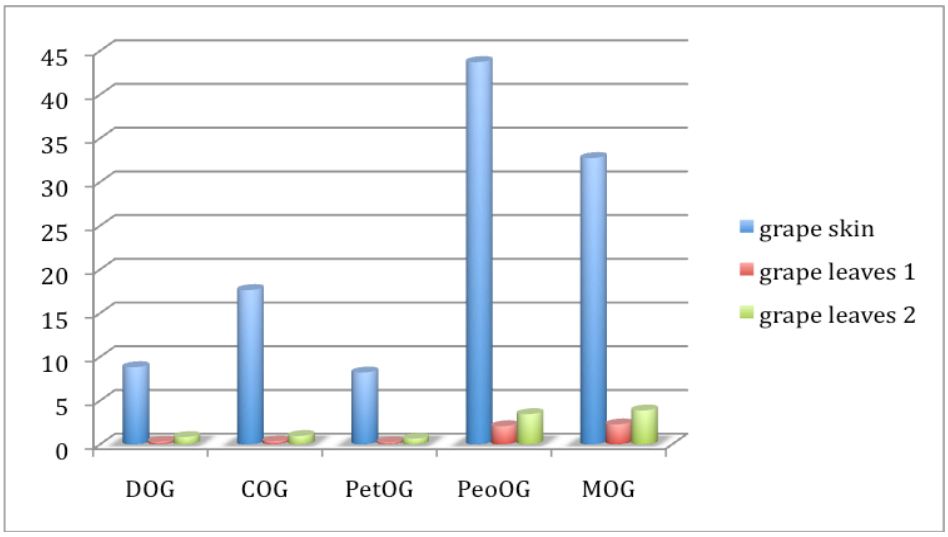


Figure 4 - Anthocyanins content in dietary supplements

Legenda

DOG = Delphinidin-3-O-glucoside, COG = Cyandin-3-O-glucoside,
PetOG = Petunidin-3-O-glucoside, PeOG =Peonidin-3-O-glucoside
MOG = Malvidin-3-O-glucoside

In grape juices and in the red wine, the most representative anthocyanins are the malvidin-3-O-glucoside and peonidin-3-Oglucoside. Red grape juice 3 contains only peonidin-3-glucoside and cyanidin 3-O-glucoside, justifying the relative low value of TAC (45.29 ± 0.08 mg/L). Among food supplements, grape skin extract has the highest content of five anthocyanins measured, with a particular abundance in peonidin 3-O-glucoside. These data confirm the hypotesis that grape skin are good sources of antioxidant compunds, so that this by-product could be a suitable ingredient for dietary supplements. Anthocyanin profile in supplements containing leaves is quite poor when compared to those containing grape skin.

CONCLUSIONS

We investigated the total polyphenol content, the antioxidant activity and the total anthocyanin content of fresh grape juices and grape dietary supplement. As expected, red wine and red fresh grape juices presented the highest TPC and AOA. These data are very important because they could justify the promotion of grape juices as a source of polyphenols and other health-promoting substances. Also red grape skin extract showed a good polyphenol profile, but this is not always quantitatively correlated to the antioxidant activity. In grape leave extracts, high levels of anthocyanins contributed poorly to the antioxidant activity. The differences in antioxidant activity could be associated with contribution of different phenolic molecules, with the polymerization degree of the anthocyanins, with the different grape varieties, the production processes and, for wine, wine-making process and ageing (Di Majo *et al.*, 2008). Moreover, the fermentation process leads to phenolic derivatives and polymeric anthocianins with higher antioxidant activity. Besides the investigation of polyphenol profile in grape derivatives, it is necessary to consider bioavailabilty of these compounds. To date, there are still few satisfactory data on this topic, but phenolic compounds in wine and grape seem not sufficiently bioavailable in vivo to be active at cellular level. Probably, the phenolic compounds, which are present as soluble forms in wine, should be more available than polymeric, insoluble and compartimentalized forms present in grape and in other fruits; this

hypotesis is still under discussion (Manach et al., 2004). Furthemore, polyphenol bioavailabilty could be affected by matrix effect of food (for example binding to proteins) or of various parameters of gut physiology (pH, intestinal fermentation, etc.) (Soleas et al., 1997), but the paucity of human data on metabolic parameters does not allow definitive conclusions.

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