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Original scientific paper

# Total phenols, stilbene and antioxidative activity in Babić and Plavac mali wines; Eficiency of pectolytic enzymes


### Abstract

*Special importance of wine polyphenols have been associated with the bioactive compounds because of their benefits on human health. Research of acceptable and safe technological processes that would enable the production of wines with a higher content of such compounds is a need and a challenge. For this reason, the aim of this study was to investigate the influence of commercial pectolytic enzymes to total phenols (UF), stilbene and antioxidative activity (AA) of Babić and Plavac mali red wines, originated from middle quality localities. Two enzymatic product with different properties were used: pectinase with celulase and hemicelulase additional activity (E1) and pectinase with inactive yeast cells (E2), in comparison to maceration without egzogenous enzymes (control, K). The quality parameters were analysed in different production stages; from the end of maceration and fermentation to wine after the second decanting and bottling. Both products affected to those quality parameters. Maceration with pectolytic enzymes (E1) provided the best extraction and production of wines with the significant highest concentrations of total phenols of both varieties, in both years. The highest values of AA were obtained in wines of treatmant E1, in both years of research and for both varieties. Significant differences were obtained for both varieties, but not in both years. Maceration with egzogenous enzymes effected enrichment of wines with stilbenes, resveratrol and piceid particularly, however the significant differences did not obtained in both years of research. The differences between varieties were noted: while the resveratrol dominated in Plavac mali wines, higher proportion were obtained for piceid in Babić wines. Despite relatively better results in comparison to control, the use of product E2 was not found to be beneficial; significant differences were not confirmed for any variety during both years of the experiment.*

*Significant positive correlation were found between the total phenols and antioksidative activity. The changes in parameters after 6 months aging were obtained in wines of both varieties, but some positive effect were reduced. The obtained differences in parameter values were not as significant as at the end of fermentation. The use of exogenous enzyme can be desirable in case of Babić and Plavac mali young wine production.*


***Key words:*** *pectolytic enzymes, stilbenes, total phenols, antioksidative activity, wine, Babić, Plavac mali*

## Introduction

Due to its complexity and impact on human life, wine is becoming an increasingly cause of interest in all areas of society. The chemical composition is of particular importance; alcohol as a potential cause of diseases, and phenolic compounds because of the potential benefits to health. The most important phenolic subgroups are flavonoids, phenolic acids and stilbene. The last two decades have been marked by studies of bioactive phenolic compounds of the leaf and grape berry, primarily stilbene resveratrol (Weiskirchen and Weiskirchen, 2016; Sirerol et al., 2016). Resveratrol is ussualy found in grape in free, aglycone form and in the glucoside form (piceid), in *cis*- and *trans*-isomeric form of both compounds. During vinification, the piceid isomers form are hydrolyzed to *trans*-resveratrol, which is responsible for health benefits. The content of *trans*-resveratrol in wine depends on many factors: varieties (Vincenzi et al., 2013), climate and soil (Bavaresco et al., 2005), and grape and wine production technologies (Bava-

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resco et al., 2012). New insights into the potential positive effects of particular polyphenolic compounds or groups of compounds on human health are accompanied by ongoing research into the potential for improving the polyphenolic composition of red wines (Bavaresco et al., 2011; Snopek et al., 2018). Some researchers attempt to produce yeast with the ability to resveratrol biosynthesis, what is not possible in nature (Halls and Ju, 2006), and to replace sulfur dioxide with resveratrol in wine production (Pastor et al., 2015). The phenolic composition of grapes and wine and changes in composition during maceration and fermentation, and during wine aging, depend on a several factors (Camerer and Carle, 2009). Among numerous factors that influence to the grape and wine production, scientists try to single out technologies that provide a better phenolic composition (Tartian et al., 2017; Champ and Kundu-Champ, 2019).

Phenolic compounds are found in the skin, juice and berry seeds and are extracted during vinification. Extraction intensity depends primarily on grape potential and conditions of maceration and fermentation (Sacchi et al., 2005). The use of the enzyme as a biocatalyst is a well-known oenological praxis to improve extraction of phenolic compounds and ensure higher and more stable wine quality; color, smell and taste (Ough and Berg, 1974; Klaus and Mojsov, 2018). The reason for the continued interest in the enzyme in commercial and scientific point of view is that the results achieved by using enzymes for wine production are not consistent. Oenological enzymes are pectinases, hemicellulases and glucosidases from *Aspergillus niger*, glucanases from *Trichoderma sp*., and ureases from *Lactobacillus fermentum*. Their production is regulated within the International Organization of Vine and Wine (OIV). The OIV recommends that detrimental side activities should be absent in commercial enzyme products and all enzymatic activities should be declared on the label (Resolution OIV/OENO 365 (2009), International Oenological Codex) (www.oiv.int). Most commercial enzymes are mixtures of glucosidases, pectinases, glucanases and proteases, and due to their composition and impurity contamination, they can have additional, mostly negative side-effects (van Rensburg and Pretorius, 2000).

An overview of the effect of exogenous enzymes on phenolic composition, expressed in phenolic index parameters or in total phenol content is presented in Table 1. Several studies have shown that the enzymes had no observable/detectable effect or improved some but not all of the quality parameters, and sometimes the effects were not long lasting (Table 1).

Unfortunately, the available data on the effect of enzymes on stilbene and AA are scarce. Some authors reported the results of the influence of technological processes during fermentation and maceration on the content of resveratrol and other stilbenes carried out on white varieties (Threlfall et al. 1999; Eder et al. 2000; Sun et al., 2003). The content of stilbene and AA in red wines depending on exogenous enzyme was presented by Sun et al. (2003), Clare et al. (2004) and Poussier et al., (2003).

Babić and Plavac mali (*Vitis vinifera* L.) are indigenous Croatian red varieties with a potential of producing wine from average to top quality and it depend primarily on environmental conditions (Mirošević et al, 2008). Previous studies have demonstrated the different influence of enzymes on phenolic quality parameters; color and specific anthocyanins in the Plavac mali wines (Alpeza et al., 2017). The aim of this research was to investigate the effect of commercial enzymes on the phenolic quality of Babić and Plavac mali wines, originating from average quality grapes. The experiment was conducted with two commercial enzyme products used during maceration compared to maceration with no enzymes added. In addition to the content of total phenols as a parameter of the phenolic potential, the emphasis was on the bioactive compounds stilbene and antioxidant activity. These parameters are very important and markers for moderate consumption of red wines and could be affirmative in promoting wines of these varieties of average quality.

**Table 1** Influence of commercial enzyme products on wine phenolic composition, literature review
**Tablica 1**. Utjecaj komercijalnih enzima na fenolni sastav vina, pregled literature

| State | Grape variety | Results, short report | Authors |
|---|---|---|---|
| Australia | Syrah | Positive effect at the end of fermentation, lacked in young wine | Clare et al. (2002) |
| France | Merlot | Positive effect at the end of fermentation, lacked in young wine | Guerrand and Gervais (2001) |
| France | Cabernet sauvignon | Positive effect at the end of fermentation, reduced during aging | Guerrand and Gervais (2002) |
| Italia | Montepulciano | Positive effect at the end of fermentation, reduced during aging | Riponni et al. (2005) |
| Italia | Aglianico | Positive effect | Gambutti et. al. (2007) |
| Italia | Sangiovese | No significant difference at the end of fermentation, positive effect in wine after 18 months | Bucelli et al. (2006) |
| Italia | Cabernet sauvignon, Nebiollo | Positive effect | Rio Segade et al. (2015) |
| SAR | Prieto Picudo | Positive effect, stable during aging | Mihnea M. et al. (2016) |
| Deutschland | Pinot crni | Positive effect | Wolz et al. (2004), Wolz (2004) |
| New Zealand | Pinot crni | Positive effect, stable during aging | Parley et al. (2001) |
| Portugal | Tinto fino | Positive effect in young wines | Gonzales San Jose M.L. i dr. (2003) |
| USA | Cynthiana (*Vitis aestivalis)* | No significant effect | Main and Morris (2007) |
| USA | Pinot crni, Cabernet sauvignon | Positive effect at the end of fermentation, reduced during aging | Watson et al. (1999) |
| Espana | Tempranillo | Positive effect | Ortega-Heras et al. (2007) |
| Espana | Tempranillo | Positive effect at the end of fermentation, stabile during aging | Guadalupe et al. (2007) |
| Espana | | No observable/detectable effect | Revilla and Gonzales-San Jose (2003.) |
| Espana | Tempranillo | Positive effect at the end of fermentation, reduced during aging | Saenz-Navajas et al. (2006) |
| Espana | Monastrell | Results vary, depending on the enzyme | Rosario-Salinas et al. (2003) |
| Espana | Monastrell | Positive effect at the end of fermentation, lacked during aging | Romero-Cascales et. al. (2006) |
| Espana | Monastrell, Syrah, Cabernet sauvignon | Results vary, depending on the enzyme and variety | Gil-Munoz et al. (2007) |
| Espana | Monastrell | Positive effect at the end of fermentation, lost during aging | Romero-Cascales et. al. (2008) |
| Espana | Mencia | Positive effect | Perez-Lamela et. al. (2007) |
| Espana | Mencia | Positive effect | Ortega-Heras M. et. al. (2012) |
| Espana | Cabernet sauvignon | Positive effect at the end of fermentation, lacked during aging | Puertolas et al. (2009) |
| Espana | Tinto fino | Positive effect at the end of fermentation, lacked during aging | Revilla I. i Gonzales-Sanjose M. L. (2003) |
| Türkiye | Üküzgözü | Positive effect | Kelebek et. al. (2007) |
| Türkiye | Üküzgözü | No signifficant effect | Borazan and Bozan (2013) |
| Uruguay | Tannat | Positive effect in young wines | Gonzales-Neves et. al. (2001, 2003) |

## Materials and methods

**Grape and wine production**. The grapes of Plavac mali (Pelješac vineyards, 500 kg in the first and 350 kg in the second harvest) and Babić (Pirovac-Skradin vineyards, 350 kg both harvests) were hand-picked at technological maturity (Table 2), and transported to the experimental cellar in Zagreb, in 15 - 20 kg boxes. The fermentation and maceration was carried out in open plastic containers during 7 days, supplemented with 15 g / kg potassium metabisulphite, 20 g / hL Lalvin EC-1118 yeast (Lallemand, Canada) and 25 g/hL Fermeid E yeast food (Lallemand, Canada), at 20-22 ° C temperature. The fermentation cap was punched down twice a day. The wines were decanted two times with metabisulphite addition each time (10 g/hL) and bottled after the second decanting cycle. The wines were stored under controlled conditions.

**Treatments.** Treatments PC (Plavac mali, Control) and BC (Babić, Control): 7-days maceration in the same conditions as well as PE1, PE2, BE1 i BE2, but without enzyme addition. Treatments PE1 i BE1: 7-days maceration with the addition of commercial product E1 (Lallemand, France). The composition of product was: 4000 Pgu/g (polygalacturonase), 1000 Peu/g (pectin esterase) and 120 Plu/g (pectin lyase), originating from *Aspergillus niger*. The enzyme was used as recommended, 2g/100 kg of grapes, dissolved in water in a 1:10 ratio. Treatments PE2 i BE2: 7-days maceration with the addition of commercial product E2 (Lallemand, Estonia), with recomended dosage 2 g/100 kg of grapes, dissolved in water in a 1:10 ratio. The B product comprises pectinases and inactive *Saccharomyces cerevisiae* yeast cells.

**Chemical analysis.** The basic parameters of the must and wine quality were analysed by OIV methods (Compendium of International Methods of Analyses of wine and Musts).

Total phenols**:** A modified Ough and Amerine spectrophotometric method (1998) was used to determine total phenols. It is based on the visual reaction of phenols with the Folin-Ciocalteau reagent, and by measuring the resulting blue intensity at 750 nm (Arnous et al., 2001).

Antioxidative activity (AA, DPPH). According to the method of Brand-Wiliams et al. (1995), antiradical activity was defined by the concentration of antioxidants required to reduce the initial concentration of DPPH by 50%, using 2,2-diphenyl-1-picrylhydrazyl (DPPH *) in methanol solution as a synthetic stable radical that reacts with various antioxidants. In its radical form, DPPH * absorbs at 515 nm, but during reduction under the influence of one or more antioxidants (AH), or some other free radical (R •), the absorption is reduced and lost. The AA results are expressed in mmol/L Trolox equivalent, a vitamin E analogue.

The linear correlation between total phenols and antioxidant activity, expressed by Pearson's correlation coefficient, with a 95% confidence level, was calculated according to standard formulas using Statistica V.7.

Stilbene. Stilbene analyzes were performed by liquid chromatography (Mattivi F., 1993), method modified by Vanzo et al. (2008), using trans-resveratrol as an external standard and a Hypersil ODS C18 20 x 2.1 mm column (Agilent Technologies, USA). Hypersil ODS C18 250 x 2.1 mm column 8Agilent Technologies, USA) was used for sample preparation. An internal standard of *trans*-4-hydroxystilben with known concentration was used to determine *trans*-resveratrol. The analysis was performed on a liquid chromatograph (Agilent HPLC 1100), with a DAD detector and NDS ChemStation (Agilent Technologies, USA).

Statistical analyses**.** All analyses were performed in three replicates, and the analysis of variance was used to test the effect of the application of pectolytic enzymes.The Duncan test was used to compare the mean values. The program Statistica V.7 (Statsoft Inc., Tulsa, OK) was used in data processing.

## Results and discussion

The values of the basic grape quality parameters are presented in Table 1. These data clearly indicate the average quality of the grapes used in this experiment. Alcoholic fermentations were carried out without delay and after 7 days fermentation all wines were dry (reducing sugars content less than 2 g/L). An indicator of controlled fermentation conditions is the low volatile acidity of all wines, less than 0.5 g/L (as acetic acid), and the free/total $SO_2$ ratio, with total $SO_2$ content between 115 and 130 mg/L.

**Table 2** The must quality
**Tablica 2.** Kakvoća mošta

| | Plavac mali, V1 | Plavac mali, V2 | Babić, V1 | Babić, V2 |
|---|---|---|---|---|
| °Oe (specific gravity) | 81 | 76 | 84 | 86 |
| Total acidity (g/L, as tartaric acid) | 5.5 | 6.5 | 6.8 | 5.9 |
| pH | 3.37 | 3.62 | 3.31 | 3.36 |

Legend: V1 i V2: vintages

**Table 3** Total phenols (mg/L, as galic acid) and antioxidative activity (mg/L, Trolox) in Plavac mali wines
**Tablica 3.** Ukupni fenoli (mg/L, kao galna kiselina) i antioksidacijski potencijal (mg/L, Trolox), u vinima Plavca malog

| | PK/V1 | PE1/V1 | PE2/V1 | PK/V2 | PE1/V2 | PE2/V2 |
|---|---|---|---|---|---|---|
| TP, young wine | $1417^{a} \pm 116$ | $1617^{b} \pm 110$ | $1529^{c} \pm 109$ | $2087^{a} \pm 125$ | $2111^{b} \pm 112$ | $2037^{c} \pm 127$ |
| TP, after bottling | $2301^{a} \pm 128$ | $2404^{b} \pm 134$ | $2334^{c} \pm 127$ | $3411^{a} \pm 134$ | $3684^{b} \pm 126$ | $3707^{c} \pm 130$ |
| AA, after bottling | $2{,}33^{a} \pm 0{,}33$ | $2{,}63^{a} \pm 0{,}35$ | $2{,}40^{a} \pm 0{,}32$ | $4{,}68^{a} \pm 0{,}52$ | $5{,}85^{b} \pm 0{,}54$ | $4{,}32^{c} \pm 0{,}46$ |

Legend: TP: total phenols, AA: antioxidative activity, PK: control, PE1: enzyme E1, PE2: enzyme E2, V1 and V2: vintages. Mean values within one line indicated by different letters were significantly different according to the Duncan test ($p < 0.05$).

**Table 4** Total phenols (mg/L, as galic acid) and antioxidative activity (mg/L, Trolox) in Babić wines
**Tablica 4.** Ukupni fenoli (mg/L, kao galna kiselina) i antioksidacijski potencijal (mg/L, Trolox), u vinima Babića

| | BK/V1 | BE1/V1 | BE2/V1 | BK/V2 | BE1/V2 | BE2/V2 |
|---|---|---|---|---|---|---|
| TP, young wine | $634^{a} \pm 33$ | $738^{b} \pm 36$ | $684^{c} \pm 26$ | $871^{a} \pm 39$ | $1034^{b} \pm 40$ | $917^{c} \pm 51$ |
| TP, after bottling | $864 \pm 26$ | $957 \pm 31$ | $894 \pm 27$ | $911^{a} \pm 26$ | $1074^{b} \pm 31$ | $1031^{c} \pm 33$ |
| AA, after bottling | $3{,}43^{a} \pm 0{,}44$ | $4{,}11^{b} \pm 0{,}55$ | $3{,}56^{c} \pm 0{,}42$ | $5{,}15^{a} \pm 0{,}33$ | $5{,}63^{a} \pm 0{,}46$ | $5{,}23^{a} \pm 0{,}26$ |

Legend: TP: total phenols, AA: antioxidative activity, BK: control, PE1: enzyme E1 , PE2: enzyme E2, V1 and V2: vintages. Mean values within one line indicated by different letters were significantly different according to the Duncan test ($p < 0.05$).

The concentrations of total phenols in wines are presented in Tables 3 and 4. Compared to other results of total phenols in the same varieties, they are significantly less than previously obtained in Babić wines (Budić Leto, 2003), but from the top quality vineyards and significantly better grape quality parameters.

Enzymatic treatments influenced to the phenol content of the wines. The lowest concentrations of total phenols was measured in control treatment wines, regardless of the variety and the technological stage of production. The wines of both enzymatic treatments were significantly richer in total phenols, compared to the control. Plavac mali young wines of E1 treatment contained 16% more total phenols compared to control and 5% more compared to E2 treatment (Table 3). After the second decanting and bottling, significantly higher concentrations of total phenols were measured in all wines. In the first vintage, there was a 62% increase in total phenols of control wines, 49% in the E1 treatment wines and 52% in the E2 treatment wines, compared to the initial values at the end of the fermentation. The differences were even greater in the second vintage; 63% in control wines, 75% in E1 treatment wines, and 82% in E2 treatment wines. However, the differences obtained between treatments were not significant in both years of experiment. A significant 95% confidence difference was obtained between control and E1 treatment in both years at both sampling stages, between control and E2 treatment at one year in both sampling stages, and between enzymatic treatments at one year. It is important to emphasize that, as the wine matured, the difference between the treatments was smaller, in both years. It was evident in the loss of significance of the influence of the treatment on the total phenols value.

The same trend of changes in phenolic composition of Babić wines was observed, during the aging and between the treatments, in both years.The significant differences were obtained in both years of study (Table 4). The content of total phenols in young wines was 16% higher in E1 wines and 8% higher in E2 wines than in control. The concentrations of total phenol increased during the aging; for 36% in the control wines and by 30 and 31% in both treatments, respectively. A statistically significant difference at the 5% level was given for both preparations with respect to control treatment in young wine. The similar results were obtained in the second vintage with differences in favor of enzymatic treatments, in both sampling time, at the statistical level. A significant difference was also found between enzymatic treatments, in favor of the E1 product. In relation to product E1 and a positive effect on phenolic extraction in both years of study with both varieties, product E2 showed a discontinuous and non-uniform influence with respect to the observed parameters. The cause may be the composition of the product. Due to the potential contamination by other enzymes, originated primarily from yeast cells and from *Asperillus niger* too, this product may have a strong secondary activity, which is manifested in changes in the polyphenolic composition. In addition to the direct effect on extraction, enzymes can also influence the stability of a number of reactive compounds; anthocyanins and tannins which participate in numerous reactions during the wine aging. This is especially important in Plavac mali production, given the complexity of its polyphenolic properties; the richness of tannins primarily.

The variability of the effect of pectolytic enzymes on polyphenolic composition was obtained by other authors also, in studies with different varieties, different commercial products of known and unknown names or producer, and different application doses (Table 1).

In some previous studies, the same product was used as in the E1 treatment. Better polyphenol extraction and better quality descriptors at the end of fermentation are reported by almost all researchers; Watson et al. (1999) in Oregon (USA), Guadalupe et al. (2007) in Spain, Guerrand and Gervais (2002), in Medoc, France, Wolz et al. (2004) in Germany, and Puertolas et. al. (2009) in Spain.

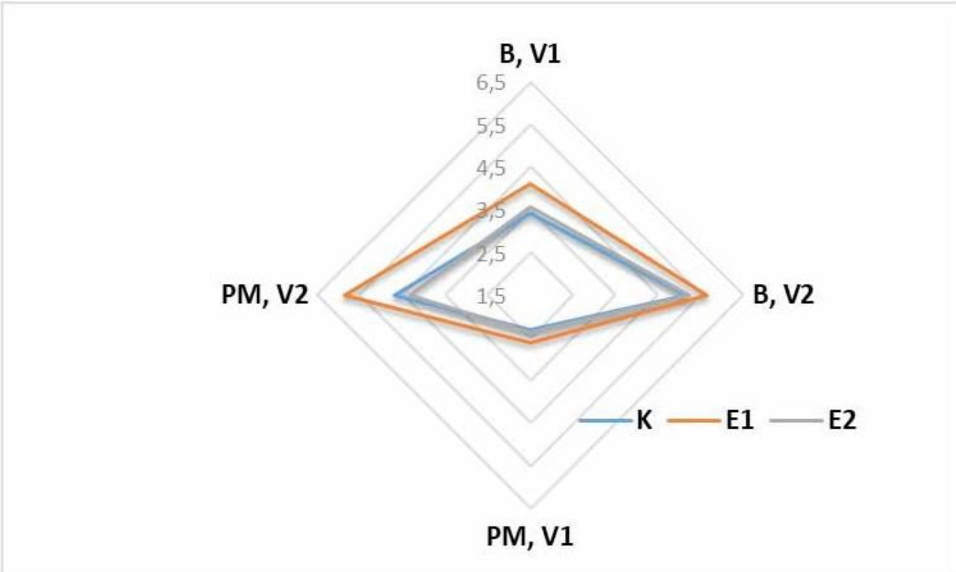
The positive effect of enzymes decreases during wine aging, depending on the variety and the time of sampling, and in most studies, despite the relative difference between treatment and control, after a few months, it is no longer significant. Guerrand and Gervais (2002) reported a positive effect of product E1 to fenolic quality one year after fermentation. However, other authors in research with Cabernet Sauvignon variety in Spain (Puertolas et al., 2009) have not confirm the efficacy of the same enzyme product. They found the differences in quality significant immediately after the fermentation,better chromatic properties and total phenols expressed through the phenolic index. However, after three months the significant differences disappeared, the differences of measurements of the mentioned parameters were less than 5%. Most authors emphasize the significant influence of exogenous enzymes on the polyphenol composition of young wines; there are only few studies in which a significant difference in quality is retained during wine maturation, especially longer than 4 months (Table 1). The polyphenol composition of the wine is very complex; in the continuous reactions of co-pigmentation, hydrolysis, condensation, precipitation, the composition and compounds relationships constantly change. Most of these reactions can be recognized through the visual changes and taste of wine. Despite numerous researches, the knowledge that could explain all the reactions and changes in polyphenolic composition during extraction and wine maturation is still insufficient (Garrido and Borges, 2013). The efficiency of the applied enzymes is conditioned primarily by the biological properties of the grape, and the contamination of the commercial enzyme products, primarily by $\beta$-glucosidase, esterases and proteases (Fia et. al., 2014). The extraction capacity depends on the solid-liquid berry ratio (berry skin/juice) which is determined by the variety (Bautista-Ortin et al. 2005; Romero-Cascales et al. 2008; Romero-Cascales et al. 2012). Furthermore, the aforementioned enzyme side-effect, caused by product contamination can be reflected in chemical changes, primarily anthocyanins, the transition to aglycone, less stable forms, and loss the color (Sacchi et al., 2005).

**Antioxidative activity (AA)**

Considering that most quality wines of Plavac mali and Babić varieties are usually put on market several months after fermentation, antioxidant activity analyzes were carried out in wines after the second decanting and bottling. The results should indicate the actual state in wine production therefore. The antioxidant activity in relation to treatments was not representative, neither for varieties nor in harvests. The highest absolute values of AA in Plavac mali, in the first vintage, was obtained in the E1 wines, but was not at the level of statistical significance. However, in the second vintage, with the same results in absolute value of AA in E1 treatment wines, the differences E1/control and E1/E2 were significant (Table 3).

In the experiment with Babić (Table 4), the best results were obtained in the E1 treatment wines, as well as in Plavac mali. A significant statistical difference was found in the first vintage between all treatments. Despite the differences in absolute values and better results in E1 treatment wines, there was no significant difference between treatments in the second year (Chart 1). The antioxidant activity is strongly related to the complex phenolic composition, and there are problems in different methods of measuring AA, and approaches in the methodology for determining individual phenolic fractions (Stratil et al. 2008). Although the E2 product is more complex because of inactive *Saccharomyces cerevisiae* yeast cells, the AA results have not been significantly better, either in Babić or Plavac mali wines.

A possible explanation for the results of AA, particular in enzyme treatments may be sought in changing of the composition and ratio of phenolic fractions that directly affect the antioxidant potential (Di Mayo et al., 2008). Moreover, this problem has been noted by other authors (Pimenta-Braz et al., 1998; Gerbauxu et al., 2002; Fisherleitner and Eder, 2008). The basic cause is probably the composition of the products, primarily the contamination of pectinase enzymes which can inactivate the underlying enzymatic activity. However, Mihnea M. et. al. (2016) obtained positive results of the effect of the added enzyme on AA, expressed as ABTS and FRAP, on the Spanish Prieto picudo variety.




Legend: K: control, E1 i E2: treatments with enzymes, V1 and V2: vintages. B: Babić, PM: Plavac mali

**Graph 1** Antioxidant activity in wines, depending on the treatments, varieties and vintages
**Grafikon 1.** Antioksidacijska aktivnost u vinima, u odnosu na tretmane, sorte i godine

Determining the linear correlation between total phenols and antioxidant activity in wines after the second decanting is important in order to check and confirm the stability of the phenolic composition during wine maturation. This ratio may vary, for each variety and year and is effected with enzyme and potential changes phenolic composition that directly affect the wine antioxidant potential. The results of the correlation analysis indicate a high degree of association between total phenols and antioxidant activity. In Plavac mali wines, the correlation coefficient was 0.98 (first vintage) and 0.88 (second vintage). The correlation coefficient in Babić wines was 0.65 (first vintage) and 0.98 in second vintage. Values greater than 0.92 provide confidence at the 99% level and values greater than 0.81 provide confidence at the 95% level. Variable values of the correlation between antioxidant potential and total phenols, as in the Babić case, have been obtained by some other authors: Orak (2007); Bajano et al. (2009); Paixao et al. (2006); Pazourek et al. (2005) and De Beer et al. (2003). According to some research (Orak, 2006; Villani et al., 2006), the best results od phenolic quality were obtained in experiments with Cabernet Sauvignon, Merlot and Syrah varieties. These results have been explained with biological properties of grape varieties; berry size, berry skin thickness, and ratio of berry size/skin surface. It provide a large active surface for the extraction of phenolic compounds as already mentioned.

**Stilbenes**

The results of *trans*- and *cis*-resveratrols and their glucoside forms *trans*- and *cis*-piceids analyse are presented in Tables 5 and 6. The use of the enzyme affected the content of stilbene in wines of both varieties.

**Table 5** Stilbene in Plavac mali wines (mg/L)
**Tablica 5.** Stilbeni u vinima Plavca malog (mg/L)

| | Young wine | | | Wine, after bottling | | |
|---|---|---|---|---|---|---|
| | PS | PE1 | PE2 | PS | PE1 | PE2 |
| *trans*-resveratrol | 5,60$^{a}$ | 5,59$^{a}$ | 4,96$^{a}$ | 4,96$^{a}$ | 5,13$^{a}$ | 3,98$^{b}$ |
| *cis*-resveratrol | 1,09$^{a}$ | 1,04$^{a}$ | 0,84$^{b}$ | 0,8$^{a}$ | 0,8$^{a}$ | 0,84$^{a}$ |
| *trans*-piceid | 3,83$^{b}$ | 4,02$^{a}$ | 3,61$^{b}$ | 3,4$^{a}$ | 3,37$^{a}$ | 3,61$^{a}$ |
| *cis*-piceid | 1,21$^{c}$ | 1,82$^{a}$ | 1,43$^{b}$ | 1,21$^{b}$ | 1,17$^{b}$ | 1,43$^{a}$ |
| total | 11,73$^{b}$ | 12,47$^{a}$ | 10,84$^{c}$ | 10,31$^{a}$ | 10,47$^{a}$ | 10,17$^{a}$ |

Legend: Legend: PS: control, PE1: enzyme E1, PE2: enzyme E2, V1 and V2: vintages. B: Babić, PM: Plavac mali . Mean values within one line indicated by different letters were significantly different according to the Duncan test (p <0.05).

**Table 6** Stilbene in Babić wines (mg/L)
**Tablica 6.** Stilbeni u vinima Babića (mg/L)

| | Young wine (V1) | | | Wine, after bottling (V1) | | | After fermentation (V2) | | | Wine, after bottling, V2) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BS | BE1 | BE2 | BSV1 | BE1 | BE2 | BS | BE1 | BE2 | BS | BE1 | BE2 |
| *trans*-resveratrol | 1,43$^{a}$ | 1,61$^{a}$ | 1,4$^{a}$ | 1,29$^{a}$ | 1,13$^{a}$ | 1,24$^{a}$ | 2,87$^{b}$ | 3,38$^{a}$ | 3,53$^{a}$ | 1,65$^{c}$ | 2,00$^{b}$ | 2,54$^{a}$ |
| *cis*-resveratrol | 0,08$^{b}$ | 0,09$^{b}$ | 0,4$^{a}$ | 0,4$^{a}$ | 0,39$^{a}$ | 0,38$^{a}$ | 0,92$^{a}$ | 0,82$^{a}$ | 0,78$^{a}$ | 0,98$^{a}$ | 1,01$^{a}$ | 0,71$^{a}$ |
| *trans*-piceid | 4,36$^{b}$ | 4,52$^{a}$ | 4,22$^{b}$ | 2,74$^{a}$ | 2,31$^{b}$ | 2,72$^{a}$ | 1,54$^{b}$ | 1,88$^{a}$ | 2,09$^{a}$ | 1,40$^{b}$ | 1,97$^{a}$ | 1,83$^{a}$ |
| *cis*-piceid | 1,23$^{a}$ | 1,28$^{a}$ | 1,24$^{a}$ | 0,93$^{a}$ | 0,85$^{a}$ | 0,96$^{a}$ | 0,69$^{a}$ | 0,87$^{a}$ | 0,88$^{a}$ | 1,18$^{a}$ | 1,03$^{a}$ | 0,61$^{b}$ |
| total | 7,1$^{a}$ | 7,5$^{a}$ | 7,26$^{a}$ | 4,78$^{b}$ | 5,76$^{a}$ | 5,3$^{a}$ | 5,99$^{b}$ | 6,95$^{a}$ | 7,27$^{a}$ | 5,21$^{b}$ | 6,01$^{a}$ | 5,70$^{a}$ |

Legenda: BS: kontrola, BE1: enzyme A, BE2: enzyme B., V1 i V2: berbe. Mean values within one line indicated by different letters were significantly different according to the Duncan test (p <0.05).

In both years of experiments with the Babić variety, both enzymatic preparations were effective. Compared to the control treatment, wines of E1 and E2 treatments had significantly higher concentrations of stilbene, with the biggest in *trans*-resveratrol. Significant differences in the concentrations of stilbene compounds were obtained in both years of the study, between the wines of both treatments and controls. The stilbene analyses of Plavac mali wines were made in one year of experiment.

The highest concentrations of stilbene were obtained in E1 treatment wines, significantly higher than in wines of treatment E2 and in control wines. The E2 product did not affect the increase of stilbene content in the wines, moreover, these wines were inferior to control. Although stilbene concentrations in wines decreased during maturing, E1 treatment wines after bottling still contained the highest stilbene concentrations, but the differences were not significant. An interesting difference was observed between the varieties in the ratio of the individual stilbene compounds. While the most desirable *trans*-resveratrol was absolutely dominated in Plavac mali wines, a significantly higher proportion of *trans*-piceids, compared to other compounds, was obtained in Babić wines, regardless of the time of sampling. The concentrations of *cis*-isomers of resveratrol and piceid increases during wine aging, as it has been observed in wines of both varieties. Since enzymatic pectolytic activity and cell wall pectin degradation cause transfer of resveratrol in juice, for each variety differently, it is necessary to carry out experiments with commercial enzymes, in relation to the variety and other technologies (Eder et al., 2004). According to the results in this research, Plavac mali may be a better source of these important bioactive compounds. The question of poor transformation of piceid into resveratrol in Babić wines should be discussed in future experiments.

Although research on the effects of various factors on stilbene, most notably resveratrol, has been numerous, there has been little published work on the effect of the use of pectolytic enzymes on their composition. Clare et al., (2002) and Poussier et al. (2003) studied the effect of pectolic enzymes on the extraction of resveratrol in red wines and found no significantl affect to the concentration of resveratrol. Italian researchers Todaro et al. (2008) conducted experiments with enzymes of different origins (*Aspergillus niger* and *Saccharomyces cerivisiae*), with Nero d´Avola and Nocera varieties. The maceration with purified commercial *ß*-glucosidase from *Aspergillus niger* increased the content of *trans*-resveratrol by 75%, without impact to the sensory properties of the wine. Gambuti et al. (2007) found that wines produced with the addition of pectolytic enzymes contained significantly higher concentrations of *trans*-resveratrol compared to prolonged maceration and wine aging barrique. They suggested direct influence of the enzyme on extraction; hydrolysis of glycosidic precursors and increase of extraction, as well as action coused by undesirable *ß* -glucosidase from the pectolytic enzyme. A possible cause may also be the impact of the synergistic effect of the endo and exoenzyme of the grapes with the additional effects of admixture originated from commercial enzyme product (van Rensburg and Pretorius, 2000), with the negative side-effect primarily by the activity of *β*-glucosidase.

## Conclusion

The enzyme preparation used in this experiment have influenced the poliphenolic extraction during maceration in production of Babić and Plavac mali red wines, but showed different effect on its stability during aging. The total phenols, stilbene profile and antioxidant activity of Babić and Plavac mali varieties during maceration and aging were differently affected by the presence of enzymes, showing a variety effect too. Maceration with pectolytic enzyme improved phenolic content, in young wines after fermentation and after six months aging, in both varieties. Maceration with the product of pectolytic yeast and inactive yeast cells combination did not significantly increase the content of total phenols, regardless of variety. Both enzymatic product influenced to the total stilbenes, and increased *trans*- and *cis*-resveratrol and *trans*-

and *cis*-piceid, in wines of both varieties. The product comprised enzymes and inactive yeast cells have shown to be less effective, and a better affinity was expressed in Plavac mali than in Babić. The values of antioxidant activity, although absolutely higher in the wines of enzymatic treatments in both varieties, compared to the control, were not significantly confirmed in both years. The changes in parameters after 6 months aging were obtained in wines of both varieties. The use of exogenous enzyme is acceptable and desirable in production of young wines of Babić and Plavac mali. Moreover, due to the phenolic composition, primarily of bioactive compounds, it is advisable to recommend the consumption of young wines, taking this as advantage in informing consumers about wine quality.

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## Ukupni fenoli, stilbeni i antioksidacijski potencijal vina Babića i Plavca malog; učinkovitost pektolitičkih enzima

**Sažetak**

*Polifenoli vina posebno su važni zbog bioaktivnih spojeva i njihovog potencijalnog pozitivnog utjecaja na ljudsko zdravlje. Istraživanja prihvatljivih i sigurnih tehnologija koje će omogućiti proizvodnju vina bogatijih ovim spojevima su i potreba i izazov. Stoga je cilj ovog istraživanja bio ispitati utjecaj komercijalnih pektolitičkih enzima na ukupne fenole (UF), stilbene i antioksidacijsku aktivnost (AA) u vinima sorata Babić i Plavac mali, podrijetlom s lokaliteta osrednje kakvoće. U istraživanju su korištena dva proizvoda različitih svojstava: pektinaze s celulazama i hemicelulazama (E1) i pektinaze s inaktivnim stanicama kvasaca (E2), uz maceraciju bez dodatka enzima kao kontrolu (K). Parametri kakvoće određivani su u različitim fazama proizvodnje; od završetka maceracije i fermentacije, do vina nakon drugog pretoka i punjenja. Oba proizvoda utjecala su na parametre kakvoće. Maceracija s pripravkom pektolitičkih enzima (E1) omogućila je bolju ekstrakciju i proizvodnju vina sa značajno većom koncentracijom ukupnih fenola, obe sorte i u obe godine. Značajna pozitivna korelacija dobivena je između ukupnih fenola i antioksidacijske aktivnosti (AA). Najveće vrijednosti AA dobivene su u vinima tretmana E1, u obe godine istraživanja, kod obe sorte. Značajne razlike dobivene su kod obe sorte, ali ne u obe godine. Oba tretmana (E1 i E2) osigurala su proizvodnju vina s većim sadržajem stilbena, i pojedinačno resveratrola i piceida, međutim značajne razlike nisu dobivene u obe godine istraživanja. Zabilježene su razlike između sorata: dok je u vinima Plavca malog dominirao resveratrol, u vinima Babića je bio veći udio piceida. Unatoč relativno boljim pokazateljima kakvoće, u odnosu na kontrolu, primjena proizvoda E2 nije pokazala opravdanu učinkovitost; dobivene značajne razlike nisu potvrđene niti za jednu sortu kroz obe godine istraživanja. Tijekom dozrijevanja zabilježene su promjene ispitivanih parametara obe sorte uz smanjenje nekih pozitivnih učinaka i gubitak značajnosti u odnosu na kraj fermentacije. Zaključno, primjena komercijalnih enzima može biti poželjna u proizvodnji vina Babić i Plavac mali, s naglaskom na mlada vina.*

**Ključne riječi:** *pektolitički enzimi, stilbeni, ukupni fenoli, antioksidacijska aktivnost, vino, Babić, Plavac mali*