Effect of Enzyme-Assisted Vinification on Wine Phenolics, Colour Components, and Antioxidant Capacity

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The aim of this study was to evaluate the impact of the macerating enzyme addition (Sihazym Extro and Vinozym Vintage) on the extraction of phenolics, colour components, and antioxidants in Babica wine. Spectrophotometry was used for determination of total phenolics, anthocyanins, and wine colour parameters (intensity, hue, and chromatic structure); the individual phenolics were detected using HPLC; while reducing and free radical scavenging activities of the samples were analysed using the FRAP and DPPH assays. The results indicate a more favourable effect of the Sihazym Extro on the extraction of phenolics, while both enzymes improved the extraction of anthocyanins during the maceration. The most abundant phenolic compounds were malvidin derivatives whose concentration continuously increased during the vinification and reached 82% of all anthocyanin derivatives in the control wine and 81% in both enzyme-treated wines. As expected, the antioxidant activity of the samples followed the trend of phenolics content growth and increased during the vinification, resulting in the higher activity of the enzyme-treated wines.

INTRODUCTION

Despite the devastation of natural potentials and strong degarization, the grape vine cultivation and wine production in Dalmatia has subsisted through the centuries. The representations of the individual grape varieties have changed significantly, but most of the indigenous species remained preserved. In the region of Central Dalmatia, especially in the coastal area from Kaštela to Trogir, Babica is the most abundant red grape cultivar. For a long time it was considered as synonym for the well-known Croatian red grape variety Babić, however, ampelographic and genetic identifications confirmed that Babica and Babić are two completely different varieties and that Babica is the direct descendant of the Plavac mali variety [Maletić et al., 2009; Zdunić et al., 2008]. According to the recent data, this grape cultivar occupies an area of approximately 18.5 ha, mostly in Kaštela-Trogir vineyards [Maletić et al., 2015], where, due to its good features and resistance to the diseases, it is a leading grape variety for the production of different types of wine [Ozmec et al., 2015].

Wine colour is an important element of wine quality and one of the most important features that influence its commercial acceptance. The compounds responsible for red wine colour and flavour are anthocyanins, that are accumulated in the grape skin, and their extraction during the maceration is an essential step during red wine production. In the traditional winemaking, during the classic maceration, only about 40% of total anthocyanins are extracted from the skins [El Darra et al., 2016], the maximal concentration is reached between 3 and 8 day of maceration and afterwards a slight decrease of anthocyanins could be noted [Ortega-Regules et al., 2006; Rio Segade et al., 2015; Romero-Cascales et al., 2012; Sacchi et al., 2005].

Today, the imperative of winemaking industry is the production of wines with a high biological value, expressed colour intensity, and improved stability during the aging so the addition of pectolytic enzymes often plays a fundamental role as their use offers quantitative (higher juice yield), qualitative (improved extraction and better organoleptic properties, colour stability during the wine aging, maturation and storage), and processing benefits (e.g. shorter maceration, filtration) [Claus & Mojsov, 2018; Kelebek et al., 2007; Mihaeta et al., 2015; Mojsov, 2013; Romero-Cascales et al., 2012].
The concentration of monomeric anthocyanins in red wines during the maturation declines constantly as a result of different mechanisms such as their adsorption by yeast, degradation and oxidation, precipitation with proteins, polysaccharides and/or condensed tannins, and the irreversible formation of more complex (oligomeric and polymeric) pigments which are crucial for colour stability [Boulton, 2001; He et al., 2012]. All these reactions are responsible for colour changes from the deep-purple colour of the young wines (due to presence of monomeric anthocyanins and reactions of self-association co-pigmentation), to the orange and brick-red tones as the wine ages [Marquez et al., 2013].

Numerous studies have been carried out into the effect of various pre-fermentative oenological practices on the yield and profile of extracted red wine phenolics, and the reported results are often contradictory. The results of our recent study on Crkvenak kaštanski variety [Generalić Mekinić et al., 2019] showed that the highest yield of phenolics was detected in wine produced without enzyme addition; no significant increase was reported in the study of El Darra et al. [2016], while studies of Pardo et al. [1999], Kelebek et al. [2007], and Romero-Cascales et al. [2012] reported a better extraction yield of phenolics by the use of enzyme preparations. In turn, Pardo et al. [1999], Kelebek et al. [2007], Mojsov et al. [2010], Bichescu et al. [2012], and Rio Segade et al. [2015] detected a higher content of anthocyanins in enzyme-treated wines, and regarding the wine chromatic and sensoric characteristics Bautista-Oritz et al. [2005] reported opposite effects of two enzyme preparations, while Pardo et al. [1999], Kelebek et al. [2007], Soto-Vazquez et al. [2010], Bichescu et al. [2012], and Romero-Cascales et al. [2012] confirmed the positive effect of enzymes on these wine parameters.

The aim of this study was to evaluate the effect of pectolytic enzyme addition during winemaking of Babica cv. on the extraction and evolution of wine phenolic compounds, colour components, and antioxidants in order to draw conclusions on choosing the optimal procedure for the production of highly-coloured red wine rich in biologically active compounds.

**MATERIALS AND METHODS**

**Chemicals and reagents**

All standards, reagents, and solvents used in this research were of adequate analytical grade and were purchased from Kemika (Zagreb, Croatia), Alkaloid AD (Skopje, Macedonia), BDH Chemicals (London, UK), Fluka (Buchs, Germany), and Sigma-Aldrich (Steinheim, Germany).

**Grape samples and vinification**

The raw materials were hand-picked grapes from *Vitis vinifera var. Babica* (from the vineyard located in Kaštela, Croatia). After the harvest, about 100 kg of grapes for each experiment were transported to winery and processed as follows: a) by the traditional vinemaking procedure (control sample), and by two procedures using enzymes (3 g/100 L); b) Vinozym Vintage® FCE (Novozymes A/S, Bagsvaerd, Denmark), and c) Sihazym Extro (Eaton Begerow Product Line, Langenlonsheim, Germany), respectively. The vinification procedure and sampling protocol were previously described by Generalić Mekinić et al. [2019]. Weighed and destemmed grapes were crushed with an MGM-940 crusher (MIO, Osijek, Croatia) and pectolytic enzyme preparations were added to fermentation tanks. The prepared mashed were treated with potassium metabisulphite (10 g/100 L) and inoculated (15 g/100 L) (SIHA®, Aktiv Hefe 8, Burgundy Yeast, E. Begerow GmbH & Co., Langenlonsheim, Germany). The maceration time was 5 days (at ~25 to 27°C), and the cap of grape solid was kept soaked using the mechanical barrier. After maceration, free run wine and the pressed wine were combined, and the pomace was removed. The produced wine was sealed with the tank’s floating lid and paraffin oil. The dynamics of the extraction was monitored daily during the maceration and after the racking (approximately 40 and 160 days after the winemaking process started).

**Phenolic compounds and wine colour parameters**

Spectrophotometric measurements were performed on a Specord 200 spectrophotometer (Analytik Jena GmbH, Jena, Germany).

The total phenolics content in the samples was determined by the colorimetric Folin-Ciocadeliu method [Singleton & Rossi, 1965] and the results are expressed as mg of gallic acid equivalents per litre (mg GAE/L).

The monomeric anthocyanins content was determined using the assay described originally by Amerine & Ough [1980]. The obtained results are expressed as mg of malvidin 3-O-glucoside equivalents per litre (mg M-3-gl/L).

Wine colour parameters as intensity (CI), hue (T), and the percentages of yellow (OD 420 = A 420 /CI × 100), red (OD 520 = A 520 /CI × 100), and blue (OD 620 = A 620 /CI × 100) were measured and calculated according to Alpeza et al. [2017].

**HPLC analysis**

The individual phenolic compounds were detected using a high-performance liquid chromatography (HPLC) system consisting of an autosampler, a binary pump, a vacuum de-gasser, a UV/VIS detector, and the Peltier column oven (all of Series 200) (Perkin Elmer, Walthamn, MA, USA). Separation, identification, and quantification of phenolic acids, flavonoids, and stilbene were performed on an UltraAqueous C18 column (250×4.6 mm, 5 μm, Restek, Bellefonte, PA, USA) while the analysis of anthocyanins was performed on the Kinetex core-shell C18 column (150×4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA).

Separation, identification, and quantification of the individual phenolics were performed by HPLC methods as described by Generalić Mekinić et al. [2019] using 0.2% phosphoric acid in water (solvent A) and 50% acetonitrile in methanol (solvent B). A gradient was applied as follows: from 96% A at 0 min to 50% A at 40 min, to 40% A at 45 min, to 20% A at 50 min, to 20% A at 53 min, then from 20 to 96% A at 54 min, and maintaining 96% A for 11 min (65 min). The flow rate was 0.8 ml/min and the injection volume was 20 μL. The detection of phenolic acids, flavonoids, and resveratrol was carried out at 280 nm on the column maintained at 25°C. The separated peaks were identified by comparing their
retention times with those acquired for corresponding standards, while quantification was performed using external calibration curves generated for each detected compound. Additionally, sample spiking was also used to assist confirmation of peak identity.

The anthocyanins were detected at 520 nm on the column maintained at 40°C. The elution solvents in this method were 0.3% perchloric acid in water (solvent A) and 0.3% perchloric acid in methanol (solvent B). The linear gradient was as follows: from 28% B to 51% B in 42 min, then to 69% in 3 min and to 80% B in 1 min 80% B for 3 min. The time of equilibration for the column to the initial gradient was 6 min. The flow rate was 0.6 mL/min and the injection volume was 10 μL. The peaks of anthocyanins were identified and quantified as described by Vanzo et al. [2008] and Budić-LeTO et al. [2018]. The compounds were eluted in order of polarity which was used for their identification (according to the retention time of each peak at 520 nm), while quantifications (concentration in mg/L) were performed using the external standard curve generated for malvidin 3-O-glucoside.

Antioxidant activity

The reducing activity of the samples was detected as the ferric reducing antioxidant power (FRAP) [Benzie & Strain, 1996] and the results are expressed as micromoles of Trolox equivalents per litre (μmol TE/L).

The free radical-scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) was investigated according to the procedure described by Katalinić et al. [2013] and the results are expressed as inhibition percentage (%).

Statistical analysis

All analyses were carried out in triplicate and the data are given as the mean ± standard deviation. Statistical analysis was performed using STATGRAPHICS® Centurion XVI (StatPoint Technologies, Inc., The Plains, Virginia, USA). Differences between the investigated parameters were analysed by one-way ANOVA followed by Fisher’s least significant difference test, while Pearson’s correlation coefficient (p<0.05) was used to determine relations between the variables.

RESULTS AND DISCUSSION

Changes in concentrations of total phenolics and anthocyanins in Babica wine sampled during the vinification, with and without enzyme addition, are presented in Figure 1. The phenolics content was continuously increasing during the vinification, with slight decreases between the first and the second racking in the control sample and in the wine produced by the addition of Vinozym Vintage. At the end of winemaking, the highest concentration of phenolics was detected in the sample produced by the addition of Sihazym Extro (1771 mg GAE/L), while the two other wine samples contained the similar amount of phenolic compounds. Figure 1b presents results obtained for monomeric anthocyanins content and they are in agreement with those reported in our previous study [Generalić Mekinić et al., 2019]. The majority of anthocyanins were extracted during the first few days of maceration, supporting the results reported by Ivanova et al. [2012] who demonstrated that extended maceration resulted in a slight decrease in the content of phenolic compounds and anthocyanins. The Vinozym Vintage sample showed the highest value at day four (180 mg M-3-gl/L) and a slight decrease on the fifth day, while the anthocyanin content of the other two samples increased. Again, a decrease of anthocyanins from the first to the second racking can be noted in all samples with the final concentration being 144 mg M-3-gl/L in the control wine, 142 mg M-3-gl/L in the Vinozym Vintage sample, and 152 mg M-3-gl/L in the Sihazym Extro sample. Statistical analysis confirmed a high correlation between the phenolic and anthocyanin content (r=0.9348, p<0.0001). If the results obtained for Babica are compared to those obtained for C. kastelanski, it can be concluded that C. kastelanski contains almost 2-fold higher concentration of total phenolics, while the content of anthocyanins is higher in Babica (especially in the samples prepared with Sihazym Extro). These results could be partially compared to those reported by Maletić et al. [2009] where two close relatives of C. kastelanski (Dobričić and Plavac mali) contained more phenolics than Babica. However, these authors reported a high concentration of anthocyanins in Babica (200 mg/L). The highest concentration of anthocyanins was found in Dobričić while the lowest among all tested cultivars was detected in Crvenak viški (50.7 mg/L) and Plavac mali (81.5 mg/L). Results of their study confirmed also a negative correlation between contents of shikimic acid, which is important in the biosynthesis of anthocyanins, and anthocyanins.

The phenolic profiles of the investigated wines are shown in Table 1. Three phenolic acids (gallic, protocatechuic, and p-hydroxybenzoic acid), two flavonoids from the group of flavan-3-ols (catechin and its epimer), flavonol quercetin, and stilbene resveratrol were detected in all samples. The gallic acid was dominant among phenolic acids with the concentration range from 16.68 to 17.80 mg/L. The protocatechuic acid content was the highest in the control wine, while enzyme addition enhanced the extraction of p-hydroxybenzoic acid. Catechin and epicatechin are the most abundant compounds among the flavan-3-ols, which are usually detected in wines. These compounds are important as they interact with the anthocyanins by the process of co-pigmentation and play a crucial role in defining the sensory properties of red wines. The extraction of these flavan-3-ols is enhanced with prolongation of the maceration process and an increase of ethanol amount [Bautista-Ortin et al., 2007; Katalinić et al., 2004]. From the presented results it can be seen that both enzymes significantly affected extraction of catechin, while Sihazym Extro had a negative effect on epicatechin and quercetin contents. The concentration of resveratrol in the samples ranged from 1.86 mg/L to 2.82 mg/L with the maximum concentration found in the Vinozym Vintage wine sample. The detected concentrations of resveratrol are several times higher than those detected in C. kastelanski wine [Generalić Mekinić et al., 2019].

Furthermore, 16 different anthocyanin derivatives (in the form of 3-O-glucosides, 3-O-acetylglicosides, 6-O-(caffeoyl)glucosides, and 6-O-coumaroyl)glucosides) were detected in the samples and the results are presented in Table 1, while the chromatograms are shown in Figure 2. Grape
Evolution of Phenolics During Vinification

Anthocyanidins are usually in the form of glycosides (primarily glucosides) in which the sugar component of the molecule increases their stability and solubility. The major forms of anthocyanins in Babica wine samples were 3-O-glucosides, with malvidin being the dominant and the most stable form. These results are consistent with those obtained in C. kaštelanski [Generalić Mekinić et al., 2019], but also with other results available in the literature [Alpeza et al., 2017; He et al., 2012; Maletić et al., 2009]. The proportion of malvidin derivatives in total anthocyanins reached 82% in the control wine and 81% in the enzyme-treated wines. The content of malvidin-3-O-glucoside after the first racking ranged from 91.62 to

FIGURE 1. Changes of a) total phenolics and b) total anthocyanins in Babica wine during the vinification without enzyme addition and with the addition of enzyme A (Vinozym Vintage) or enzyme B (Sihazym Extro).

GAE- gallic acid equivalents, Enzyme A – Vinozym Vintage, Enzyme B – Sihazym Extro, M-3-gl- malvidin 3-O-glucoside, Rack 1/Rack 2- 40/160 day of winemaking process. Different letters (a-c) above bars denote statistically significant difference (p<0.05) among wine samples.
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95.13 mg/L (data not showed), while at the end of the vinification process those values significantly decreased. A significant correlation was observed between concentrations of malvidin-3-O-glucoside and monomeric anthocyanins (r=0.5982, p=0.0042).

The colour of red wine is influenced by numerous wine-growing and processing factors affecting transfer of pigments during wine-making. Moreover, during the maturation and aging, the red wine colour changes from an intense red hue characteristic for anthocyanins to a more red-orange hue which comes from different anthocyanin-derived compounds (pyranoanthocyanins) [He et al., 2012]. Wines with higher co-pigmentation and higher amount of acylated forms of non-malvidin compounds have deeper colour [Boulton, 2001]. Although these colour changes can be observed with the naked eye, they are usually easily determined spectrophotometrically by measuring such parameters as CI and T, as well as OD 420, OD 520, and OD 620.

### TABLE 1. Phenolic compounds content of Babica young wine (mg/L) produced by traditional vinification without and with the addition of enzymes Vinozym Vintage (Enzyme A) or enzyme Sihazym Extro (Enzyme B).

<table>
<thead>
<tr>
<th>Group</th>
<th>Phenolic compound</th>
<th>No enzyme</th>
<th>Enzyme A</th>
<th>Enzyme B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic acids</td>
<td>Gallic acid</td>
<td>16.68±0.08</td>
<td>17.80±0.07</td>
<td>17.39±0.23</td>
</tr>
<tr>
<td></td>
<td>Protocatechuic acid</td>
<td>2.12±0.03</td>
<td>1.89±0.05</td>
<td>1.79±0.06</td>
</tr>
<tr>
<td></td>
<td>p-Hydroxybenzoic acid</td>
<td>1.48±0.11</td>
<td>2.30±0.14</td>
<td>2.12±0.05</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>31.79±0.22</td>
<td>44.34±0.25</td>
<td>42.27±0.38</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Epicatechin</td>
<td>62.02±1.01</td>
<td>65.10±2.23</td>
<td>38.46±0.53</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>1.39±0.02</td>
<td>1.65±0.02</td>
<td>1.24±0.02</td>
</tr>
<tr>
<td>Stilbenes</td>
<td>Resveratrol</td>
<td>1.86±0.01</td>
<td>2.82±0.04</td>
<td>2.17±0.02</td>
</tr>
<tr>
<td></td>
<td>Delphinidin-3-O-glucoside</td>
<td>3.27±0.01</td>
<td>3.22±0.02</td>
<td>2.66±0.04</td>
</tr>
<tr>
<td></td>
<td>Cyanidin-3-O-glucoside</td>
<td>0.14±0.01</td>
<td>0.15±0.01</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td></td>
<td>Petunidin-3-O-glucoside</td>
<td>6.15±0.13</td>
<td>6.30±0.36</td>
<td>6.13±0.62</td>
</tr>
<tr>
<td></td>
<td>Peonidin-3-O-glucoside</td>
<td>4.04±0.37</td>
<td>4.27±0.17</td>
<td>4.47±0.21</td>
</tr>
<tr>
<td></td>
<td>Malvidin-3-O-glucoside</td>
<td>59.90±0.25</td>
<td>58.86±0.02</td>
<td>61.91±0.20</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>Delphinidin-3-O-acetylglucoside</td>
<td>0.82±0.05</td>
<td>0.56±0.00</td>
<td>1.10±0.09</td>
</tr>
<tr>
<td></td>
<td>Cyanidin-3-O-acetylglucoside</td>
<td>0.03±0.00</td>
<td>0.03±0.01</td>
<td>0.07±0.00</td>
</tr>
<tr>
<td></td>
<td>Petunidin-3-O-acetylglucoside</td>
<td>0.13±0.00</td>
<td>0.14±0.01</td>
<td>0.10±0.00</td>
</tr>
<tr>
<td></td>
<td>Peonidin-3-O-acetylglucoside</td>
<td>0.25±0.00</td>
<td>0.27±0.01</td>
<td>0.23±0.00</td>
</tr>
<tr>
<td></td>
<td>Petunidin-(6-O-caffeoyl)glucoside</td>
<td>0.11±0.00</td>
<td>0.10±0.00</td>
<td>0.24±0.07</td>
</tr>
<tr>
<td></td>
<td>Malvidin-3-O-acetylglucoside</td>
<td>2.59±0.01</td>
<td>2.70±0.02</td>
<td>2.90±0.13</td>
</tr>
<tr>
<td></td>
<td>Malvidin-(6-O-caffeoyl)glucoside</td>
<td>1.16±0.02</td>
<td>0.91±0.02</td>
<td>0.98±0.01</td>
</tr>
<tr>
<td></td>
<td>Cyanidin-(6-O-coumaryoyl)glucoside</td>
<td>0.27±0.00</td>
<td>0.30±0.00</td>
<td>0.20±0.00</td>
</tr>
<tr>
<td></td>
<td>Petunidin-(6-O-coumaryoyl)glucoside</td>
<td>0.04±0.00</td>
<td>0.05±0.00</td>
<td>0.06±0.00</td>
</tr>
<tr>
<td></td>
<td>Peonidin-3-(6-O-coumaroyl)glucoside</td>
<td>0.58±0.00</td>
<td>0.72±0.02</td>
<td>0.65±0.01</td>
</tr>
<tr>
<td></td>
<td>Malvidin-3-(6-O-coumaroyl)glucoside</td>
<td>4.30±0.00</td>
<td>4.82±0.03</td>
<td>4.37±0.02</td>
</tr>
</tbody>
</table>

Enzyme A – Vinozym Vintage; Enzyme B – Sihazym Extro. Different letters (a-c) in superscript in the row denote statistically significant difference (p<0.05) between concentrations of detected phenolics in wine samples.
CI, usually defined as the colour amount that indicates wine darkness, is a parameter that is mostly determined by the content and structure of the anthocyanins present in wine [Glories, 1984]. The CI of Babica wines was successively increasing and the highest values were detected in all samples after the second racking (Figure 3). Ivanova et al. [2012] investigated the effect of the maceration on the extraction of wine colour components and found a correlation between the concentration of total anthocyanins and the CI. In this study, a correlation was also detected between the value of this pa-
parameter and total phenolics content \((r=0.9416, p<0.0001)\) as well as between CI and monomeric anthocyanins content \((r=0.9150, p<0.0001)\). T is a parameter that indicates the development of a colour towards orange tones and it increases during wine aging. Its highest values were recorded after the second racking (range from 0.96 to 0.99). Ribèreau-Gayon et al. [2006] reported that the normal T values for young red wines are between 0.5 and 0.7 but they increase during wine ageing up to 1.3. Figure 4 shows the shares of the three basic colour components (yellow, red and blue) in the overall Babica wine colour during the vinification without and with enzyme addition. The highest absorption was detected at the wavelength of 520 nm where red wines have the maximal absorption. According to Glories [1984], the most optimal ra-
**FIGURE 4. Colour composition of the three basic colour components in Babica wine during the vinification without enzyme addition (a) and with Vinozyme Vintage (b) and Sihazym Extro. Rack 1/Rack 2- 40/160 day of winemaking process.**

The evolution of phenolics during vinification is shown in Figure 2. The optical density values for OD 420 (yellow), OD 520 (red), and OD 620 (blue) were measured over a period of 5 days for wines treated with enzymes and control samples in Babica wine. The results show a significant increase in antioxidant properties, especially CI, correlated with the phenolic profile and chromatic characteristics. The use of pectolytic enzyme preparations in winemaking is a well-established practice for improving wine quantity and quality. The results of this study contribute to a better understanding of the mechanism of enzyme actions and their effect on the phenolic profile, chromatic characteristics, and antioxidant properties of wine.
FIGURE 5. Comparison of the antioxidant properties of Babica wine samples with and without enzymes during vinification obtained by FRAP (a) and DPPH (b) method.

Enzyme A – Vinozym Vintage, Enzyme B – Sihazym Extro, TE– Trolox equivalents. Rack 1/Rack 2- 40/160 day of winemaking process. Different letters (a-c) above bars denote statistically significant difference (p<0.05) among wine samples.
RESEARCH FUNDING

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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