Determination of *trans*- and *cis*-Resveratrol in Serbian Commercial Wines

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Abstract

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a phytoalexin produced by grapevines in response to fungal infection, particularly to Botrytis cinerea. It has been shown that it possess various biological effects such as prevention of cardiovascular diseases and anti-inflammatory and anticancerogenic properties. Red wines are a primary source of resveratrol. Although a number of investigations have focused on the determination of resveratrol in wines of different countries, there is no similar study about the wines produced in Serbia. As authors are aware, the only study concerning resveratrol content in wine in the Balkan region was conducted in Greece. In this study, the *trans*- and *cis*-resveratrol content in samples obtained from 18 commercial Serbian wines (10 red, 7 white, and 1 rose) were analyzed. Analyses were performed after solid-phase extraction by high-performance liquid chromatography with a diode array detection system using an RP-C_{18} column with gradient elution [solvent A: acetonitrile–acetic acid–water (20:2:78 v/v), solvent B: acetonitrile–acetic acid–water (90:2:8 v/v)]. Detection of *trans*- and *cis*-resveratrol was performed on 306 and 286 nm, respectively. It was clearly established that there was a presence of *trans*-resveratrol isomers in all analyzed wines (0.11–1.69 mg/L) except in one white wine. *Cis*-resveratrol was present in 12 from 18 samples in different amounts (0.12–1.49 mg/L).

Introduction

It is well-known that grapes and related products contain large amounts of phenolic compounds, which have multiple positive effects on human health. Phenolic compounds present in grape berry skins influence the sensory characteristics of wine such as color, flavor, and astringency. As well as many other plant phenolics, they have been reported to have multiple biological effects including antifungal, antimicrobial, and disease-resistant properties (1,2). At high concentrations (1000–1800 mg/L), a large part of phenolics found in wines may act as antioxidants (3). Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a phytoalexin present in a wide variety of plant species including mulberries, peanuts, and grapes (4,5). Phytoalexins are a group of low-molecular-mass substances with microbial inhibitory activity (6), and they are produced by the plant as a defense response to some exogenous stimuli, such as UV radiation, chemical stressors, and particularly, microbial infections (4). Grapes and related products are considered as the most important dietary sources of resveratrol (7,8).

Resveratrol exists in two isomeric forms. The *trans*-isomer occurs in the berry skins of most grape cultivars, and its synthesis is stimulated by UV light, injury, and fungal infection (4,9,10). Red wine seems to be the main dietary source of *trans*-resveratrol because it is present in much higher level in red than in white wines and grapes. (11)

Cis-isomer is produced by UV irradiation of the *trans*-isomer (11). It is generally absent or only slightly detectable in grapes, but both isomers are present in variable amounts in commercial wines (11,12).

Resveratrol accounts for 1% or less of total red wine polyphenols. Concentration of this compound in wine increases during fermentation on the skins, but the extracted amount depends on the variety and enological conditions (13). Accordingly, through acquiring knowledge on absorption, metabolism, and biochemical activity in humans, resveratrol concentration is linked to the selectivity and sensitivity of analytical methods (14).

So far, many investigations have been conducted that suggest multiple positive effects of resveratrol on human health. Resveratrol may be responsible in part for the decreased mortality by cardiovascular diseases (15) and linked to the reduced cardiac disease rates observed in moderate and regular wine-consuming populations (16). This effect is the so-called French paradox, and it was a trigger for the wave of interest in monitoring the presence of resveratrol in wine. Some authors claim that phenolic compounds are present only in red wine but not in white and contribute to lower incidence of cardiovascular disease in French people (17). But, in recent publications, it is suggested that white wine consumption also affords benefits due to the presence of other antioxidants, which are also found in olive oil (18).

Also, the studies earlier reported support a role of dietary resveratrol in the prevention of cardiovascular diseases. This

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potential effect could be due to the ability of resveratrol to inhibit the oxidation of human low-density lipoprotein (19), block platelet aggregation (20), eicosanoid synthesis (21), and produce vasodilatation (22). Numerous lines of evidence suggest that resveratrol exibit antioxidative activity and that it can protect LDL against peroxidative degradation. (23) However, in recent studies, some authors suggest that protective effect of red wine polyphenols including resveratrol is independent of any antioxidant action of these compounds (24). Moreover, resveratrol possesses anticancer properties, and as phytoestrogen, it may favorably influence several physiological processes (14). The carcinopreventive activity of resveratrol has been also proved (25). It has been shown that resveratrol has cancer chemopreventive activity in assays representing three major stages of carcinogenesis (initiation, promotion, and progression) and that it has great potential in the prevention and therapy of wide variety of tumors (26,23).

Cis-resveratrol is not reported as a natural constituent of grape berries (27,28). However, recently, Moreno et al. detected *cis*-isomer in red grapes skin (11). Still, *cis*-resveratrol and its glucoside have been detected in many of analyzed wines so far, regardless of the origin and the technology applied. More likely, *cis*-resveratrol is derived from its *trans*-isomer during vinification, although there are no clear suggestions on this subject (29). Additionally, there is no evidence about factors that could facilitate such conversion during winemaking (30).

Trials conducted under different conditions show that *trans*-resveratrol is stable for months when protected from light, except when it is in high-pH buffers. However, *cis*-resveratrol was stable only near pH neutrality when completely protected from light (12). There has been no specific information on the properties of *cis*-resveratrol, and its light sensitivity has made it difficult to purify. Physiological activity of *cis*-resveratrol has only been described once and was shown to have potential anticancer activity, as do the *trans*-isomers, by inhibiting kinase activity, a cancer-related factor (31). It has not been clear yet whether biological activity of *cis*-form differs from *trans*-isoform.

The Serbian vineyards consist mainly of native cultivars (*V. vinifera* L.) with unknown potential of resveratrol biosynthesis. As far as the authors are aware, the resveratrol content of monovarietal Serbian appellation of origin wines has not been determined until present. Hence, it was of great interest to perform a survey of *trans*- and *cis*-resveratrol content of some wines from this region. This study presents the first report concerning the concentration of resveratrol in Serbian wines. Analysis of *trans*- and *cis*-resveratrol content in 18 commercial wines (10 red, 7 white, and 1 rose) produced in Serbia was carried out by high-performance liquid chromatography (HPLC).

Materials and Methods

Chemicals

Methanol and acetonitrile used were HPLC-grade (JT Baker, Deventer, Holland), and acetic acid was p.a.-grade (99.5%, Polskie Odczynniki Chemiczne, Gliwice, Poland). Ultra-pure water was generated with TKA water purification system (Germany, Niederelbert), including reverse osmosis, activated carbon, and ion-exchange cartridges. *Trans*-resveratrol (99%) was purchased from Sigma Chemicals (Steinheim, Germany). *Cis*-resveratrol was obtained in a laboratory from *trans*-resveratrol by photochemical transformation (10 h sunlight exposure of the *trans*-isomers).

Instrumentation

An HPLC consisting of an autosampler Hitachi 655A-40, two pump system Hitachi 655A-11 with gradient controller Hitachi L-5000 (Pleasanton, California), and UV detector Gilson (Middleton, Wisconsin) was used. Data processing as well as on-line quantification was performed by using CSW32 software (DataApex, Prague, Czech Republic).

Samples

Serbian bottled wines, 10 red, 7 white, and 1 rose, from the 2000–2004 vintage were analyzed for their *trans*- and *cis*-resveratrol content. The wines were purchased from wine producers and kept at 4°C in darkness until analyzed. Wines were filtered immediately after opening using teflon filters (0.45 µm, Agilent, Wilmington, Delaware). Before analysis extraction was performed on C_{18} solid-phase. Wine samples (5 mL) were applied to LiChrolut RP_{18} extraction cartridges (Merck, Darmstadt, Germany) after conditioning with 5 mL of methanol and 5 mL water, washed with water (5 mL) and eluted with methanol (1 mL). This solution was directly analyzed by HPLC.

Analytical HPLC procedure

Resveratrol content in wine was measured after separation by Bischoff Hyperchrome ODS Hypersyl reversed-phase HPLC column (250 × 4.6 mm i.d., 5µm, Leonberg, Germany). Binary gradient consisted of solvent A: acetonitrile–acetic acid–water (20:2:78 v/v) and solvent B: acetonitrile–acetic acid–water (90:2:8 v/v) as follows: linear gradient from 0% to 10% B in 8 min, 10–15% B in 12 min, 15–30% B in 10 min, 30–50% B in 5 min, 50–100% B in 50 min, and 2 min 100% B isocratic. The column was thermostated at 25°C, flow rate was 1.0 mL/min, injection volume 50 µL, and detection wavelength was 306 nm and 286 nm for *trans*- and *cis*-resveratrol, respectively. Analyses were performed in duplicate.

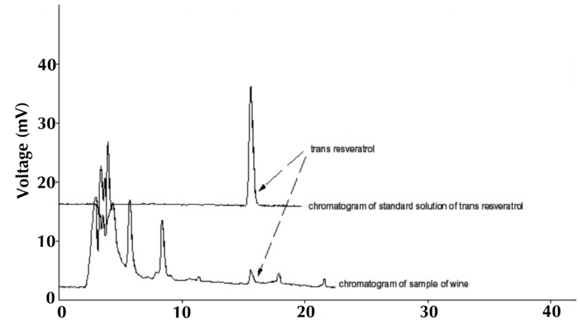

Figure 1. Chromatogram of wine sample and standard solution of *trans*-resveratrol.

Identification and quantification

Identification of resveratrol in wine was performed by comparing retention time and UV spectras of eluted *trans*- and *cis*-resveratrol with corresponding resveratrol standards. Solutions of *trans*-resveratrol in methanol were prepared in concentration range 0.2–15 mg/L. Standard calibration curve for *trans*-resveratrol was established after HPLC analysis of these solutions on 306 nm by plotting the area of peaks against different concentrations. In order to obtain standard calibration curve for *cis*-resveratrol, same standard solutions of *trans*-resveratrol were exposed to day light for 10 h. They were analyzed every 2 h using the same chromatographic conditions with detection on 286 nm (32).

Calibration curve for *cis*-resveratrol was in the range of 0.25–10 mg/L. In order to determine limit of detection (LOD), different amounts of *trans*- and *cis*-resveratrol were added to wine samples that did not contain resveratrol. After extraction on solid-phase, the signal/noise ratio was measured.

Results and Discussion

The method described can be used to determine both *trans*- and *cis*-isomer of resveratrol, as applied in this study. Under these experimental conditions, the *trans*- and *cis*-resveratrol are well-separated, and no compound present in the wine interferes in determination. In analyzed wines, the range of concentration of *trans*-resveratrol was from 0.11 mg/L to 1.69 mg/L, and the same value for *cis*-isomer was from 0.12 mg/L up to 1.49 mg/L.

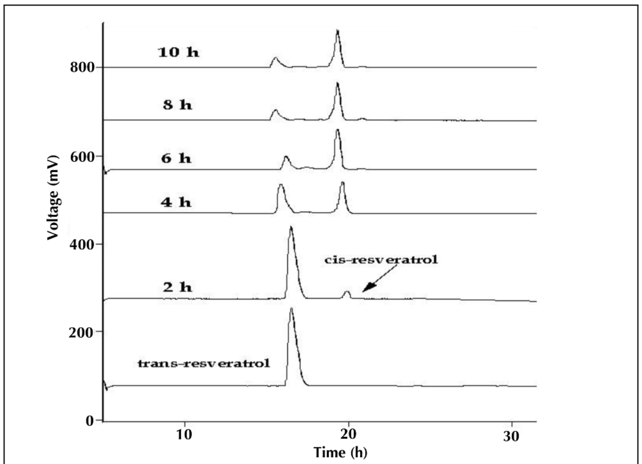

Figure 2. Chromatograms of standard solution of *trans*-resveratrol analyzed every 2 h during 10 h daylight exposure.

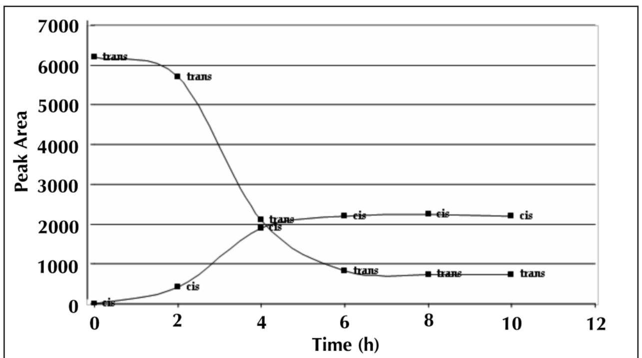

Figure 3. Isomerization of *trans*- to *cis*-resveratrol by daylight exposure.

Analytical method

Trans- and *cis*-isomers were identified in the chromatogram of wine samples (Figure 1) by comparison of its spectra and retention time with the standards. The LOD found for *trans*-resveratrol was 0.02 mg/L and for *cis*-resveratrol 0.05 mg/L, considering the LOD as the analyte concentration giving a signal three times higher than the noise. These values are considerably lower than reported for both isomers using the same type of detector (33). Chromatograms obtained during isomerization of *trans*- to *cis*-resveratrol by daylight exposure are shown in Figure 2 and Figure 3. As it can be seen from Figure 3, equilibrium between two isomers was reached after 6 h exposure to daylight.

Concentration of each compound was determined by external standard method using the calibration curves for *trans*- and *cis*-resveratrol and the measured peak areas of both isomers obtained after analysis of wine samples. Duplicate injections were performed, and average value of peak areas were used for the quantitative analysis. The concentrations of obtained *cis*-resveratrol solution used for establishing the standard calibration curve were calculated by using molar apsorptivities of both isomer [ε_{trans}(MeOH), 306 nm = 25400, ε_{cis}(MeOH), 286 nm = 12000] (34), peak area of analyzed *cis*-isomer and peak area as well as concentration of corresponding *trans*-resveratrol, precursor for each solution [$C_{cis} = (C_{trans} \times P_{cis}286 \times \varepsilon_{trans}$ (MeOH), 306 nm) / ($P_{trans}306 \times \varepsilon_{cis}$ (MeOH), 286 nm)]. The calibration results are shown in Table I. Each point of calibration is the mean value from two independent analyses.

Accuracy and repeatibility of analytical method were determined by consecutive injections (n = 6) of standard solutions of *trans*- and *cis*-resveratrol and expressed as relative standard deviation (RSD) (Table I). After five consecutive injections of wine extract and determination of concentrations for both isomers in wine, confidence interval of ± 0.04 for probability 95 % (F = 4, n = 5) was obtained. In order to determine the recovery of extraction, red wine sample was analyzed after direct injection, and the concentrations of both, *cis*- and *trans*-isomer were determined. The same sample was analyzed under the identical experimental

Table I. Calibration Results for Determing *trans*- and *cis*-Resveratrol in Wines

Resveratrol	Equation	RSD (%)	R	LOD (mg/L)	Range (mg/L)
trans-	C(mg/L) = 0.0139 × P(mVs)	3.3	0.9998	0.02	0.2–15
cis-	C(mg/L) = 0.0303 × P(mVs)	5.8	0.9994	0.05	0.25–10

Table II. Extraction Efficiency

Resveratrol (mg/L)					
Direct injection		Injection after SPE		Recovery (%)	
Trans-	*Cis-*	*Trans-*	*Cis-*	*Trans-*	*Cis-*
1.47	0.974	1.39	0.965	94.56	95.61

conditions after previous preparation by solid-phase extraction. The obtained results were compared in order to calculate the recovery. Recovery of extraction was 94.56 % for *trans*-resveratrol and 95.61 % for *cis*-resveratrol (Table II).

Wine analysis

In this study the total of 18 Serbian wines (10 red, 7 white, and 1 rose wine) were analyzed for their *trans*- and *cis*-resveratrol content. The wines were obtained from six Serbian wine producers and were made from nine different cultivares (Grasevina, Chardonnay, Sauvignon, Cabernet Sauvignon, Merlo, Pinot Noir, Royal, Vranac, Rose) grown at different cultivation sites. All results are summarized in Tables III and IV. Only resveratrol aglycones were analyzed. The concentration of *trans*-resveratrol ranged from 0.11 to 1.69 mg/L, that of *cis*-isomer from 0.12 to 1.49 mg/L. The total amount reached up to 2.5 mg/L. A considerable difference exists in *trans*- and *cis*-resveratrol content between analyzed wines. These findings are consistent with previously reported results (35,36). It was also observed that considerable variability in resveratrol concentrations exist even in wines produced by the same grape variety. This result is in agreement with a previously reported study (36). More specifically, grapes of the same cultivar variety that have been grown at different cultivation sites produced wines that contain different amounts of phenolic compounds presumably because environmental and growth factors also influence the concentration of these substances (37).

Table III. *Trans*- and *cis*-Resveratrol Content in Red and Rose Serbian Commercial Wines

		resveratrol (mg/L)			
Red wine varieties	Produced by	*trans-*	*cis-*	Sum	*trans/cis*
Cabernet Sauvignon	Navip, Zemun	0.61	0.27	0.88	2.26
Cabernet Sauvignon	Podrum Radovanovic, Krnjevo	1.69*	0.81	2.5‡	2.09
Cabernet Sauvignon	Radmilovac, Faculty of Agriculture	0.18	0.19	0.37	0.95
Cabernet Sauvignon	Rubin, Kruševac	0.36	0.42	0.78	0.86
Cabernet Sauvignon	Vino vita, Trstenik	1	1.49†	2.49	0.67
Merlo	Radmilovac, Poljoprivredni fakultet	0.11	0.12	0.23	0.92
Merlo "Dionis"	Navip, Zemun	1	0.53	1.53	1.89
Pinot Noir	Rubin, Kruševac	1.31	0.82	2.13	1.6
Royal	Navip, Zemun	0.72	0.66	1.38	1.09
Vranac	Rubin, Kruševac	0.84	0.2	1.04	4.2
Average value		0.782	0.551	1.333	
Rose wine varietal					
Rose		0.29	–	0.29	–

* Red wine with the highest content of *trans*-resveratrol.
† Red wine with the highest content of cis-resveratrol.
‡ Red wine with the highest total content of resveratrol.

Table IV. *Trans*- and *cis*-Resveratrol Content in White Serbian Commercial Wines

		Resveratrol (mg/L)			
White wine varieties	Produced by	*trans-*	*cis-*	Sum	*trans/cis*
Graševina	Erdevik, Erdevik	0.33	0.49†	0.82‡	0.67
Chardonnay	Erdevik, Erdevik	0.34*	0.12	0.46	2.83
Chardonnay	Podrum Radovanovic, Krnjevo	0.29	–	0.29	–
Chardonnay	Radmilovac, Poljoprivredni fakultet	–	–	-	–
Chardonnay	Rubin, Kruševac	0.15	–	0.15	–
Sauvignon	Rubin, Kruševac	0.11	–	0.11	–
Sauvignon blanc	Navip, Zemun	0.19	–	0.19	–
Average value		0.235		0.34	

*White wine with the highest content of *trans*-resveratrol.
†White wine with the highest content of *cis*-resveratrol.
‡White wine with the highest total content of resveratrol.

Trans-resveratrol

As shown in Tables III and IV, there are variations of *trans*- and *cis*-resveratrol content in analyzed wines as well as of the concentration ratio of these two isomers (*trans*/*cis* resveratrol). As their amount in wines depends on different factors (growing conditions, climate, temperature, UV irradiation, production technology, etc.), these observations are expected. *Trans*-resveratrol was detected in 17 from 18 analyzed samples while the highest amount of this compound was found in Cabernet Sauvignon from Krnjevo (1.69 mg/L), which has also the highest amount of total resveratrol content (sum of both isomers).

The range of concentration of *trans*-resveratrol in analyzed red wines was from 0.11 to 1.69 mg/L. The average value (0.78 mg/L) appeared to be lower than those reported for red wines from France, Canada, and the U.S., although still lower values have also been reported (30,38–40). Some mean values of red wines *trans*-resveratrol concentrations reported in literature range from 0.132 mg/L to 2.46 mg/L (41,42) considering various mean values as 0.157 mg/L in Japan (40), 0.77 mg/L in Canada (43), 0.873 mg/L in Greece (30), 0.998 mg/L in California (44), 1.00 mg/L in Portugal (45), and 1.21 mg/L in Chile/Argentina (35). These differences could be attributed to environmental conditions such as humidity and fungal disease, which are factors influencing the production of *trans*-resveratrol by grapevines (35). The authors are aware this is the only investigation of resveratrol content in wine in the region, except the one involving botrytized Hungarian wines. Total resveratrol content was from < 0.003 to 7.8 mg/mL with a mean value of 2.5 mg/mL (46). In this case, high fungus pressure caused by Botrytis cinerea showed slightly higher content of resveratrol, which may be due to the long skin contact during production. Generally, average concentration of *trans*-resveratrol for Serbian red wines is anticipated because the average red wine can be expected to contain 1.9 ± 1.7 mg/L *trans*-resveratrol (47).

Trans-resveratrol was detected in all analyzed white wines except the Chardonnay from Radmilovac in amounts from 0.11 to 0.34 mg/L. The average *trans*-resveratrol concentration in white wines (0.23 mg/L) was, as expected, considerably lower than that of red wines.

This level seems to be similar with those reported for some Greek white wines (48), which is lower in comparison with values reported for other white wines (49). The low *trans*-resveratrol values in white cultivars noted might equally be due to limited pathogen attachment or shading of the fruit (48). In rose wine, *trans*-resveratrol was present in concentrations of 0.29 mg/mL.

There are no correlations in resveratrol content among the same cultivares. For example, Cabernet Sauvignon from Krnjevo has the highest concentration of *trans*-resveratrol from all analyzed wines while the wine made from the same grape varietal, Cabernet Sauvignon from Radmilovac, has one of the lowest value measured. The similar result is observed concerning *trans*-resveratrol content in five samples of Chardonnay obtained from different producers.

Cis-resveratrol

Cis-resveratrol was present in all analyzed red wines (Table III). It has been reported that even though this compound generally is not a natural constituent of grape berries (27,28), it has been detected in almost all wines analyzed so far, regardless of the origin and the technology applied. In some analyzed wines (Grasevina and two samples of Cabernet Sauvignon), the concentration of *cis*-resveratrol is higher than of *trans*-isomer, which is not so unexpected (32) and depends mainly on wine-making technology.

As it can be seen (Table III) in analyzed red wines the highest concentration of *cis*-resveratrol was found in Cabernet Sauvignon, Trstenik (1.49 mg/L) and the lowest in Merlo, Radmilovac (0.12 mg/L), the red wine with the lowest quantified content of both resveratrol isomers. The average value of *cis*-resveratrol concentration in red wines (without considering the wine in which the *cis*-isomer was not detected) was 0.55 mg/L. In white wines, *cis*-isomer was quantified only in two samples from Erdevik, Chardonnay (0.12 mg/L) and Grasevina (0.49 mg/L). *Cis*-isomer was absent from analysed rose wine sample.

This result corroborates other studies that show which wines from various countries and regions have a range concentration of *cis*-resveratrol between 0.1–5.0 mg/L. For 16 wines from nine different countries or regions, this value was reported to be from 0.02 mg/L up to 4.0 mg/L (50). In some analyzed commercial wines, the *cis*-resveratrol content was reported to be in a range of 0.6–5.1 mg/L for Czech wines (32) and up to 0.9 mg/L for French wines (51).

Conclusion

Various Serbian wines purchased from wine producers (10 red, 7 white, and 1 rose) were analyzed for their concentration in *trans*- and *cis*-resveratrol. Analysis was carried out by HPLC, and the results of the chromatographic analyses are summarized in Tables III and IV. The concentration of these substances seems to vary considerably. *Trans*-resveratrol content ranged from 0.11 to 1.69 mg/L with a mean value of 0.54 mg/L. While *trans*-resveratrol was detected in all except one analyzed wine, *cis*-isomer was present in 12 from 18 analyzed samples. It was detected in concentration from 0.12 to 1.49 mg/L. Mean value, considering samples containing *cis*-resveratrol, was 0.51 mg/mL.

The differences between resveratrol content in analyzed wines could be attributed to environmental conditions, fungal disease, wine-making techniques, storage, and other factors influencing the production of *trans*-resveratrol by grapevines (35). These great numbers of factors are also responsible for the wide range of resveratrol content reported by different authors. In analyzed Serbian wines, variability in resveratrol concentrations was considerable even in wines produced by the same grape variety, which corroborate with observations reported previously. Also, accordingly to Soleas (52), comparisons between concentration values of *trans*-resveratrol have to take into account not only the statistical parameters but also the sample preparation (direct injection or not) as well as the chromatographic method (gas chromatography or HPLC). Additionally, in red wines, the concentration of this substance seems to vary considerably because it depends on diverse factors, while in white wines *trans*-resveratrol occurs mostly in low levels. Similar results have been reported for other white cultivars too.

This is the first report concerning the determination of resveratrol content in Serbian wines. In Eastern Europe, similar investigations had been conducted only in Hungary while in the Balkan region only wines from Greece (30,53) were analyzed. Additional and more expanded and detailed examinations are required for better understanding of the factors that influence resveratrol content in Serbian wines.

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