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Free resveratrol monomers in varietal red and white wines from Dalmatia (Croatia)

VIŠNJA KATALINIĆ[1]*
IVICA LJUBENKOV[2]
IVAN PEZO[3]
IVANA GENERALIĆ[1]
OLIVERA STRIČEVIĆ[1]
MLADEN MILOŠ[1]
DARKO MODUN[4]
MLADEN BOBAN[4]

[1]Faculty of Chemistry and Technology
University of Split, Teslina 10
21000 Split, Croatia

[2]SMS-Nutrition Development Center
Kurtovići bb, 21231 Klis, Croatia

[3]Institute of Adriatic Crops and Carst
Reclamation, Put Duilova 11
21000 Split, Croatia

[4]School of Medicine, University of Split
Šoltanska 21000 Split, Croatia

Correspondence:
Višnja Katalinić
Faculty of Chemistry and Technology
University of Split
Teslina 10, 21000 Split, Croatia
E-mail: visnja.katalinic@ktf-split.hr

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Abstract

Introduction: *Resveratrol is considered to be one of the major antioxidant constituents in red wine. In this article we investigated the presence of, and relation between* trans- *and* cis-*resveratrol monomers in the most characteristic varietal wines from Dalmatia (Croatia), produced according to the Croatian appellation of origin system.*

Materials and Methods: *The wines of red grape varieties (*Plavac mali, Merlot, Cabernet sauvignon, Babić, Plavina, Trnjak, Vranac *and* Lasin*) and white grape varieties (*Cetinka, Pošip, Maraština, Debit, Kujundžuša, Malvasija Dubrovačka, Vugava, Medna *and* Zlatarica*), vintage 2004, were analyzed in June 2005. Standard methods of analysis for general wine components were used for preliminary control of the selected wines. Resveratrol monomers in wine were measured by HPLC.*

Results: *Significant differences were found in the phenolic composition even between the wines produced from same grape varieties, but originating from different localities. Mean concentration of free resveratrol monomers was 0.43 mg/L (range 0.11–1.04) in white wines, and 2.98 mg/L (range 0.5–8.57) in red wines. The average relative amount of* cis-*resveratrol in white wines was almost 2-fold higher in comparison with red wines. A significant impact of grape variety on resveratrol content in wine was confirmed. Except in wine Merlot, high concentrations of free resveratrol monomers were found in red wines produced from autochthonous grape variety* Plavac mali*. Among white wines, the highest concentrations were detected in wine Zlatarica.*

Conclusion: *According to the obtained results, some red wines from the region of Dalmatia could be a good dietary source of resveratrol.*

INTRODUCTION

Stilbenoids are phenol-based plant metabolites widely represented in nature and implicated in benefits for human health regarding problems such as cancer, inflammation, neurodegenerative disease, and heart disease *(1–10)*. Among stilbenes, the phytoalexin resveratrol (*trans*-3,5,4'-trihydroxystilbene) has attracted immense attention due to its numerous biological properties. It is the parent compound of a family of molecules, including glucosides and polymers, existing in *cis* and *trans* configurations in a narrow range of spermatophytes of which vines, peanuts and pines are the prime representatives. Its synthesis from *p*-coumaryl CoA and malonyl CoA is induced by stress, such as injury, infection or UV-irradiation, and it is classified as a phytoalexin

anti-fungicide conferring disease resistance in the plant kingdom *(11–13)*. Resveratrol has been identified from a number of dietary sources including red wines and berry fruits. It is also consumed in the forms of botanical dietary supplements and herbal formulations used in traditional Chinese medicine and Indian Ayurvedic medicine where it is commonly used as an active ingredient *(14)*. In the western world the interest in stilbene derivatives present in grapevines was stimulated when epidemiological studies showed an inverse correlation between moderate wine consumption and the incidence of cardiovascular diseases, the so called »French paradox» *(6, 15)*. Resveratrol is considered to be one of the major antioxidant constituents in red wine *(16)*. In grape berries resveratrol synthesis is primarily located in the skin and seed cells and it is absent or low in the fruit flesh. The variety of grapes also plays an important role in resveratrol synthesis which may be genetically controlled *(17, 18)*. The most important factors affecting the content of stilbenes in wine are their concentrations in grape, the winemaking technology and their changes during wine aging process *(19–21)*. Despite their biological importance, there is a lack of systematic investigations of *cis*- and *trans*-resveratrol content in the Croatian varietal wines. In this article we investigated the presence of, and relation between *trans*- and *cis*-resveratrol monomers in the most characteristic varietal wines from Dalmatia (Croatia), produced according to the Croatian appellation of origin system.

MATERIAL AND METHODS

2.1. Materials and chemicals

Chemicals. All of the chemicals and reagents were of pro analysis purity and were purchased from Sigma-Aldrich (Steineheim, Germany), Merck (Darmstadt, Germany) and Kemika (Zagreb, Croatia). The *trans*-resveratrol was acquired from Sigma (Milwaukee, USA). The stock solution of resveratrol *cis*-isomer was prepared by UV irradiation at 254 nm of alcoholic solution of *trans*-resveratrol according to Romero-Perez *at al.* (1996).

Material. We analyzed 20 samples of red and 15 samples of white wines of various grape varieties (Red: *Plavac mali, Merlot, Cabernet sauvignon, Babić, Plavina, Trnjak, Vranac, and Lasin*; White: *Cetinka, Pošip, Maraština, Debit, Kujundžuša, Malvasija Dubrovačka, Vugava, Medna and Zlatarica*) from the region of Dalmatia (Croatia). The samples were derived from pure grape varieties. Excluding *Merlot, Cabernet sauvignon* and *Vranac* which are introduced red vine cultivars, the selected wines were produced from native Dalmatian grape cultivars. All samples were wines from vintage 2004, produced according to the Croatian appellation of origin system, and were obtained thanks to the Institute of Adriatic Crops and Carst Reclamation, Split, Croatia, which conducts control over the production of the mentioned wines. Wine samples were analyzed in June 2005, after the wine processing had been completed.

2.2. Methods

Standard methods of analysis of common analytical wine components (wine density, alcohol, total and sugar free extract, total and volatile acidity, pH, and total and free SO_2) were used for preliminary control of the quality of selected wines. Controlled wine components (results not presented) were in accordance with Croatian wine quality regulations and Croatian appellation of origin system. To determine the phenolic composition of wines, total phenol content, phenol index, as well as flavonoid and anthocyanin (unpolymerized pigments) content were analyzed. The separation, identification and quantification of *trans*- and *cis*-resveratrol monomers were performed using reversed-phase high-performance liquid chromatography (HPLC-RP).

2.2.1 Total phenols, flavonoids, anthocyanins

Total phenolic compounds and phenolic fractions including flavonoids and anthocyanins were determined using spectrophotometric methods *(22–24)* on UV–VIS spectrophotometer (double beam) Specord 200, Analytik Jena GmbH, Germany.

Total phenols. Total phenolic concentration in selected wine samples was determined spectrophotometrically according to the Folin-Ciocalteu (FC) colorimetric method *(22, 23),* with calibration against gallic acid standards and expression of results as mg of gallic acid equivalents (GAE)/L. Data presented are average of three measurements.

Flavonoids. Red wine samples were treated with formaldehyde to precipitate flavonoid phenolic compounds. The method was developed by Kramling and Singleton, 1969 *(22)*. Formaldehyde reacts with 6- or 8-position on 5,7-dihydroxy flavonoids forming a methylol derivative that will attach to another 6- or 8-position on another flavonoid and so on. These condensed molecules were removed by filtration. The remaining phenolics (non-flavonoids) were analyzed by the Folin-Ciocalteu procedure *(22)*. The amount of flavonoids was calculated as a difference between total phenols and nonflavonoids in wine (also reported in mg GAE /L of wine). The presented data are the average of three measurements.

Anthocyanins. The anthocyanin content in wines was determined using bisulfite bleaching method *(24, 25)*. Sulfur dioxide additions cause changes in absorbance in unpolymerized pigments but not in condensed or polymerized pigments *(22)*. Data presented are average of three measurements.

2.2.2. Determination of free resveratrol monomers

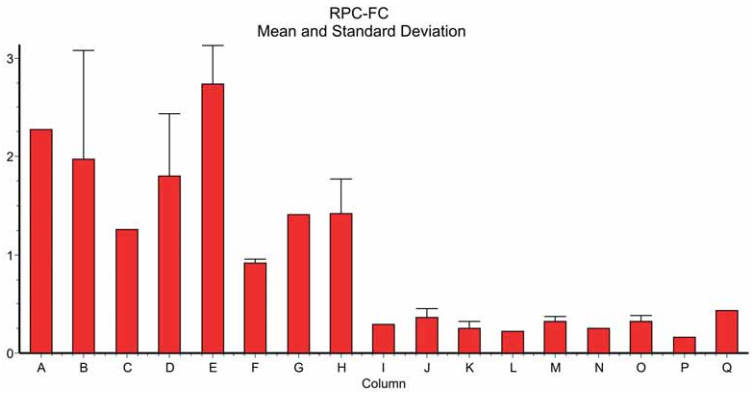
The concentrations of free *trans*- and *cis*-resveratrol monomers in selected wines were determined using a direct injection isocratic UV-HPLC method *(26)*. The HPLC system used was composed by a Varian UV-VIS PDA 330 detector, a ternary gradient liquid Pro Star 230 pump, column heater model 500 and Star chromatography workstation version 6.0. Free resveratrol monomers

TABLE 1

Total phenols, flavonoids, anthocyanins, cis-resveratrol and trans-resveratrol in the analyzed wine samples from Dalmatia (Croatia).

		Color	Location	Concentrationof phenols (mg/L)			Stilbenes (R–resveratrol)		
Cultivars	Number of samples			Total phenols	Flavonoids	Anthocyanins	*trans-* R	cis-R	*cis-* + *trans-*R
Babić	N =1	R	Primošten, Bucavac	2269	1611	455	2.66	0.24	2.90
Cabernet S.	N=2	R	Vrgoračko polje	2757	2329	481	0.98	0.10	1.08
			Dubrovnik Konavle	1189	832	245	0.48	0.03	0.51
Lasin	N=1	R	Imotski	1258	707	298	1.53	0.34	1.87
Merlot	N=5	R	Vrgorac, Vrgoračko polje	2460	1994	376	5.98	1.40	7.38
			Drniš, Petrovo polje	825	420	227	0.91	0.22	1.13
			*Drniš, Oklaj	1761	1139	551	5.95	2.09	7.04
			*Drniš, Petrovo polje	1715	1164	455	1.63	0.44	2.00
			Dubrovnik Konavle	2249	1769	551	6.74	1.83	8.57
Plavac mali	N =6	R	Pelješac, Dingač	3028	2577	376	1.15	0.52	1.67
			Pelješac, Postup	3068	2692	341	4.32	0.64	4.96
			Pelješac, spec. položaj	1997	1790	324	4.42	0.58	5.00
			*Hvar, Sv. Nedilja	2711	2310	341	0.14	1.10	1.24
			Hvar, Sv. Nedilja	2877	2467	306	0.08	0.96	1.04
			Brač, Murvica	2752	2392	358	1.97	1.03	3.00
Plavina	N=2	R	Šibenik, Donje polje	941	489	88	1.11	0.09	1.20
			Drniš, Petrovo polje	890	458	158	1.74	0.38	2.12
Trnjak	N=1	R	Imotski, Lokvičići	1413	945	411	0.54	0.14	0.68
Vranac	N=2	R	Vrgorac, Vrgoračko polje	1670	1180	289	2.72	0.24	2.96
			Dubrovnik Konavle	1177	636	420	1.79	0.14	1.93
Cetinka	N=1	W	Korčula, Blato	291	56	n.d.	0.31	0.10	0.41
Debit	N=2	W	Drniš, Oklaj	306	68	n.d	0.31	0.19	0.50
			Drniš, Petrovo polje	424	97	n.d	0.22	0.04	0.26
Kujundžuša	N=1	W	Imotsko polje	198	34	n.d.	0.31	0.19	0.50
			Imotski, Proložac	303	63	n.d.	0.16	0.19	0.35
Malvasija D.	N=1	W	Dubrovnik Konavle	219	50	n.d.	0.34	0.08	0.42
Maraština	N=4	W	Šibenik, Donje polje	268	68	n.d.	0.10	0.06	0.16
			Korčula, Čara	379	92	n.d.	0.52	0.27	0.79
			Drniš, Oklaj	355	74	n.d.	0.40	0.27	0.67
			Benkovac, Pristeg	286	63	n.d.	0.13	0.02	0.15
Medna	N=1	W	Vrgorac, Kozica	252	45	n.d.	0.09	0.02	0.11
Pošip	N=2	W	Korčula, Smokvica	270	58	n.d.	0.30	0.19	0.49
			Korčula, Čara	364	70	n.d.	0.45	0.26	0.71
Vugava	N=1	W	Vis, Viško polje	161	24	n.d.	0.24	0.15	0.39
Zlatarica	N=1	W	Vrgorac, Kozica	431	82	n.d.	0.74	0.30	1.04

* barrique wine



Red: A – Babić (N=1); B – Cabernet sauvignon (N=2), C – Lasin (N=1); D – Merlot (N=5); E – Plavac mali (N=6); F – Plavina (N=2); G – Trnjak (N=1); H – Vranac (N=2);
White: I – Cetinka (N=1); J – Debit (N=2); K – Kujundžuša(N=2); L – Malvasija Dubrovačka (N=1); M – Maraština (N=4); N – Medna (N=1); O – Pošip (N=2), P – Vugava (N=1); Q – Zlatarica (N=1)

Figure 1. *The reducing power capacity (RPC-FC) in monovarietal red and white wines.*

were separated on an octadecyl column Microsorb-MW 100 (150 mm L x 4.6 mm i.d., 5-µm particle size diameter; purchased from Varian) maintained at 35 °C. Wine samples were filtered in 0.45 µm membrane and directly injected through a 20 µL fixed loop into a guard C18 column. Red wine samples were 6- times diluted with an eluent prior to injection. Reversed phase HPLC was performed with an isocratic elution (1.0 mL min^{-1} flow rate) using H_2O: Acetonitril (75:25) solution as an eluent. The pH of solution was adjusted to 3.00 by using concentrated H_3PO_4. The signal was monitored at 306 nm wavelength. *trans*-Resveratrol and *cis*-resveratrol peaks were identified by their retention times using internal standard method. The stock solution of *cis*-isomer was prepared according to Romero-Perez *at al.,* 1996 *(27)*. Each sample was injected three times in the chromatographic system. The amount of resveratrol was calculated as a sum of free *trans*- and *cis*-resveratrol monomers.

2.3. Reducing power capacity of selected wines – RPC-FC

There are several methods proposed for routine assessment of antioxidant capacity (AOC) in foods, botanicals, nutraceuticals and other dietary supplements *(28)* The Folin-Ciocalteu (FC) method is an electron transfer based assay for determination of reducing capacity which is usually expressed as phenolic content based on electrons' chemical reducing capacity (relative to an equivalent reducing capacity of gallic acid) *(28)*. Reducing power capacity RPC-FC of controlled wine samples was calculated as a ratio between A1 and A2 at 765 nm. A1 is absorption of wine sample and A2 is absorption of a standard (1000 mg/L gallic acid) after oxidation of phenols by molybdotungstate reagent using FC method. RPC-FC was used for quick comparison of AOC of tested wine samples.

2.4. Statistical analysis

The t-test was used to compare whether the mean of a variable differs between red wines and white wines. The direction and magnitude of correlation between variables was done using the analysis of variance (ANOVA) and quantified by the correlation factor »r«. The P-value ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Results of determining phenolic composition of selected monovarietal red and white wines from Dalmatia (Croatia) made by traditional winemaking technologies, vintage 2004, are presented in Table 1. and Figures 1–5. As expected there was a wide range of phenol concentrations in selected wines. Selected red wines had significantly higher amounts of total phenols, flavonoids and anthocyanins compared to white wines (Table 1). This is due to a greater grape skin and seed contact time and temperature for the fermentation process for red wines. The mean level of total phenols in red wines was 1950±765 mg GAE/L (range 825 –3068), and flavonoids 1495 mg GAE/L (range 420–2692). The average relative amount of flavonoids in phenolic composition of red wines was almost 80 %. Especially rich in phenolic compounds were the Plavac wines, produced from the autochthonous grape variety *Plavac mali,* cultivated on special localities on Pelješac peninsula. Selected white wines had significantly lower content of total phenols (mean 301±79 mg GAE/L; range 161–431), and flavonoids (mean 63 mg GAE/L; range 24–97). The ability of polyphenolic compounds to act as antioxidants has been well documented and is related to the presence of numerous low-molecular mass phenols, mainly flavonoids. The levels of total phenols in wines determined according to the FC method are not absolute measurements of the amounts of phenolic materials but are, in fact, based on their chemical reducing capacity relative to the equivalent reducing capacity of gallic acid. For that reason this method is proposed for routine assessment of reducing antioxidant capacity (RPC-FC) by Prior *et al.,* 2005 *(28)*. Also, extremely significant linear (Pearson) correlation ($P<0.0001$) was confirmed between total phenolics and

related antioxidant capacity of plant extracts determined as ferric reducing/antioxidant power (FRAP) *(29)*. According to the obtained RPC-FC results (Figure 1) it is obvious that red wines have greater reduction power capacity than white wines. The mean RPC-FC for selected red wines was 2.01±0.74, and for selected white wines 0.30±0.08. Significant differences in reduction power capacity of some red wines can be related to differences in flavonoid content, especially anthocyanins which represent a numerous flavonoid subgroup of very efficient free radical scavengers with confirmed excellent antioxidant properties (Table 1, Figure 1).

It is known that the pathways of flavonoid and stilbene synthesis are very similar. The two closely related polyketide synthases: chalcone synthase (CHS) and stilbene synthase (STS) are key enzymes in the biosynthesis of flavonoids and stilbene phytoalexins, respectively. Both enzymes use the same substrates with the same stoichiometry, so the relationship between CHS and STS may potentially be a competitive one *(30)*. A question arises about interrelation of the level of flavonoid or certain flavonoid subgroups level and stilbene level in wines. Through statistical data analysis, significant linear (Pearson) correlation was confirmed between the levels of anthocyanins and related levels of resveratrol in selected red wines ($P<0.05$), which implies that the activity of CHS does not inhibit the activity of STS.

Selected white wines had significantly lower content of total phenols (mean 301±79 mg GAE/L; range 161–431), and flavonoids (mean 63 mg GAE/L; range 24–97), their average RPC-FC was significantly lower and they cannot be considered as a rich source of polyphenolic antioxidants in nutrition.

Stilbenes belong to a non-flavonoid class of phenolic compounds. The interest in this group of phenolic compounds in wines was stimulated when epidemiological studies showed an inverse relationship between consumption of red wine and incidence of cardiovascular diseases (2). It is generally accepted that polyphenols in wine are responsible for this benefit. In recent years there has been growing interest in stilbenes and, among stilbene monomers, *trans*-resveratrol has been the most widely studied grapevine phytoalexin for its impact on human

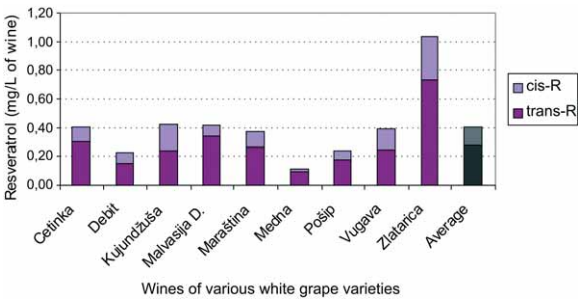


Figure 2. *The concentrations of the free* cis- *and* trans- *resveratrol* (cis- *and* trans-*R) in white wines, vintage 2004, produced from native Dalmatian (Croatia) grape cultivars. Wines were produced according to the Croatian appellation of origin system.*

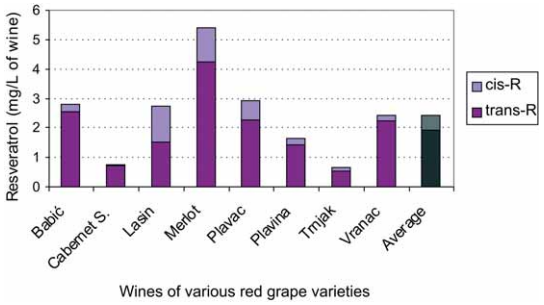


Figure 3. *The concentrations of the free* cis- *and* trans- *resveratrol (cis- and trans-R) in red wines, vintage 2004, produced from native* (Babić, Lasin, Plavac mali, Plavina, Trnjak) *and introduced* (Cabernet Sauvignon, Merlot and Vranac) *grape cultivars from Dalmatia (Croatia). Wines were produced according to the Croatian appellation of origin system.*

health. Over 1000 citations have indicated that *trans*-resveratrol has a great potential for treatment of a wide variety of diseases. Many studies have been carried out to analyze the concentration of *cis*- and especially *trans*--resveratrol in red and white wines: French *(31),* Italian *(32),* Brazilian *(26),* Slovenian *(33),* Californian *(34),* Greek *(17),* Aragon *(35)* and many others *(11, 27)*. As far as authors are aware, there are no reports in the literature about the presence of, and relation between *trans*- and *cis*-resveratrol in wines from Dalmatia (Croatia).

Results of *trans*- and *cis*- resveratrol contents in selected monovarietal red and white wines are shown in Table 1. Significant differences were found in phenolic composition, even between the wines produced from same grape varieties but originating from different localities. The concentration of free resveratrol monomers (*cis*- and *trans*-) in white and red wines ranged from 0.11 – 1.04 mg/L (mean 0.46±0.25) in white wines, and from 0.5 – 8.57 mg/L (mean 2.92±2.40) in red wines. The average concentrations of resveratrol in selected wines as a function of grape variety are shown in Figure 2 and Figure 3. The impact of grape variety on resveratrol content in wines can be noticed. According to the obtained results, the highest levels were found in wines produced from grapes *Merlot* followed by *Plavac mali, Babić, Lasin, Vranac,* and *Plavina* cultivars. The lowest levels of resveratrol were found in Cabernet sauvignon and Trnjak wines. The mean content of this important bioactive compound in selected red wines from Dalmatia was similar or higher to that reported by other researchers *(35–37)*. Among white wines, the highest concentrations were detected in wine Zlatarica.

Results also point to differences in resveratrol level even in wines produced from the same grape variety, but different wine producers. For example, resveratrol concentration in red wines produced from autochthonous grape variety *Plavac mali* ranged from 1.04 to 5.00 mg/L, with the highest resveratrol contents found in the famous red wine *Postup* and wine *Plavac* from a special micro-locality. Resveratrol concentration in *Merlot* wines ranged from 1.13 to 8.57 mg/L. These differences were not unexpected since it is known that wine-making techniques af-

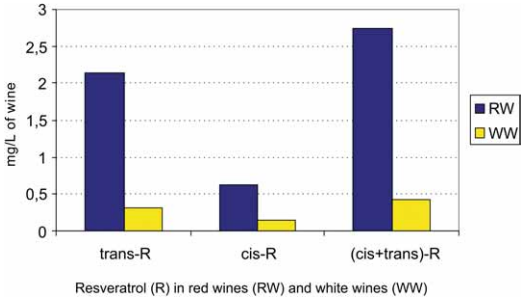


Figure 4. *The average content of* cis- *and* trans- *resveratrol monomers in red (RW) and white (WW) Dalmatian wines.*

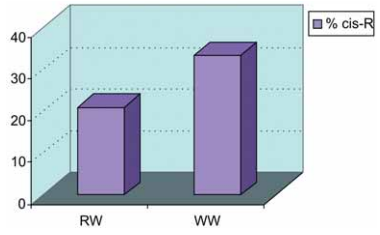


Figure 5. *The average relative amount of* cis-*resveratrol isomer in selected 20 red and 15 white wines from Dalmatia (RW – red wines; WW – white wines).*

fect resveratrol content of wines. The results emphasize the need of optimizing the vinification process to obtain resveratrol-rich wine.

The dominant monomer was *trans*-resveratrol, as expected (Figure 4). The mean level of trans-resveratrol was 2.34±2.05 mg/L of red wine (range 0.08–6.74), and 0.31±0.17 mg/L of white wine (range 0.09–0.74). The average relative amount of *cis*-resveratrol in selected white wines was significantly higher in comparison to selected red wines (Figure 5). As it is generally accepted that resveratrol is produced as a *trans*-isomer and the *cis*-isomer is derived by isomerization of the trans-resveratrol mainly during fermentation *(38),* it can be supposed that other phenolic compounds have a »protective« effect regardless of the *trans*-isomer stability in wine.

CONCLUSION

According to the obtained results, some red wines from the region of Dalmatia (Croatia), rich in polyphenolic compounds, could be a good dietary source of resveratrol. Special attention in future investigation should be paid to native red grape cultivars like Babić, Lasin or Plavina, and especially to the autochthonous grape cultivar Plavac mali which yields anthocyanin-rich wines with high reducing power capacity. In this cultivator certain producers have managed to preserve free resveratrol monomers in the amount sufficient to make them interesting nutritional products.

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