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ANTIOXIDATIVE ACTIVITY OF RED WINE WITH THE INCREASED SHARE OF PHENOLIC COMPOUNDS FROM SOLID PARTS OF GRAPE

The structure and amount of phenolic compounds in the wine depend on the grapevine variety, agroecologic conditions and a way of vinification. The influence of pomace enrichment with solid parts of grape (stem and grape seeds) during maceration on the antioxidative activity of red wines was investigated. The antioxidative activity of red wines towards DPPH$^{\bullet}$ and hydroxyl ($^{\bullet}$OH) radicals was determined by the electron spin resonance (ESR) spectroscopy. The addition of stem to the pomace had no significant influence on the antioxidative wine activity increase, whereas enriching of pomace with 120 g seeds/kg of pomace resulted in the increase of antioxidative capacity of a wine. In the wine enriched with tannins and flavan-3-ols from the seeds, the antioxidative activity towards DPPH$^{\bullet}$ ($AA_{DPPH^{\bullet}}$) was 100%. None of the applied clarifiers showed a significant influence on the antioxidative activity of these wine samples. The antioxidative activity, measured as DPPH$^{\bullet}$ scavenging activity, of the wine supplemented by seeds remained unchanged, showing 100% efficiency after the treatment by all tested fining agents. A significant difference in antioxidative activities towards hydroxyl radicals ($AA^{\bullet}_{OH}$) between the two wines was found. The antioxidative activity of the wine Merlot was higher than the antioxidative activity of the wine Cabernet sauvignon.

Key words: red wine; stem; seed; fining agents; antioxidative activity; ESR spectroscopy.

Free radicals are extremely harmful to living organisms because they attack different constituents of the cell, leading to acceleration of the ageing process and sometimes even to destruction, or, if the DNA is affected, to irreversibile malfunctions [1]. Oxygen-derived species such as superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}$OH) play an important role in a tissue damage, causing the oxidative degradation of proteins, unsaturated lipids, carbohydrates, and nucleic acids [2]. A growing evidence of the role of free radicals and antioxidants in health and ageing has focused great interest on later compounds. The current research of free radicals has confirmed that food rich in antioxidants plays an essential role in the prevention of cardiovascular diseases, cancers, and neurodegenerative diseases, the most well-known of which are Parkinson's and Alzheimer's diseases [3-5].

Phenolic compounds are essential for sensoric characteristic of red wines, most of all for colour, bitternes and astringency. They are derived in two groups - nonflavonoids and flavonoids. The phenomenon known as "Franch paradox" may be explained by the presence of phenolic compounds in the red wine [6]. Types and amount of phenolic compounds of the wine depend on cultivar, vintage, as well as on conditions of vinification [7,8].

It is now widely recognised that the phenolic compounds of the wine have very high free radical scavenging potential. The protective effect of the wine is mainly due to the anthocyanic fraction although the results do not exclude the possibility of a sinergistic action among the different classes of polyphenols [9]. Moreover, according to Kerry and Abbey [10], the antioxidant property of the red wine is due predominantly to monomeric catechins, procyanidins, monomeric anthocyanidins and phenolic acids. It was determined that the antioxidative activity of red wines is strictly correlated with the content of gallic acid, (+)-catechin, (-)-epicatechin, and total phenols [11,12].

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The antioxidative activity of phenolic compounds, usually present in red wines, has already been studied by the electron spin resonance (ESR) spectroscopy. In those studies, a high antioxidative activity was strongly correlated with total phenol contents of red wines [13-15], and grape seed extracts [16]. When producing red wines, it is of great importance to obtain a good and clear soft wine with a good stable colour for a prolonged period of time. Not only turbid wines are fined, but also those inclined to turbidity and precipitation. Agents of different origin are used for fining but they must not affect or cause such changes of a chemical composition that would negatively reflect on the wine quality. Several agents are recommended for fining red wines: bentonite, gelatine, albumin and casein. It is also important to use it at optimal dosages. In addition, a treatment by polyvinylpolypyrrolidone (PVPP) is recommended in red wines for the fixation and consequent reduction of the most reactive tannins which caused colloid instability of wine [17].

The aim of this study was to examine the influence of addition of seed and stem during maceration, and also fining agents on the content of total phenols, tannins, anthocyanins and flavan-3-ols in supplemented red wines. Further, the antioxidative activity of these red wines was evaluated by ESR spectroscopy.

MATERIALS AND METHODS

Sample preparation

Wines were produced by fermentation of Cabernet sauvignon and Merlot grape grown in the vineyards of Sremski Karlovci, Faculty of Agriculture, Novi Sad, Serbia. Grape was harvested at its maturity required for the wine production: sugar content (determined by refractometer) and total acid content (determined by titration with NaOH and expressed as tartaric acid) were 20.40% (w/v) and 8.20 g/l, respecttively (for Cabernet sauvignon) and 20.5 % (m/v), *i.e.*, 7.60 g/l in Merlot. Five kg of grape was crushed to obtain a wine sample after the microvinification. Pomace was treated with $K_2S_2O_5$ (200 mg/kg grape). The fermentation started after inoculation with selected pure-culture yeast *Saccharomyces cerevisiae* (0.2 g/kg). Microvinification was conducted at 28-30 °C during the period of 9 days. The control wine was produced from pomace with the original content of the seed, while the stem was separated. The total weight of the stem obtained from 5 kg of the grape was 0.5 kg, and the seed content was determined to be 40 g/kg grape.

Other wine samples were produced from pomace enriched in a solid phase, according to the scheme: A - grape pomace supplemented by 50% of the previously separated stem, B - grape pomace supplemented by 120 g seeds/kg. After 9 days of fermentation, the pomace was pressed and after a spontaneous sedimentation the wines were racked off and bottled. Two months later fining agents: albumin (0.2 g/l), bentonite (0.75 g/l), gelatine (0.2 g/l) and PVPP (0.2 g/l) were added to the wines and after four days the wines were separated from the precipitate and analyzed.

Spectrophotometrical analysis of phenolic compounds

Total phenols (TP) were analyzed by the spectrophotometry method with Folin-Ciocalteu reagent [18], with gallic acid as standard, and the results were expressed as g gallic acid equivalent (GAE) per l of wine. Total tannins were determined according to Bourzeix, Dubernet and Heredia [19] spectrophotometrically measuring the absorption at 280 nm: total tannins = $A_{280}\times100$. The total anthocyanins were determined spectrophotometrically, with bisulphite bleaching [20]. The vanillin index served to determine catechins and proanthocyanidins reacting with the vanillin, according to the vanillin-HCl method [21].

DPPH˙ assay

Blank probe was obtained by mixing 400 µl 0.4 mM methanolic solution of DPPH˙ and 200 µl 12% ethanol. The influence of wine on transformation of DPPH˙ was analyzed in the mixture of 15 µl wine sample, 185 µl 12% ethanol and 400 µl 0.4 mM methanolic solution of DPPH˙. The mixture was stirred for 2 min and transferred to a quartz flat cell ER--160FT. The ESR spectra were recorded on an ESR spectrometer Bruker 300E (Rheinstetten, Germany) under following conditions: field modulation 100 kHz, modulation amplitude 0.226 G, time constant 40.96 ms, conversion time 327.68 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23 °C. The antioxidative activity (AA_{DPPH}•) of the wine was defined as: AA_{DPPH}• = $100(h_0 - h_x)/h_0$ (%), where h_0 and h_x are the height of the second peak in the ESR spectrum of DPPH˙ of the blank and the probe, respectively.

Hydroxyl radical assay

Hydroxyl radicals were obtained by the Fenton reaction in the system: 100 µl 10 mM H_2O_2, 100 µl 10 mM $FeCl_2{\cdot}4H_2O$, 200 µl 12% ethanol and 400 µl 80 mM DMPO, as spin trap (blank). The influence of wine on the formation and transformation of hydroxyl radicals was investigated by adding 200 µl of wine to the Fenton reaction system. ESR spectra were recorded after 5 min, with the following spectrometer settings: field modulation 100 kHz, modulation ampli-

tude 0.226 G, time constant 81.92 ms, conversion time 327.68 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23 °C. The antioxidative activity ($AA\bullet_{OH}$) of the wine was defined as: $AA\bullet_{OH} = 100(h_0 - h_x)/h_0$ (%), where h_0 and h_x are the height of the second peak in the ESR spectrum of DMPO-OH spin adduct of the blank and the probe, respectively.

RESULTS AND DICUSSION

The effect of wine supplementation on the total phenol content, anthocyanin content, total tannins, vanillin index and antioxidative activity

Before fermentation, grape pomace was supplemented by seed and stem. The results obtained by analyzing the anthocyanins, phenolic compounds, tannins content, vanillin index, as well as DPPH$^\bullet$ and hydroxyl radical antioxidative activities are presented in Table 1.

The antioxidative activity of the wine was investigated by the spectrometrical ESR analysis, as the only technique for a direct determination of free radicals. The structure of the stable DPPH$^\bullet$ in ESR spectrum is the result of the interaction of an unpaired electron and two ^{14}N atoms (1 = 1). It consists of five lines, with relative intensities 1:2:3:2:1. The value of the splitting constant is a_N = 9.03 G. Comparing the ESR spectra of blank and investigated wine samples it can be noticed that the structure of the spectra is preserved, and the line intensities of ESR signals decrease.

Fenton's reaction system for the generation of $^\bullet$OH for the determination of the antioxidative activity of wine towards $^\bullet$OH was prepared. The generated reactive $^\bullet$OH in the presence of spin-trap (DMPO) form stable nitroxide radicals (DMPO-OH spin-aducts) which have relatively long life-time and are suitable for ESR detection. The ESR spectrum of DMPO-OH spin-aduct is characterized by its 1:2:2:1 quartet of lines and splitting constants a_N and a_H = 14.9 G.

Comparing the ESR signal intensities of DMPO--OH spin aducts of blank and samples it was found that wines with the higher content of total phenolic compounds from the solid parts of grape (stem and seeds) had the higher inhibitory effect on the formation of $^\bullet$OH.

A high level of a positive correlation between the total phenolic compounds content and the individual portions of gallic acid, (-)epicatechin, (+)-catechin and the antioxidative activity of wine is presented in a number of papers [11,22-25].

Several authors attributed the highest level of antioxidative activity to compounds from the anthocyanins group [9], not considering the fact that the antioxidative effect increases the synergistic action of anthocyanins and tannins from wine and grape skin compared to pure anthocyanins [1].

A close connection of wine antioxidative activity and total phenolic compounds content and vannilin index was found. However, this connection was not found for anthocyanins (Table 1). The enrichment of grape pomace in solid phase by addition of 50% of previously removed stem resulted in the increased total phenols content by 60% compared to the control wine. The presence of the stem increased the total tannin content, expressed as A_{280} index, for approximately 1.3 times. The antioxidative activity towards DPPH$^\bullet$ ($AA_{DPPH}\bullet$) is 10% higher in the Cabernet sauvignon wine, enriched with the stem (wine A). The addition of 120 g seeds/kg grape pomace also caused a 2.2 times higher content of total phenolic compounds in comparison to the control wine and, at the same time, the total tannin content was improved by 85% which consequently led to a higher antioxidative activity. The wine produced with the addition of 120 g seeds/kg pomace reduces completely the ESR signal ($AA_{DPPH}\bullet$ = 100%). The antioxidative activity of the wine towards hydroxyl radicals ($AA\bullet_{OH}$) increased for 25% compared to the control wine disregarding the enrichment with seeds or stem.

The values presented in the same table show that Merlot wine exhibits a significantly higher antioxidative activity towards hydroxyl radicals ($AA\bullet_{OH}$), compared to Cabernet sauvignon wine, independently on the solid phase portion in the pomace. A decrease

Table 1. The effect of wine supplementation on anthocyanin content, total phenol content (TP), total tannin content (A_{280}), vanillin index and antioxidative activity measured as $AA_{DPPH}\bullet$ and $AA\bullet_{OH}$

Wine	Sample	Anthocyanins, mg/l	*TP* / g gallic acid l^{-1}	$A_{280}\times100$	Vanillin index, mg/l	$AA_{DPPH}\bullet$ / %	$AA\bullet_{OH}$ / %
Cabernet sauvignon	control wine	319.40	1.569	33.20	268.70	85.32	38.09
	wine A	245.60	1.521	32.70	282.00	93.65	47.79
	wine B	210.20	3.527	61.50	1271.30	100	47.02
Merlot	control wine	329.10	1.460	38.40	368.30	83.73	70.30
	wine A	226.00	1.601	39.40	717.00	84.13	66.30
	wine B	258.60	2.385	58.30	2370.00	100	71.04

of the antioxidative activity towards hydroxyl radicals, for 4% was recorded after the addition of the wine produced with addition of 50% of stem, although the content of total phenolic compounds and vanillin index was increased by 10% and 95%, respectively. This change of the antioxidative activity could be explained by the lowest anthocyanin content in the mentioned wine, most probably due to the reaction with tannins extracted from the stem.

The change of the antioxidative activity of Merlot wine towards DPPH$^{\bullet}$ (AA_{DPPH}•) was not significant due to the addition of stem, compared to the control wine. The value AA_{DPPH}• = 100% was achieved with the wine produced with the addition of a tripple amount of seed compared to the natural content and this also affected the increase of the total phenolic compounds content for 49% and 6-fold increase of the vanillin reacting phenol content, *i.e.*, flavan-3-ols.

Considering the previous results and discussion, the addition of the solid phase (stem and seeds) to pomace was justified. Total phenol and tannin contents were significantly improved. Moreover, increased phenolic concentrations remained at high level after the treatment by fining agents in most cases, which means that the enrichment was stable. The increased level of total phenols and tannins benefited a higher quality of red wines especially those which are planned for ageing with relevance to sensoric properties [26].

The effect of some fining agents on the content of phenolic compounds and antioxidative activity of red wines

The effect of fining agents on the content of total phenolic compounds, total tannins and antioxidative activity of wines towards DPPH$^{\bullet}$ and $^{\bullet}$OH was investigated. The results are presented in Tables 2, 3 and 4.

Table 2. The effect of fining agents on total phenol content and the antioxidative activity of the control wine

Wine	Parameter	Before fining	Bentonite	Albumin	Gelatine	PVPP
Cabernet sauvignon	*TP* / g gallic acid l^{-1}	1.569	1.425	1.579	1.606	1.596
	A_{280}×100	33.20	33.10	31.00	32.40	34.80
	AA_{DPPH}• / %	85.32	83.33	82.94	76.98	83.73
	$AA_{•OH}$ / %	38.09	30.60	21.64	23.88	29.10
Merlot	*TP* / g gallic acid l^{-1}	1.460	1.390	1.470	1.306	1.496
	A_{280}×100	38.40	35.10	37.00	34.40	35.80
	AA_{DPPH}• / %	83.73	81.94	82.14	79.37	82.14
	$AA_{•OH}$ / %	70.30	62.38	67.33	55.45	61.88

Table 3. The effect of fining agents on total phenol content and the antioxidative activity of the wine supplemented with stem (wine A)

Wine	Parameter	Before fining	Bentonite	Albumin	Gelatine	PVPP
Cabernet sauvignon	*TP* / g gallic acid l^{-1}	1.521	1.319	1.346	1.489	1.473
	A_{280}×100	32.70	32.70	32.70	30.20	31.00
	AA_{DPPH}• / %	93.65	82.94	81.75	73.81	77.78
	$AA_{•OH}$ / %	47.79	28.36	11.19	33.58	27.61
Merlot	*TP* / g gallic acid l^{-1}	1.601	1.520	1.436	1.489	1.573
	A_{280}×100	39.40	37.70	36.70	33.20	35.00
	AA_{DPPH}• / %	84.13	80.95	79.37	73.80	83.73
	$AA_{•OH}$ / %	66.34	48.51	59.41	60.89	53.96

Table 4. The effect of fining agents on total phenol content and the antioxidative activity of the wine supplemented with grape seed (wine B)

Wine	Parameter	Before fining	Bentonite	Albumin	Gelatine	PVPP
Cabernet sauvignon	*TP* / g gallic acid l^{-1}	3.527	3.064	3.048	2.500	3.080
	A_{280}×100	61.50	61.40	61.30	57.80	62.10
	AA_{DPPH}• / %	100	100	100	100	100
	$AA_{•OH}$ / %	47.02	31.34	31.72	39.55	35.07
Merlot	*TP* / g gallic acid l^{-1}	2.385	1.865	1.948	1.650	1.980
	A_{280}×100	58.30	58.40	57.30	57.60	54.10
	AA_{DPPH}• / %	100	98.48	88.69	92.38	92.06
	$AA_{•OH}$ / %	71.04	67.82	68.32	64.85	63.12

The results in Table 2 demonstrate no significant effect of fining agents on the content of phenolic compounds, except bentonite in both red wines and gelatine in Merlot wine.

Also, according to the results presented, it can be concluded that all investigated fining agents decreased the antioxidative activity of both red wines towards both free radical species. Observing the influence of fining agents on the antioxidative activity towards hydroxyl radicals it is evident that the most intesive effect on Cabernet sauvignon wine was provided by albumin, and by gelatine on Merlot wine. The measured $AA\bullet_{OH}$ value for Merlot wine was about two times higher compared to Cabernet sauvignon wine, independently on the applied enologic agent. The antioxidative activity of wines with added enological agents towards DPPH$^{\bullet}$ was similar to that of the control wines.

The investigated fining agents decreased the content of phenolic compounds in both red wines, except for total tannins in Cabernet sauvignon supplemented with the stem treated with bentonite or albumin where the value remained unchanged. In accordance with this, the antioxidative activity of these wines in both tested systems was decreased.

The addition of gelatine affected most intensively a decrease of antioxidative activity of wine towards DPPH$^{\bullet}$ for both wines produced with addition of stem, for 23% for Cabernet sauvignon wine and 12% for Merlot wine. Albumin showed a similar effect on a decrease of antioxidative activity of wine but less pronounced compared to gelatine. This is in accordance with the fact that both enologic agents of proteinaceous nature are reacting with phenolic compounds, tannins in the first place. Regarding this, the structure of phenolic compounds in Merlot wine could be considered as more stable towards the action of applied enologic agents.

The most significant decrease of antioxidant activity towards $^{\bullet}$OH was determined for Merlot wine treated with bentonite ($AA\bullet_{OH}$ = 48.51%), and for Cabernet sauvignon wine treated with albumin ($AA\bullet_{OH}$ = = 11.19%). It is reported that bentonite has higher affinity towards free anthocyanins, catechins and dimers of proanthocyanidins [27]. Besides, the secondary binding of tannins on the surface of bentonite flocules coated with proteins takes place.

In the case of both red wines supplemented with grape seeds fininig agents generally influenced the loss of phenols. The loss of total tannins was minor compared to total phenols.

Both analyzed wines produced with higher seed content in the pomace had a high antioxidative activity towards DPPH$^{\bullet}$ where all tested Cabernet sauvignon wines had $AA_{DPPH\bullet}$ = 100%. On the other hand, Merlot wine with lower content of total phenols showed a significant decrease of antioxidative activity after the treatment with enologic agents. The treatment with albumin resulted in the decrease of antioxidative activity for almost 12%, compared to other agents, 2–8%. This is in accordance with findings of other authors [27,28], who found that the loss of catechins and proanthocyanidins affected by fining agents depended on the initial concentration of these compounds. A relative loss in wines with the contents of these compounds is smaller compared to the wines with lower contents of phenolic compounds.

A substantial difference in antioxidtive activities towards $^{\bullet}$OH between the two wines was found where the antioxidative activity of Merlot wine was higher compared to Cabernet sauvignon wine. This relative relation is not affected by the applied enologic agent. The lowest antioxidative activity was detected for Merlot wine treated with PVPP ($AA\bullet_{OH}$ = 63.12%), most probably due to high afinity of this agent towards tannins [29].

CONCLUSION

The results obtained in this study showed that seed supplementation of grape pomace significantly affected the increase of phenolic compounds in wine and improved the antioxidative activity of wine on DPPH$^{\bullet}$ to the maximal value of 100% in the investigated system. The analyzed wines were produced and treated in the same way, but from the grape of two different grapevine sorts. In Cabernet sauvignon wine, produced with the supplement of 120 seeds/kg pomace, a higher content of total phenols and a lower vanillin index were found compared to Merlot wine. The antioxidative activities of two wines were also different. The antioxidative activity of Merlot wine towards hydroxyl radicals ($AA\bullet_{OH}$) was 50% higher compared to Cabernet sauvignon wine. The addition of stem to grape pomace increased the antioxidative activity of the wine less than the addition of seeds. The applied fining agents had no influence on the antioxidative activity of Cabernet sauvignon wine produced with the seed supplementation of pomace on DPPH$^{\bullet}$ ($AA_{DPPH\bullet}$ = 100%), while the antioxidative activity of Merlot wine was decreased for about 2% (bentonite) and for almost 12% after the treatment with albumin. The antioxidative activity of both wines towards hydroxyl radicals was significantly reduced after the treatment with fining agents, both for control wine and the wine produced with the addition of stem. Merlot wine produced after the supplementation with seeds exhibited similar values of antioxidative activi-

ties towards hydroxyl radicals ($AA\bullet_{OH}$) after the treatment with fining agents. The $AA\bullet_{OH}$ value of this wine was decreased for only about 4% after the treatment with albumin and for about 11% after the treatment with PVPP.

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NAUČNI RAD

ANTIOXIDATIVNI POTENCIJAL CRVENOG VINA SA POVEĆANIM UDELOM FENOLNIH JEDINJENJA IZ ČVRSTIH DELOVA GROZDA

Struktura fenolnih jedinjenja vina zavisi od sorte vinove loze, agroekoloških uslova i načina vinifikacije. U radu je ispitan uticaj povećanog sadržaja čvrstih delova grozda (šepurine i semenki) u fazi maceracije, na antioksidativnu aktivnost crvenih vina. Antioksidativna aktivnost crvenih vina na DPPH˙ i hidroksil (˙OH) radikale, određena je primenom elektron-spin rezonantne (ESR) spektroskopije. Dodatak šepurine u kljuk nije značajno uticao na porast antioksidativne aktivnost vina, dok je obogaćenje kljuka sa 120 g semenki/kg, rezultovalo povećanjem antioksidativne aktivnosti. Antioksidativna aktivnost vina obogaćenog taninima i flavan-3-olima semenki, na DPPH˙ radikale ($AA_{DPPH\cdot}$), iznosila je 100%. Nijedno primenjeno sredstvo za bistrenje nije značajno uticalo na antioksidativnu aktivnost ispitivanih uzoraka vina. Nakon obrade vina sredstvima za bistrenje, antioksidativna aktivnost ($AA_{DPPH\cdot}$) ostala je nepromenjena. Između dva ispitivana vina utvrđena je razlika antioksidativne aktivnosti na hidroksil radikale ($AA_{\cdot OH}$). Antioksidativna aktivnost vina Merlot bila je viša od antioksidativne aktivnosti vino Cabernet sauvignon.

Ključne reči: crveno vino; šepurina; semenke; sredstva za bistrenje; antioksidativna aktivnost; ESR spektroskopija.