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Changes in the Composition of Aroma and Phenolic Compounds Induced by Different Enological Practices of Croatian White Wine

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The aim of this research was to investigate the effects of pre-fermentative maceration, *Saccharomyces* and non-*Saccharomyces* yeasts during alcoholic fermentation, and antioxidant additions (sulfur dioxide and glutathione) at bottling on the compositions of aroma and phenolic compounds of white wine Pošip (*Vitis vinifera* L.). Additionally, for the first time the insight in volatile thiol precursors in Pošip grape was given, wherein higher concentrations of glutathionylated thiol precursors in comparison to cysteinylated ones were determined. Regarding the applied practices, significant differences among produced wines were established: pre-fermentative maceration resulted in a decrease of 3-sulfanylhexyl acetate (3SHA) and a slight increase of C6 compounds; indigenous yeasts produced higher concentrations of terpenes and esters, while sequential fermentation with *Torulaspora delbrueckii* also influenced higher concentrations of esters. Most abundant phenolic compound was caffeic acid, except for wine produced by indigenous yeasts where *trans*-caftaric acid was predominant. Finally, combination of higher SO_2 and glutathione resulted in higher concentrations of thiols.

INTRODUCTION

The composition of wine and, consequently, its overall quality depends on numerous factors and their interactions. During grape ripening, significant changes occur in the chemical composition of grapes where, primarily, increase of sugar levels along with changes in phenolic and aroma compound profiles take place. Among them, significant increase of volatile thiol precursors, namely 3-*S*-cysteinylhexan-1-ol (Cys-3SH), 3-*S*-glutathionylhexan-1-ol (Glut-3SH), 4-*S*-cysteinyl-4-methylpentan-2-one (Cys-4MSP), and 4-*S*-glutathionyl-4-methylpentan-2-one (Glut-4MSP) also occur during this vine-growing phase [Jeffery, 2016; Roland *et al.*, 2011b]. Recent investigations suggested the presence of new thiol precursors such as cysteinyl-glycine S-conjugate (CysGly) and γ-glutamyl-cysteine S-conjugate (γGluCys) [Bonnaffoux *et al.*, 2017, 2018], as well as *S*-3-(hexanal)-glutathione (Glut-3SH-Al) and its bisulfite (Glut-3SH-SO_3) [Thibon *et al.*, 2016]. Generally, these precursors are odorless compounds that undergo enzymatic cleavage during alcoholic fermentation which result in volatile thiols release. Liberated compounds: 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexan-1-ol (3SH), 3-sulfanylhexyl acetate (3SHA), are very desirable and important varietal aroma compounds, especially for Sauvignon blanc wines, since they contribute to the enticing boxwood, grapefruit, and passionfruit nuances [Tominaga *et al.*, 1998]. However, it is demonstrated that only a small portion (less than 5%) of thiol precursors are cleaved [Roland *et al.*, 2011b].

Despite the certain concentrations in grapes, transfer of the aroma compounds and their precursors to the must and, subsequently, in wine during wine production is strongly affected by the applied process techniques. For example, in early stage of winemaking, the application of maceration could significantly affect their extraction into grape juice and could lead to modifications in resulting wine. It is known that application of maceration technique could result in improved quality and stability of white wines, due to the increased extraction of aroma compounds and precursors, such as volatile thiols precursors [Olejar *et al.*, 2015], as well as phenolic compounds [Di Lecce *et al.*, 2013]. However, it is necessary to conduct this process under strictly controlled conditions, in order to reduce excessive phenolics extraction and consequently to reduce browning of white wines. Furthermore, release of volatile thiols by yeasts during alcoholic fermentation, as well as

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formation of the new aroma compounds as a result of yeast metabolism, significantly affect the final aroma of wine, where the selected yeast strain could have detrimental effect [Belda *et al.*, 2017; Renault *et al.*, 2016; Sadoudi *et al.*, 2012; Zott *et al.*, 2011]. So far, the use of *Saccharomyces cerevisiae* as the most important yeast in winemaking, has been extensively studied in order to understand its role for wine properties [Swiegers *et al.*, 2005]. Recently, non-*Saccharomyces* yeast were arisen as innovative tools for industrial wine production as it has been demonstrated that these yeasts could improve aromatic complexity and distinction of the wines [Azzolini *et al.*, 2012; Loira *et al.*, 2014; Renault *et al.*, 2016]. Several studies showed the advantages of *Torulaspora delbrueckii* in winemaking, such as low production of acetic acid, contribution to the overall aroma profile through the higher esters and volatile thiols production, as well as decreasing the perception of vegetal flavor [Azzolini *et al.*, 2012; Renault *et al.*, 2015, 2016]. Finally, it is well-known that the overall wine aroma depends on the physicochemical reactions that occur during aging, where the oxidation reactions play a significant role and result in a loss of fresh and fruity character of wines. Lately, the importance of antioxidants additions during bottling is increasingly emphasized in a view of longer protection of wine, where the addition of glutathione is particularly highlighted due to its protective effect against aroma loss and browning in white wines [Kritzinger *et al.*, 2013].

The Pošip cultivar is a native grape variety of *Vitis vinifera* L. grown in Croatia southern vine-growing region, primarily on the island of Korčula and, economically, it is the most important white wine variety in this region. Recently, it has provoked the great interest of the winemakers due to its fruity, citrus aroma that resembles the scent often described as 'Sauvignon aroma', presumably due to the presence of volatile thiols, compounds that will be evaluated in this research. The aim of this study was to evaluate the effect of different enological practices on the compositions of aroma and phenolic compounds of produced Pošip wines: the pre-fermentative maceration technique, indigenous yeast strains fermentation, *T. delbrueckii* sequential fermentation and commercial *S. cerevisiae* fermentation, along with antioxidant additions (sulfur dioxide and glutathione) at the bottling. Apart from that, the content of thiol precursors in Pošip grapes will be evaluated for the first time ever.

MATERIALS AND METHODS

Chemicals

Deionized water was produced by a Millipore Milli Q system (Bedford, MA, USA). Ethanol was HPLC grade and purchased from J.T. Baker (Deventer, The Netherlands); sodium chloride p.a., ethyl acetate p.a., hydrochloric acid (37% v/v) and sodium sulfate anhydrous were purchased from Carlo Erba (Val de Reuil, Spain); sodium acetate trihydrate from Gram-mol (Zagreb, Croatia); acetic acid from Alkaloid (Skopje, Macedonia); liquid nitrogen from Messer Croatia (Zagreb, Croatia). Methanol HPLC grade, glutathione reduced, *N*-acetyl-cysteine, 2-aminoethanol, *o*-phthalaldehyde, cysteine hydrochloride hydrate, dichloromethane, *p*-hydroxymercurybenzoate (*p*-HMB), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), Dowex exchange resin, formic acid and sulfuric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethylendiaminotetraacetic acid disodium salt dehydrate (EDTA) p.a. was purchased from Kemika (Zagreb, Croatia); sodium tetraborate decahydrate p.a. and sodium acetate p.a. from Merck (Darmstadt, Germany). The aroma reference standards, with the highest available purity (minimum of 98%), were purchased from Merck, Sigma Aldrich, Fluka (Seelze, Germany) and SAFC (St. Louis, MO, USA) except of 4-methyl-4-sulfanylpentan-2-one (4MSP) and 3-sulfanylhexyl acetate (3SHA) which were purchased from Oxford Chemicals (Hartlepool, UK), as well as 3-sulfanylhexanol (3SH) and 4-methoxy-2-methyl-2-sulfanylbutane which were purchased from Penta Manufacturing Company (Livingston, MONT, USA). Deuterated [$^2H_2$]-3-sulfanylhexyl acetate (d3SHA) and [$^2H_2$]-3-sulfanylhexan-1-ol (d3SH) standards were obtained from University of Auckland, New Zealand. Cysteinylated and glutathionylated thiol precursor standards, together with deuterium labeled analogues (Cys-4MSP-d6 and Glut-4MSP-d10) were synthesized according to the procedures described in details by Vanzo *et al.* [2017]. Chemicals, used for precursor synthesis were analytical or higher grade purity and obtained from Sigma Aldrich, as well as hydroxycinnamic acids reference standards and GRP.

Grapes sampling

Grape samples were collected from the Croatia southern vine-growing region, microlocation Čara, island Korčula. This region is classified in C_3 viticultural climatic zone, as well as in C II wine-growing zone of the European Union, with yearly insolation up to 2700 h and annual precipitation of around 730–1050 mm. The grapes were sampled at their technological maturity; the grape harvest and wine production were carried out the same day (end of August 2015). The physicochemical characteristics of harvested grapes were: reducing sugars 206.5±3.8 g/L; total acidity 5.3±0.1 g/L with pH 3.4±0.1. The berry sampling was conducted in a random way, where three replicates of 100 berries were picked from three different plots of 25 randomly selected vines within the vineyard. Herein, berries were picked from top, the center, and the tip of selected clusters, both exposed to the sun and in shade. Immediately after picking, grapes were rapidly frozen in the liquid nitrogen and transferred to the laboratory in dry ice and stored at -80°C prior to analysis.

Wine production

The grapes were hand-harvested at technological maturity (physicochemical characteristic previously noted), placed in plastic boxes, transported to winery and immediately processed. Winemaking was carried out in Krajančić winery (island Korčula, Croatia). After the grapes were destemmed and crushed, vinification was carried out in four distinct processes, where the first process represents the control wine (wine PK): destemmed and crushed grapes were pressed under inert atmosphere of nitrogen (N_2) with the addition of SO_2 at 50 mg/L, addition of 25 mL/hL of clarifying agent (Hydroclar 30, Enartis, San Martino, Italy) and flotated with nitrogen as the flotation agent. Clarified must was transferred to a stainless steel tank and inoculated with *Saccharomyces cerevisiae* Lalvin

TABLE 1. Physicochemical properties of produced wines.

	Wine PK	Wine MK	Wine MT	Wine MI
Alcohol (%, v/v)	13.1±0.1^{bc}	13.0±0.1^{c}	13.5±0.2^{ab}	13.6±0.2^{a}
Total acidity (g of tartaric acid equivalents/L)	5.3±0.1^{b}	5.7±0.2^{ab}	5.5±0.1^{ab}	5.9±0.2^{a}
Volatile acidity (g of acetic acid equivalents/L)	0.5±0.0^{b}	0.4±0.0^{c}	0.4±0.0^{bc}	0.6±0.1^{a}
pH	3.6±0.0^{ab}	3.6±0.0^{a}	3.5±0.0^{b}	3.6±0.0^{ab}
Reducing sugars (g/L)	2.1±0.1^{b}	3.0±0.1^{a}	2.6±0.2^{a}	2.8±0.2^{a}
Total extract (g/L)	21.0±0.9^{b}	23.8±1.1^{ab}	23.2±1.2^{ab}	23.9±1.1^{a}
Malic acid (g/L)	2.2±0.3^{a}	2.6±0.3^{a}	1.9±0.3^{a}	2.5±0.3^{a}
Lactic acid (g/L)	nd	nd	nd	nd

Concentrations expressed as mean ± standard deviation (n=3). Abbreviations: P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; nd – not detected. Means with different superscript letters in the same row differ significantly ($p<0.05$).

EC-1118 yeasts (25 g/hL, Lallemand, Montreal, Canada) and kept with fermentation temperature below 18°C. Further processes consisted of destemming and crushing and sulfuring (at 50 mg/L), followed by pre-fermentative maceration for 12 h at temperatures below 10°C, pressing under inert atmosphere (N_2) at the same pressing program as in the case of wine PK, addition of 25 mL/hL of a clarifying agent (Hydroclar 30), and flotation with nitrogen. After flotation, clarified must was divided into three stainless steel tanks of 1000 L volume and each tank was subjected to fermentation with different yeasts: (i) first tank (wine MK), was inoculated with 25 g/hL *Saccharomyces cerevisiae* Lalvin EC-1118 yeasts, same as control, PK wine; (ii) second was subjected to sequential inoculation of *Torulaspora delbrueckii* with *Saccharomyces cerevisiae* (25 g/hL, LEVEL2 TD, Lallemand, Montreal, Canada) (wine MT); and finally (iii) third tank was subjected to indigenous yeasts fermentation (wine MI). During sequential fermentation, *T. delbrueckii* was inoculated first followed 48 h later by inoculation of *S. cerevisiae*. Prior to fermentation with indigenous yeasts, a small portion of wine (approx. 60 L) was transferred into a separate tank and slightly heated, at approximately 25°C, in order to accelerate yeast propagation phase. After fermentation started, wine was transferred to the main tank. This procedure assured that *S. cerevisiae* were predominant yeasts for alcoholic fermentation. As in the case of control wine, alcoholic fermentation temperature was kept under 18°C. After fermentation was finished (residual sugars below 3 g/L), SO_2 was added at a concentration level of 20 mg/L. Approximately 50 days after alcoholic fermentation, wines (PK, MK, MT, MI) were bottled with the four distinctive variants of antioxidant additions and closed by screw-cap closures. Physicochemical properties of the produced wines are summarized in Table 1. The antioxidants addition variants were: (i) standard free SO_2 concentration (free SO_2 30 mg/L), (ii) higher SO_2 concentration (free SO_2 45 mg/L), (iii) addition of 20 mg/L of glutathione (free SO_2 30 mg/L), and (iv) higher SO_2 concentration with 20 mg/L of glutathione (free SO_2 45 mg/L). The schematic illustration of wine production is presented in Figure 1. Each variant was bottled in triplicate and the wines were analyzed after 6 months of storage at cellar temperature (15–18°C).

Grape analysis

Analysis of volatile thiol precursors, glutathione (GSH), and oxidized glutathione (GSSG) in grapes

The analysis of volatile thiol precursors, GSH, and GSSG in grapes was carried out using the method described by Vanzo *et al.* [2017]. Briefly, a 10 g aliquot of pulverized frozen grapes was rapidly transferred into cold, deoxygenated methanol (1:4, w/v), spiked with deuterium-labelled internal standards (Cys-4MSP-d6 and Glut-4MSP-d10), vortexed, extracted, and centrifuged. A small aliquot of the extract was filtered and directly injected onto 1290 infinity UHPLC system coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) and separated by 100 mm × 2.1 mm. 1.8 μm column (Acquity HSS T3, Waters, Milford, MA, USA). The chromatographic and mass spectrometry parameters used, were identical as stated in previously mentioned research. Direct injection allowed quantification of GSH and GSSG together with Glut-3SH, which was present in Pošip grape extract above limit of quantification (LOQ). To determine Glut-4MSP, Cys-4MSP, and Cys-3SH which were below LOQ by direct injection, grape extracts were concentrated and purified according to the procedure described in previously mentioned research. Recovery of Glut-4MSP-d10 was used for quantification of Glut-4MSP, whereas recovery of Cys--4MSP-d6 was used for quantification of Cys-3SH and Cys--4MSP. Analyses were conducted in triplicate.

Wine analyses

Glutathione analysis

Concentrations of GSH in wines were analyzed by Agilent 1200 Series high-performance liquid chromatography with fluorescence detection (HPLC-FLD) (Agilent Technologies) and on-line column derivatization. Separation was performed at 25°C using a Synergi Fusion-RP 80A column (150 mm × 2.0 mm, 4 μm) (Phenomenex Inc., Aschaffenburg, Germany) according to the method in detail described by Janeš *et al.* [2010]. Wine samples were, immediately after bottle open-

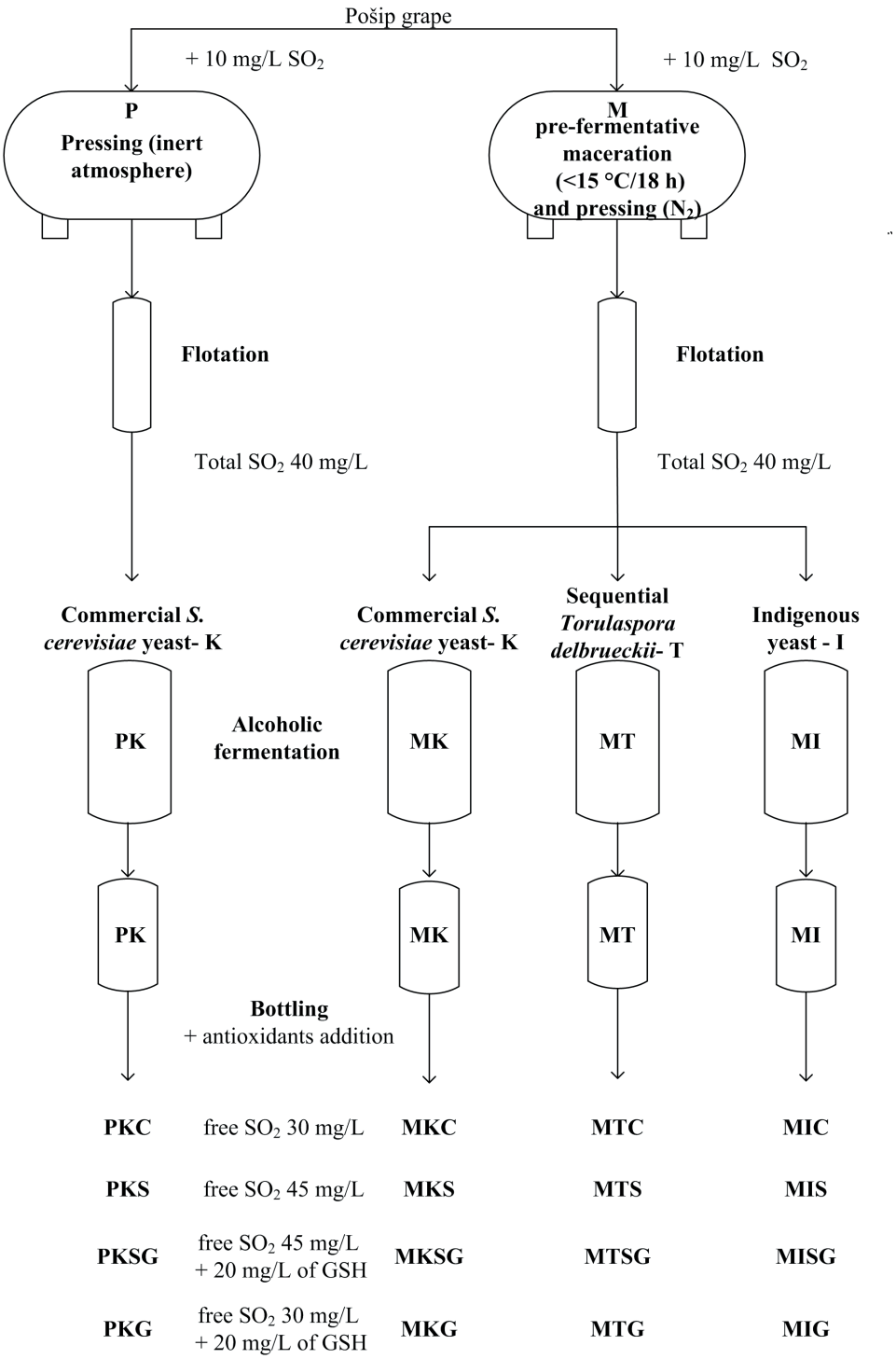

FIGURE 1. Schematic diagram of Pošip wine production.

ing, placed in methanol (1:10), with *N*-acetyl-l-cysteine as the internal standard, filtered through 0.45 μm Minisart RC 25 filters Sartorious (Goettingen, Germany), diluted 1:1 with a 5 mM sodium acetate buffer containing 0.1 mM EDTA, and analyzed as previously described [Janeš *et al.*, 2010]. Analyses were conducted in triplicate.

Volatile thiols analysis

Three thiols: 4MSP, 3SH, and 3SHA, were determined in wines by slightly modified method of Tominaga *et al.* [1998]. The modified method was in detail described by Šuklje *et al.* [2013]. Identification and quantification was performed with a gas chromatograph (Agilent Technologies 7890A) equipped with the MPS 2 automatic sampler (Gerstel, Mülheim an der Ruhr, Germany) and coupled with mass spectrometric detector (Agilent Technologies 5973C upgraded with Triple Axis detector). For quantification of analyzed thiols, single point calibration was used, where water solution of 4MSP at concentration of 70 ng/L, 3SHA at concentration 660 ng/L, and 3SH at concentration of 1654 ng/L was extracted with

the same extraction procedure as samples. Analyses were conducted in triplicate.

Terpenes analysis

Prior to GC/MS analysis, terpenes were extracted and concentrated by headspace solid-phase microextraction (SPME) technique using polydimethylsiloxane/divinylbenzene fiber (Supelco, Bellefonte, PA, USA). Identification and quantification was performed with a gas chromatograph (Agilent Technologies 7890A) equipped with the MPS 2 automatic sampler (Gerstel, Mülheim an der Ruhr, Germany) and coupled with mass spectrometric detector (Agilent Technologies 5973C upgraded with Triple Axis detector). Single point calibration was used for quantification. Sample preparation, as well as extraction and chromatographic conditions were in detail reported by Bavčar *et al.* [2011]. Analyses were conducted in triplicate.

Minor volatiles (without terpenes) analysis

A discontinuous liquid-liquid extraction (LLE) with dichloromethane was used for the extraction of volatile compounds, primarily esters, according to the previously described method [Bavčar *et al.*, 2011]. Identification and quantification were performed with a gas chromatograph (Hewlett Packard 6890, Agilent Technologies) coupled with mass spectrometric detector (Hewlett Packard 5973). For quantification single point calibration was used. Analyses were conducted in triplicate.

Hydroxycinnamic acids and their tartrate esters analysis

Hydroxycinnamic acids and their tartrate esters (HCA), namely *cis*- and *trans*-caftaric, coutaric, fertaric, caffeic, *p*-coumaric and ferulic acids, together with glutathione derivative of caftaric acid (GRP) were analyzed using previously described methods (HPLC analysis) [Šuklje *et al.*, 2012; Vanzo *et al.*, 2007], with the exception of the injection volume of 10 μL. The sample was filtered through a 0.25 μm CA filter (Millipore, Bedford, USA). Separation was performed by Agilent 1100 HPLC with DAD detection (Agilent Technologies), using a 250 × 2.1 mm, 5 μm, ODS Hypersil C18 column (ThermoFisher Scientific, Waltham, MA, USA). The identification of HCA and GRP was carried out by comparison of their spectra and retention time with those of standards, as described by Šuklje *et al*. [2012]. The HCA for which no standards were available (*cis*-caftaric, *cis*-coutaric, and *cis*-fertaric acid) were identified by their retention time and spectral parameters, as reported in previous investigations [Bengoechea *et al.*, 1995; Pena-Neira *et al.*, 2000; Vanzo *et al.*, 2007]. Quantitative determinations were made by using the external standard method with *trans*-caftaric acid, and the respective concentrations of HCA and GRP in the samples were expressed as *trans*-caftaric acid equivalents. As described in Šuklje *et al*. [2012], a calibration curve was prepared by injecting a standard of *trans*-caftaric acid in the range from 1.05 to 500 mg/L. Analyses were conducted in triplicate.

Statistical analysis

The statistical data analysis was carried out using Statistica V.10 software (Statsoft Inc., Tulsa, OK, USA). The one-way analysis of variance (ANOVA) was performed on all independent variables of the analyzed aroma compounds. In order to compare variable means of concentrations of aroma and phenolic compounds, Tukey's HSD test was used when samples were significantly different after ANOVA ($p<0.05$). In order to examine the significance of applied enological treatments, the main effects ANOVA was conducted, while the principal component analysis (PCA) was performed on the correlation matrix using the attributes of all analyzed compounds in order to examine any possible grouping of samples by different applied treatments.

RESULTS AND DISCUSSION

Thiol precursors

Content of four thiol precursors, namely Cys-4MSP, Cys-3SH, Glut-4MSP, Glut-3SH, as well as contents of GSH and GSSG in Pošip grapes are presented in Table 2. The highest content was determined in the analyzed grapes for Glut-3SH and it amounted 7.53 μg/kg, while the Glut-4MSP was found in the lowest content of 0.09 μg/kg. Regarding the contents of these compounds in other grape varieties, only a few investigations deal with their presence in grapes, while the most of the presented studies are focused on their levels in grape juices and musts. Nevertheless, based on available data it can be seen that contents of individual precursors in Pošip grapes, analyzed in the present study, are lower than of those found in Sauvignon blanc grapes. For example, the average contents of thiol precursors in Sauvignon blanc grapes from different vintages (2013–2015) were in the range of 8–16 μg/kg for Glut-3SH, 1–6 μg/kg for Cys-3SH, 1–4 μg/kg for Cys-4MSP, and 0.3 μg/kg for Glut-4MSP [Vanzo *et al.*, 2017]. Furthermore, sum of their contents determined in Sauvignon blanc grapes skin and pulp were in the range of 15–85 μg/kg for Cys-3SH and 17–112 μg/kg of Glut-3SH, while the content of Glut-4MMP amounted 8–11 μg/kg [Roland *et al.*, 2011a]. Furthermore, Capone *et al.* [2011] determined contents of 3SH precursor diastereomers (*R*-/*S*-Glut-3SH, *R*-/*S*-Cys-3SH) at the time of harvest, which amounted around 200 μg/kg of Glut-3SH and 30 μg/kg of Cys-3SH, representing more than 20-fold higher content than those obtained in Pošip grape. But, according to recent investigations [Jeffery, 2016; Vanzo *et al.*, 2017], lower contents are most likely due to the sampling method, where rapid freezing immediately after picking results in significantly lower contents due to the lack of oxygen or enzymatic reaction that impede

TABLE 2. Content of glutathione (mg/kg), oxidized glutathione (mg/kg), and volatile thiol precursors (μg/kg) in Pošip grapes.

Compound	Concentration in Pošip grapes
Glutathione (GSH)	68.37±1.72
Oxidized glutathione (GSSG)	0.75±0.03
Cys-4MSP	0.16±0.00
Cys-3SH	0.30±0.05
Glut-4MSP	0.09±0.00
Glut-3SH	7.53±0.75

Concentrations expressed as mean ± standard deviation (n=3).

precursors formation. Moreover, regarding the proportions among the analyzed precursors, inconsistent results could be found in previous research. Namely, according to Roland *et al.* [2010] cysteinylated precursors were more abundant than glutathionylated ones in Sauvignon blanc grapes, while higher amounts of glutathionylated precursors were found in Australian grape samples [Capone *et al.*, 2010] as well as in Melon B. and Sauvignon blanc grapes from Loire Valley and Montpellier [Roland *et al.*, 2011a]. The latter findings are in accordance with the results obtained in this research. Higher contents of GSH precursors could be due to the higher levels of glutathione in the grape berries, which could participate in Glut-3SH biogenesis [Roland *et al.*, 2011a]. Besides available glutathione, other factors, such as water deficit, assimilable nitrogen, as well as infection of grapes by *Botrytis cinerea* also modulate the final concentrations of precursors [Thibon *et al.*, 2009].

Besides the thiol precursors, concentrations of GSH and GSSG were also determined in the analyzed grape. Generally, GSH is the most abundant thiol compound of low molecular weight and its contents in grapes are closely related to the vine nitrogen status where nitrogen-deficient vines are characterized with its significantly lower levels [Choné *et al.*, 2006]. As presented in Table 2, GSH contents in Pošip grapes reached 68.37 mg/kg, representing a moderate level since it is determined in grapes in ranges of up to 114 mg/kg [Kritzinger *et al.*, 2013]. As stated previously [Roland *et al.*, 2011a], the latter authors [Kritzinger *et al.*, 2013] also indicated the possible relation of a higher GSH content with a higher content of glutathionylated thiol precursors, especially Glut-3SH, which is in accordance with our research. Furthermore, the high GSH:GSSG ratio, as stated in Kritzinger *et al.* [2013], indicates that no oxidative stress occurred in grape berries during the sampling as well as during sample preparation.

Glutathione in wines

The concentrations of GSH, determined in Pošip wines after six months of aging in bottles, are presented in Figure 2, where significant differences among wines could be observed. Primarily, those differences are related to various antioxidant additions at the bottling. For example, in all produced wines, the addition of glutathione (variant G), as well as combination of glutathione with higher SO_2 (variant SG) resulted in significantly higher concentrations of GSH six months after bottling, with more than 2-fold higher concentrations determined in the mentioned variants when compared with wine bottled with only SO_2 additions (variants C and S). It was previously

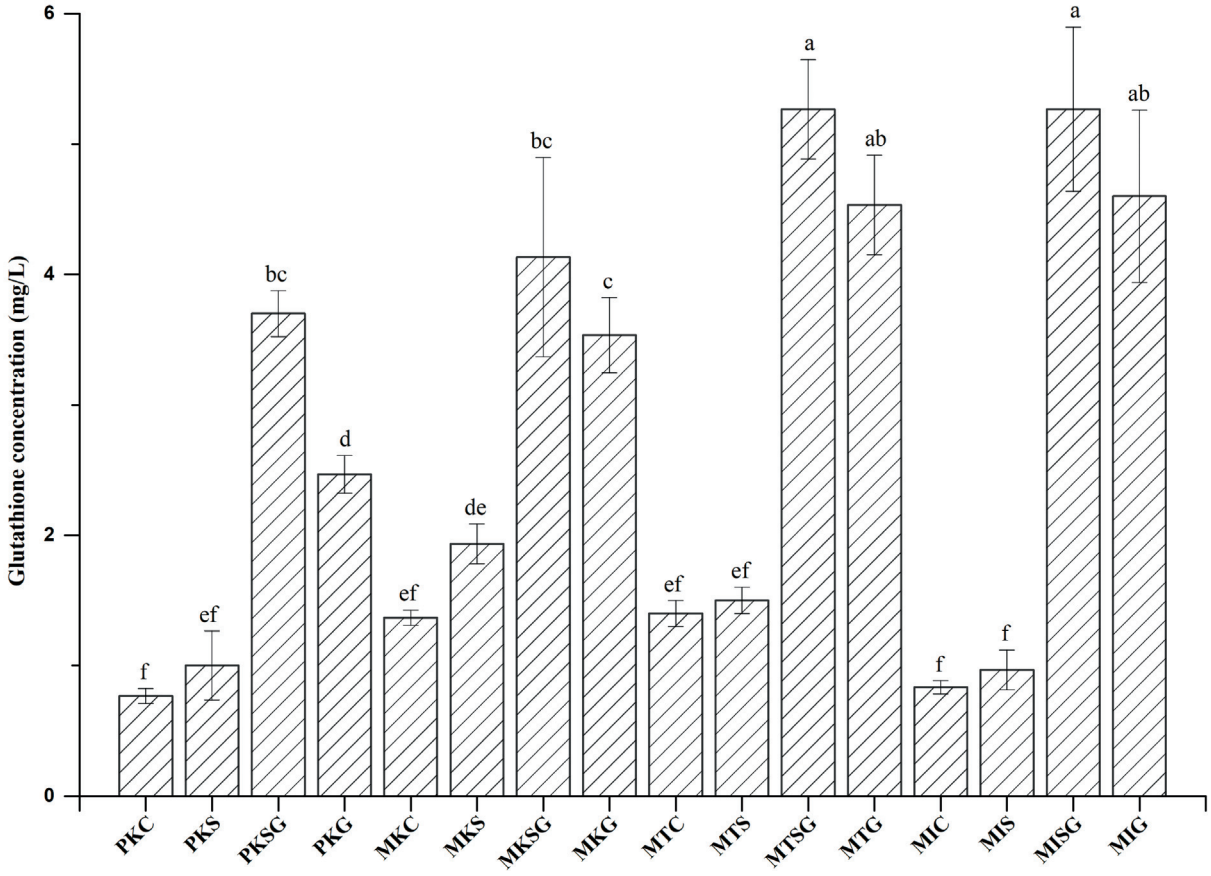

FIGURE 2. Concentration of glutathione (mg/L) in Pošip wines.

Data presented as means ± standard deviation (n=3). P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *T. delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione. Different letters indicate significant differences among wines (Tukey's test, $p<0.05$).

demonstrated that the combination of SO_2 and GSH acted synergistically due to the accelerated consumption of oxygen in white wines [Fracassetti *et al.*, 2013]. Furthermore, it can be seen that wines produced by sequential alcoholic fermentation of *T. delbrueckii* with *S. cerevisiae*, as well as wines fermented by indigenous yeasts resulted in slightly higher concentrations of GSH in comparison to wines fermented by commercial *S. cerevisiae* yeasts. Differences in GSH concentration in wines fermented by distinct yeast strain were also reported previously [Lavigne *et al.*, 2007], implying the importance of selected yeast strain on the final quality of wine. In comparison to grapes, concentrations of GSH in wines were significantly lower. This was expected since only a part of the GSH present in grapes is transferred to the must and wines, as well as considering the fact that its concentration significantly lowers during aging [Fracassetti *et al.*, 2013]. In fact, according to Ferreira-Lima *et al.* [2016], GSH practically disappears after 8 months of wine aging, which is in line with results of our study.

Aroma compounds

Regarding the effect of enological practices on wine aroma, this paper represents a further investigation of the previously published research [Tomašević *et al.*, 2017]. The present study involves different vintage and microlocation, as well as the use of additional enological practices, primarily *Torulaspora delbrueckii* yeasts for sequential alcoholic fermentation. Herein, different aroma compounds were identified in produced Pošip wines, and the results are shown in Tables 3 and 4, wherein Table 3 mostly represents the concentrations of aroma compounds (volatile thiols and terpenes) along with C6 compounds as grape-derived compounds, while Table 4 shows concentrations of esters, as contributors of fermentation aroma. Among aroma compounds, nine compounds were identified: 3 volatile thiols (4MSP, 3SHA and 3SH), four terpenes (linalool, α-terpineol, citronellol, and geraniol), and two C6 compounds (1-hexanol and *cis*-3-hexen-1-ol) (Table 3). Concentrations of the analyzed thiol compounds were in ranges of 29.6–48.0 ng/L (4MSP), 63–157 ng/L (3SHA), and 719–1273 ng/L (3SH). These concentrations are above their perception thresholds, namely 0.8 ng/L (for 4MSP), 4.2 ng/L (for 3SHA), and 60 ng/L (for 3SH) [Tominaga *et al.*, 1998], implying a potential positive influence of these compounds on the sensory characteristics of the produced Pošip wines. Generally, the concentrations of 3MH have been found to be particularly high, up to 18680 ng/L in Sauvignon blanc wines [Lund *et al.*, 2009], while lower concentrations, similar to these determined in Pošip wines, were determined in other grape cultivars, *e.g.* up to 970 ng/L in Riesling wines [Tominaga *et al.*, 2000] and up to 1200 ng/L in Pinot Noir wines [Capone *et al.*, 2015]. Other two compounds were detected in lower concentrations, up to

TABLE 3. Concentration of volatile thiols (ng/L), terpenes (μg/L), and C6 compounds (mg/L) in Pošip wines.

Samples	Volatile thiols			Terpenes				C6 compounds	
	4MSP	3SHA	3SH	Linalool	α-Terpineol	Citronellol	Geraniol	1-Hexanol	*cis*-3-Hexen-1-ol
PKC	43.0±3.3^{a}	104±9^{cd}	1245±27^{a}	40.9±1.5^{bc}	53.0±1.3^{f}	17.1±0.8^{ab}	62.4±5.3^{fgh}	0.70±0.02^{ab}	0.12±0.00^{d}
PKS	33.1±5.9^{a}	100±8^{d}	1161±92^{a}	37.3±0.6^{def}	53.6±1.5^{f}	14.8±0.6^{d}	57.8±5.6^{gh}	0.69±0.03^{ab}	0.12±0.01^{d}
PKSG	38.8±2.3^{a}	100±12^{d}	1273±46^{a}	34.3±0.7^{gh}	56.8±1.4^{f}	13.4±0.3^{e}	53.5±2.4^{gh}	0.66±0.03^{b}	0.11±0.00^{d}
PKG	40.2±8.6^{a}	96±4^{d}	1120±58^{a}	31.5 ±1.0^{h}	61.9±0.6^{e}	12.5±0.7^{a}	55.4±2.2^{gh}	0.67±0.02^{b}	0.11±0.00^{d}
MKC	43.3±4.2^{a}	63±3^{e}	1169±147^{a}	42.6±0.1^{b}	82.5±1.5^{c}	18.1±0.2^{bc}	75.6±5.3^{cde}	0.87±0.03^{a}	0.14±0.00^{ab}
MKS	36.7±6.5^{a}	65±6^{e}	1097±36^{a}	39.4±0.7^{bcd}	82.9±0.9^{c}	16.7±0.4^{cd}	65.4±2.8^{efg}	0.84±0.01^{a}	0.14±0.00^{bc}
MKSG	44.9±7.1^{a}	65±17^{e}	1176±60^{a}	35.1±0.8^{ef}	91.0±0.9^{b}	15.4±0.6^{d}	72.7±3.9^{def}	0.86±0.03^{a}	0.14±0.00^{ab}
MKG	48.0±9.5^{a}	65±9^{e}	1163±116^{a}	34.0±0.6^{gh}	91.9±0.9^{b}	15.2±0.2^{k}	75.2±6.7^{cde}	0.85±0.02^{a}	0.14±0.00^{b}
MTC	37.8±7.3^{a}	99±6^{d}	719±53^{c}	19.1±0.5^{h}	78.6±1.3^{c}	3.6±0.3^{hij}	50.4±2.0^{h}	0.46±0.01^{c}	0.12±0.00^{d}
MTS	39.6±8.6^{a}	100±5^{d}	721±35^{c}	42.0±1.4^{b}	64.8±1.2^{de}	7.1±0.3^{ij}	95.8±4.7^{ab}	0.47±0.03^{c}	0.12±0.01^{d}
MTSG	29.6±3.6^{a}	106±4^{bcd}	739±14^{bc}	38.2±2.5^{cde}	67.5±3.0^{d}	6.4±0.6^{j}	83.3±2.6^{cd}	0.45±0.00^{c}	0.12±0.00^{d}
MTG	31.5±6.1^{a}	104±12^{cd}	763±100^{bc}	35.8±0.5^{ef}	68.8±2.0^{d}	5.9±0.2^{f}	73.5±3.0^{def}	0.48±0.00^{c}	0.12±0.00^{cd}
MIC	43.5±2.8^{a}	134±18^{ab}	1016±193^{ab}	46.4±2.0^{a}	90.6±2.1^{b}	9.5±0.3^{f}	86.7±0.6^{bc}	0.73±0.02^{ab}	0.15±0.00^{ab}
MIS	38.4±5.3^{a}	155±10^{a}	1162±165^{a}	40.7±1.5^{bcd}	91.9±1.2^{b}	8.4±0.3^{fg}	79.3±2.6^{cd}	0.73±0.01^{ab}	0.15±0.00^{ab}
MISG	40.7±6.8^{a}	157±3^{a}	1152±12^{a}	37.4±0.7^{bcde}	94.1±2.9^{ab}	7.7±0.4^{gh}	75.7±0.9^{cde}	0.75±0.04^{a}	0.15±0.01^{d}
MIG	43.7±1.6^{a}	133±15^{abc}	1165±35^{a}	34.5±0.5^{gh}	98.0±1.0^{a}	7.3±0.1^{ghi}	105.5±6.2^{a}	0.70±0.02^{ab}	0.15±0.00^{ab}

Data presented as means ± standard deviation (n=3). Different superscript letters indicate statistical differences among wines (Tukey's test, $p<0.05$). Abbreviations: 4MSP, 4-methyl-4-sulfanylpentan-2-one; 3SHA, 3-sulfanylhexyl acetate; 3SH, 3-sulfanylhexan-1-ol; P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione.

2500 ng/L for 3SHA and up to 50 ng/L for 4MMP [Benkwitz *et al.*, 2012; Piano *et al.*, 2015; Ribéreau-Gayon *et al.*, 2006]. As can be seen in Table 3, there are slight, but not significant differences ($p \geq 0.05$) among samples regarding the concentration of 4MSP, while significant differences ($p < 0.05$) could be observed in 3SHA and 3SH concentrations. Additionally, regarding the applied enological practices, several trends can be drawn. Firstly, the pre-fermentative maceration in combination with commercial *S. cerevisiae* yeasts resulted in lower concentrations of 3SHA, while did not affected other thiols. Secondly, higher concentrations of the previously mentioned thiols were influenced by indigenous yeast fermentation, while sequential fermentation with *T. delbrueckii* resulted in higher concentrations of 3SHA, but lower ones of 3SH when compared to the fermentation by commercial *S. cerevisiae* yeasts. Thirdly, no uniform tendency could be observed regarding the antioxidant additions. But, in majority of the macerated wine samples, the highest concentrations of these compounds, especially of 3SHA, were determined in wines bottled with the combination of higher SO_2 and glutathione addition (variant SG). Regarding the pre-fermentative maceration, it is expected that its application enhances the varietal character of wines due to the improved extraction of aroma compounds and their precursors from grapes [Olejar *et al.*, 2015]. But, as can be seen in the presented results, in our case this process affected lower concentrations of 3SH and 3SHA (wine MK) and practically no changes in concentrations of 4MSP. This could likely be due to the increased extraction of phenolic compounds, and oxidation as well, which occurs during the applied maceration process. These compounds undergo oxidation reactions and form *o*-quinones, being very reactive compounds that easily react with thiols and, subsequently, result in their decrease and loss of varietal aroma [Nikolantonaki & Waterhouse, 2012]. Also, Mattivi *et al.* [2012] have shown that, despite inert pressing, the oxidative loading of grapes into the press resulted in removing a large part of glutathione, and subsequently resulted in lower concentrations of thiols. Since the wine production in our case was similar to the previously described procedure regarding the grape loading, this could be a reason for the obtained trend. Considering the yeast species, previous researches reported different conclusions, primarily regarding the influence of *T. delbrueckii.* For example, several studies investigated the ability of *T. delbrueckii* strain to produce 3SH and 3SHA in sequential as well as in simultaneous inoculation with *S. cerevisiae* and demonstrated that their lower concentrations were produced in comparison to single inoculation with *S. cerevisiae* [Zott *et al.*, 2011]. On the other hand, a recent study showed higher concentrations of 3SH and 4MSP in wines fermented by this yeast [Belda *et al.*, 2017], in comparison to fermentation by *S. cerevisiae.* Moreover, there are different conclusions regarding the assimilation of precursors by *T. delbrueckii* strains. Firstly, this yeasts was reported to be incapable of assimilating cysteinylated precursors [Renault *et al.*, 2016], but in more recent study this conclusion was rebutted [Belda *et al.*, 2017]. These findings suggest that the production of volatile thiols is strain-dependent, since different commercial starter cultures were used in the mentioned investigations. Besides the *T. delbrueckii*, musts were subjected to indigenous yeasts fermentations, as well, and these wines were characterized by more than 2-fold higher concentrations of 3SHA, compared to the wines fermented by commercial *S. cerevisiae* culture. These results are contrary to our previous research regarding the influence of yeast strain on the aroma of Pošip wine [Tomašević *et al.*, 2017], where the commercial *S. cerevisiae* resulted in higher concentrations of thiols in final wine in comparison to indigenous yeasts. The reason of this disagreement could be the starter cultures used, wherein the one used in the current research (Lalvin EC-1118) is characterized by ester-forming ability, while the one used in the previous study (Zymaflore X5) as a thiol-releasing strain. Nevertheless, a higher concentration of 3SHA after indigenous yeast fermentation is most probably due to the higher production of acetate esters by this yeasts.

Among the terpenes, α-terpineol was determined in the highest concentration, ranging from 53.0 to 98.0 μg/L, while citronellol was found in the lowest concentrations, amounting up to 18.1 μg/L (Table 3). The pre-fermentative maceration induced higher concentrations of most of the analyzed terpenes, especially of geraniol. Despite their increased concentrations, only linalool was determined in concentrations above its perception threshold of 25 μg/L [Escudero *et al.*, 2007]. But, even though terpenes are present in concentrations below their perception thresholds, their olfactory impact is synergistic [Ribéreau-Gayon *et al.*, 2006], meaning they could have an important role in the overall aroma of Pošip wine. Regarding the yeast strain used for alcoholic fermentation, commercial yeasts resulted in higher concentrations of citronellol and indigenous yeasts in a higher concentration of α-terpineol, while concentrations of other terpenes were yeast-independent. Generally, terpenes undergo significant changes during alcoholic fermentation, wherein the major transformation concerns the degradation of nerol and geraniol by the enzymatic activity of *S. cerevisiae* yeasts and their reduction to citronellol, α-terpineol, and linalool [Darriet *et al.*, 2012]. This finding was not in line with trends observed in our investigation since both, geraniol and α-terpineol, were determined in higher concentrations, which could probably be due to the different enzymatic activity of indigenous yeasts.

The last group of aroma compounds presented in Table 3 were C6 compounds: 1-hexanol and *cis*-3-hexen-1-ol. The pre-fermentative maceration slightly increased their concentrations, except when *T. delbrueckii* yeasts were used, but in a much lesser extent than that determined in our previous work and other investigations [Cejudo-Bastante *et al.*, 2011; Ribéreau-Gayon *et al.*, 2006; Tomašević *et al.*, 2017], where their concentrations increased significantly after the applied pre-fermentation maceration. This was probably due to the inert conditions which caused a reduction in enzymatic lipid oxidation and thus a decrease in the production of C6 compounds [Petrozziello *et al.*, 2011]. Furthermore, based on the obtained results and reported literature, regarding the perception thresholds, it can be concluded that these compounds have a limited influence on the aroma of the analyzed wines, since it is known that they contribute to the wine aroma in concentrations above 1.1 mg/L (1-hexanol) and 0.4 mg/L (*cis*-3-hexen-1-ol) [Peinado *et al.*, 2004]. Despite the potentially insignificant effect on the sen-

TABLE 4. Concentration of esters (mg/L) in Pošip wines.

Samples	Ethyl butanoate	Ethyl hexanoate	Ethyl octanoate	Ethyl decanoate	Ethyl dodecanoate	Ethyl hexadecanoate	Diethyl butanedioate	3-Methylbutyl acetate	Hexyl acetate	2-Phenyl-ethyl acetate
PKC	0.25±0.01^{cde}	0.54±0.02^{b}	0.96±0.03^{d}	0.39±0.01^{c}	0.02±0.00^{a}	0.06±0.01^{a}	0.54±0.02^{fg}	0.93±0.03^{c}	0.07±0.00^{c}	0.20±0.01^{c}
PKS	0.25±0.01^{de}	0.54±0.01^{b}	0.92±0.01^{d}	0.38±0.02^{c}	0.03±0.00^{a}	0.06±0.02^{a}	0.51±0.01^{g}	0.91±0.02^{c}	0.07±0.00^{cd}	0.20±0.01^{c}
PKSG	0.23±0.05^{e}	0.53±0.01^{b}	0.91±0.01^{d}	0.38±0.01^{c}	0.04±0.02^{a}	0.06±0.01^{a}	0.49±0.00^{g}	0.89±0.09^{c}	0.07±0.00^{cd}	0.20±0.00^{c}
PKG	0.24±0.03^{e}	0.54±0.00^{b}	0.92±0.01^{d}	0.38±0.00^{c}	0.02±0.00^{a}	0.05±0.00^{ab}	0.55±0.01^{efg}	0.91±0.05^{c}	0.07±0.00^{cd}	0.20±0.00^{c}
MKC	0.26±0.00^{abcde}	0.53±0.02^{b}	1.00±0.05^{d}	0.40±0.02^{c}	0.02±0.00^{a}	0.01±0.00^{cd}	0.64±0.03^{d}	1.03±0.03^{c}	0.06±0.00^{cd}	0.21±0.01^{c}
MKS	0.25±0.00^{cde}	0.53±0.01^{b}	0.98±0.01^{d}	0.39±0.00^{c}	0.03±0.01^{a}	0.01±0.00^{cd}	0.61±0.01^{def}	1.00±0.00^{c}	0.06±0.00^{d}	0.20±0.00^{c}
MKSG	0.27±0.01^{abcde}	0.54±0.02^{b}	1.00±0.02^{bcd}	0.40±0.00^{c}	0.02±0.00^{a}	0.02±0.01^{cd}	0.63±0.02^{de}	1.04±0.04^{c}	0.06±0.00^{cd}	0.21±0.00^{c}
MKG	0.26±0.01^{bcde}	0.53±0.01^{b}	0.99±0.01^{d}	0.38±0.00^{c}	0.02±0.00^{a}	0.01±0.00^{cd}	0.64±0.01^{d}	1.01±0.02^{c}	0.06±0.00^{cd}	0.20±0.00^{c}
MTC	0.29±0.01^{abcd}	0.64±0.01^{b}	1.16±0.01^{d}	0.45±0.01^{b}	0.02±0.00^{a}	0.02±0.00^{cd}	0.77±0.01^{bc}	2.98±0.08^{b}	0.11±0.00^{b}	0.73±0.01^{a}
MTS	0.30±0.01^{abc}	0.68±0.03^{a}	1.20±0.02^{a}	0.46±0.03^{b}	0.03±0.01^{a}	0.03±0.01^{bc}	0.75±0.02^{c}	3.10±0.13^{b}	0.11±0.01^{b}	0.75±0.04^{a}
MTSG	0.30±0.01^{abc}	0.66±0.01^{a}	1.16±0.07^{a}	0.45±0.00^{b}	0.02±0.00^{a}	0.02±0.00^{cd}	0.76±0.00^{c}	3.07±0.09^{b}	0.11±0.00^{b}	0.73±0.01^{a}
MTG	0.30±0.01^{a}	0.64±0.01^{a}	1.16±0.02^{a}	0.45±0.01^{b}	0.02±0.00^{a}	0.02±0.00^{cd}	0.90±0.03^{a}	2.99±0.02^{b}	0.11±0.00^{b}	0.72±0.01^{a}
MIC	0.29±0.01^{abc}	0.66±0.02^{a}	1.12±0.03^{a}	0.54±0.04^{a}	0.03±0.01^{a}	0.01±0.00^{cd}	0.84±0.04^{a}	3.42±0.07^{a}	0.23±0.01^{a}	0.32±0.01^{b}
MIS	0.29±0.00^{abcd}	0.65±0.01^{a}	1.10±0.07^{abc}	0.53±0.01^{a}	0.03±0.00^{a}	0.01±0.00^{cd}	0.84±0.02^{a}	3.38±0.02^{a}	0.23±0.00^{a}	0.31±0.01^{b}
MISG	0.30±0.01^{ab}	0.65±0.02^{a}	1.11±0.02^{ab}	0.54±0.01^{a}	0.03±0.01^{a}	0.01±0.00^{cd}	0.83±0.05^{ab}	3.48±0.14^{a}	0.23±0.01^{a}	0.32±0.01^{b}
MIG	0.29±0.01^{abcd}	0.65±0.02^{a}	1.10±0.05^{abc}	0.53±0.02^{a}	0.03±0.00^{a}	0.01±0.00^{d}	0.84±0.02^{a}	3.43±0.12^{a}	0.20±0.01^{a}	0.32±0.01^{b}

Data presented as means ± standard deviation (n=3). Different superscript letters indicate statistical differences among wines (Tukey's test, $p<0.05$). Abbreviations: P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione.

TABLE 5. Concentration of hydroxycinnamic acids (mg/L) in Pošip wines.

Samples	*cis*-Caftaric acid	*trans*-Caftaric acid	GRP	*cis*-Coutaric acid	*trans*-Coutaric acid	*cis*-Fertaric acid	*trans*-Fertaric acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Sum (without GRP)
PKC	2.80±0.47^{efg}	2.74±0.11^{e}	17.05±0.09^{c}	6.76±0.06^{f}	nd	0.68±0.39^{a}	3.31±0.20^{cd}	55.78±0.59^{b}	10.97±0.22^{f}	3.23±0.16^{ab}	86.26±1.56^{d}
PKS	2.63±0.42^{gh}	2.82±0.10^{e}	18.12±0.06^{c}	7.01±0.33^{f}	nd	1.01±0.29^{a}	3.63±0.19^{bcd}	56.38±0.45^{b}	11.31±0.34^{ef}	3.37±0.12^{a}	88.17±0.18^{d}
PKSG	2.19±0.00^{h}	2.90±0.04^{e}	18.01±0.10^{c}	6.66±0.01^{f}	nd	0.44±0.29^{a}	3.43±0.50^{cd}	55.47±1.03^{b}	11.77±0.27^{e}	3.27±0.03^{a}	86.14±1.13^{d}
PKG	2.99±0.03^{cdefg}	2.79±0.10^{e}	18.03±0.04^{c}	7.11±0.29^{f}	nd	0.58±0.32^{a}	3.02±0.39^{d}	55.29±0.87^{b}	11.00±0.17^{f}	3.43±0.18^{a}	86.21±1.63^{d}
MKC	3.44±0.05^{abcd}	5.42±0.04^{c}	24.54±0.09^{b}	9.83±0.97^{cde}	nd	0.59±0.61^{a}	3.52±0.57^{cd}	56.63±1.37^{b}	14.17±0.28^{d}	2.30±0.14^{de}	95.91±3.90^{c}
MKS	2.94±0.06^{cdefg}	5.58±0.03^{c}	25.19±1.13^{b}	10.15±0.47^{bcd}	nd	0.53±0.58^{a}	3.80±0.59^{bcd}	57.33±1.13^{b}	15.03±0.33^{abc}	2.38±0.11^{cde}	97.76±3.19^{abc}
MKSG	2.89±0.09^{defg}	5.49±0.06^{c}	25.93±0.12^{b}	10.31±0.29^{bcd}	nd	0.22±0.03^{a}	3.53±0.03^{cd}	56.95±0.10^{b}	14.93±0.12^{bc}	2.25±0.11^{e}	96.60±0.60^{bc}
MKG	3.50±0.06^{abc}	5.44±0.04^{c}	25.78±0.07^{b}	10.28±0.31^{bcd}	nd	0.61±0.49^{a}	3.64±0.54^{bcd}	57.23±0.98^{b}	14.59±0.25^{cd}	2.38±0.08^{cde}	97.67±1.93^{abc}
MTC	3.56±0.10^{ab}	4.25±0.38^{d}	15.33±0.60^{d}	7.92±0.46^{ef}	nd	0.99±0.51^{a}	4.19±0.50^{bc}	63.49±1.31^{a}	14.86±0.05^{bc}	2.73±0.06^{cd}	102.00±2.48^{abc}
MTS	2.92±0.12^{defg}	4.50±0.46^{d}	16.56±0.69^{cd}	7.82±0.29^{f}	nd	0.76±0.46^{a}	4.44±0.56^{bc}	63.96±1.50^{a}	15.41±0.20^{ab}	2.76±0.11^{bcd}	102.57±2.99^{ab}
MTSG	2.71±0.19^{fgh}	4.52±0.33^{d}	16.75±0.82^{cd}	8.39±1.16^{def}	nd	0.81±0.22^{a}	4.72±0.16^{b}	64.48±0.51^{a}	15.64±0.17^{a}	2.77±0.17^{bc}	104.05±2.38^{a}
MTG	3.70±0.26^{ab}	4.06±0.15^{d}	16.90±1.19^{cd}	8.02±0.13^{ef}	nd	0.69±0.24^{a}	4.18±0.56^{bc}	63.17±1.41^{a}	14.89±0.25^{bc}	2.73±0.21^{cd}	101.44±2.89^{abc}
MIC	3.76±0.04^{ab}	42.01±0.70^{b}	27.09±0.03^{a}	12.02±1.16^{ab}	9.02±0.40^{a}	0.99±0.02^{a}	6.10±0.07^{a}	12.52±0.25^{c}	9.98±0.18^{g}	2.45±0.07^{cde}	98.85±1.46^{abc}
MIS	3.27±0.12^{bcdef}	43.11±0.37^{a}	28.03±0.22^{a}	11.59±1.06^{abc}	9.38±0.44^{a}	0.86±0.02^{a}	6.31±0.05^{a}	12.42±0.11^{c}	10.19±0.17^{g}	2.53±0.03^{cde}	99.66±0.44^{abc}
MISG	3.35±0.05^{bcde}	43.20±0.08^{a}	28.14±0.09^{a}	12.34±1.14^{a}	9.59±0.37^{a}	0.88±0.06^{a}	6.33±0.03^{a}	12.48±0.03^{c}	10.19±0.10^{g}	2.56±0.14^{cde}	100.93±1.40^{abc}
MIG	4.01±0.06^{a}	42.85±0.46^{ab}	28.22±0.16^{a}	13.52±0.26^{a}	9.55±0.44^{a}	1.05±0.02^{a}	6.21±0.08^{a}	12.24±0.18^{c}	9.88±0.14^{g}	2.64±0.15^{cde}	101.95±1.73^{abc}

Data presented as means ± standard deviation (n=3). Different superscript letters indicate statistical differences among wines (Tukey's test, $p<0.05$). Abbreviations: GRP, glutathione derivative of caftaric acid; P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione; nd – not detected.

sory properties of wine, it is important to note the differences in concentrations produced by different yeast species. Thus, the commercial *S. cerevisiae* yeast strain, as well as indigenous yeasts, resulted in their higher concentrations, while sequential fermentation with *T. delbrueckii* resulted in their lowest concentrations, possibly implying that this species uses these compounds as precursors to form other compounds, *e.g.* hexyl acetate through alcohol acetyl transferase activity during fermentation [Sumby *et al.*, 2010]. Finally, regarding the antioxidant additions, no significant influence could be observed on the concentrations of these compounds.

Table 4 represents concentrations of esters determined in wine samples, wherein ten different compounds were quantified. Among them, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, 3-methylbutyl acetate, and 2-phenylethyl acetate are the most significant compounds for the aroma of Pošip wine since they were determined in concentrations above their perception thresholds [Swiegers *et al.*, 2005]. As can be seen in the presented results, the maceration effect is not pronounced (except for ethyl hexadecanoate) although higher concentrations of these compounds in the macerated wines fermented by sequential fermentation and indigenous yeast could be observed, in comparison to the directly pressed sample. These significant differences ($p<0.05$) are probably related to the yeast species used since indigenous yeast as well as sequential fermentation with *T. delbrueckii* resulted in higher concentration of most of the analyzed ester compounds, especially 3-methylbutyl acetate, hexyl acetate, and 2-phenylethyl acetate that were determined in 3-fold and 2-fold higher concentration when compared to commercial *S. cerevisiae* yeasts, respectively. Besides them, indigenous yeast fermentation also resulted in higher concentrations of ethyl hexanoate, ethyl decanoate, and diethyl butanedioate, while sequential fermentation with *T. delbrueckii* resulted in higher concentrations of ethyl hexanoate, ethyl octanoate, and diethyl butanedioate, but in a lesser extent than the previously stated. Similar results, concerning the higher concentration of esters, primarily 3-methylbutyl acetate, were also demonstrated in previous research regarding the Pošip wine aroma where the significant effect of indigenous yeasts was also established [Tomašević *et al.*, 2017]. In relation to the sequential fermentation with *T. delbrueckii*, different results can be found in previous investigations. For example, Azzolini *et al.* [2015] investigated the influence of this strain on aroma of Soave and Chardonnay white wines in comparison to fermentation by *S. cerevisiae* strain and opposite results were obtained, where the higher concentrations of 3-methylbutyl acetate, hexyl acetate, ethyl hexanoate, and ethyl octanoate were determined in wines fermented by single fermentation with *S. cerevisiae.* Also, similar results were obtained in earlier investigation [Azzolini *et al.*, 2012]. On the other hand, higher concentrations of desirable esters produced by *T. delbrueckii*, as presented in this paper, were also previously documented [Loira *et al.*, 2014; Renault *et al.*, 2015]. Finally, as can be seen in the presented results (Table 4) and, similarly to the trend established for aroma compounds, there were no differences among samples according to the different bottling variants.

TABLE 6. Effect of the applied enological practices on the analyzed compounds as indicated by ANOVA.

Compound	Pre-fermentative maceration	Yeast species	Antioxidant addition
4MSP	ns	*	ns
3SH	***	***	ns
3SHA	ns	***	ns
Linalool	ns	*	ns
α-Terpineol	***	***	***
Citronellol	***	***	***
Geraniol	***	***	ns
1-Hexanol	ns	***	ns
cis-3-Hexen-1-ol	***	***	ns
Ethyl butanoate	***	***	ns
Ethyl hexanoate	***	***	ns
Ethyl octanoate	***	***	ns
Ethyl decanoate	***	***	ns
Ethyl dodecanoate	ns	*	ns
Ethyl hexadecanoate	***	***	ns
Diethyl butanedioate	***	***	***
3-Methylbutyl acetate	***	***	ns
Hexyl acetate	***	***	ns
2-Phenyl-ethyl acetate	***	***	*
cis-Caftaric acid	***	***	***
*trans-C*aftaric acid	***	***	ns
GRP	***	***	ns
cis-Coutaric acid	***	***	ns
Coutaric acid	***	***	ns
cis-Fertaric acid	ns	**	ns
Fertaric acid	***	***	ns
Caffeic acid	***	***	ns
p-Coumaric acid	***	***	***
Ferulic acid	***	***	ns

***$p\leq0.001$; **$p\leq0.01$; *$p\leq0.05$; ns, not significant.

Hydroxycinnamic acids and their tartrate esters

Ten HCA were identified and quantified in wine samples: *cis*-caftaric, *trans*-caftaric, *cis*-coutaric, *trans*-coutaric, *cis*-fertaric, *trans*-fertaric, caffeic, *p*-coumaric, and ferulic acid, along with GRP, and the results are shown in Table 5. In most of the analyzed wines the most abundant compound was caffeic acid, except for the wine produced by fermentation with indigenous yeast where the *trans*-caftaric acid was determined in the highest concentration. Generally, it is well-known that hydroxycinnamic esters, such as caftaric, coutaric and fertaric

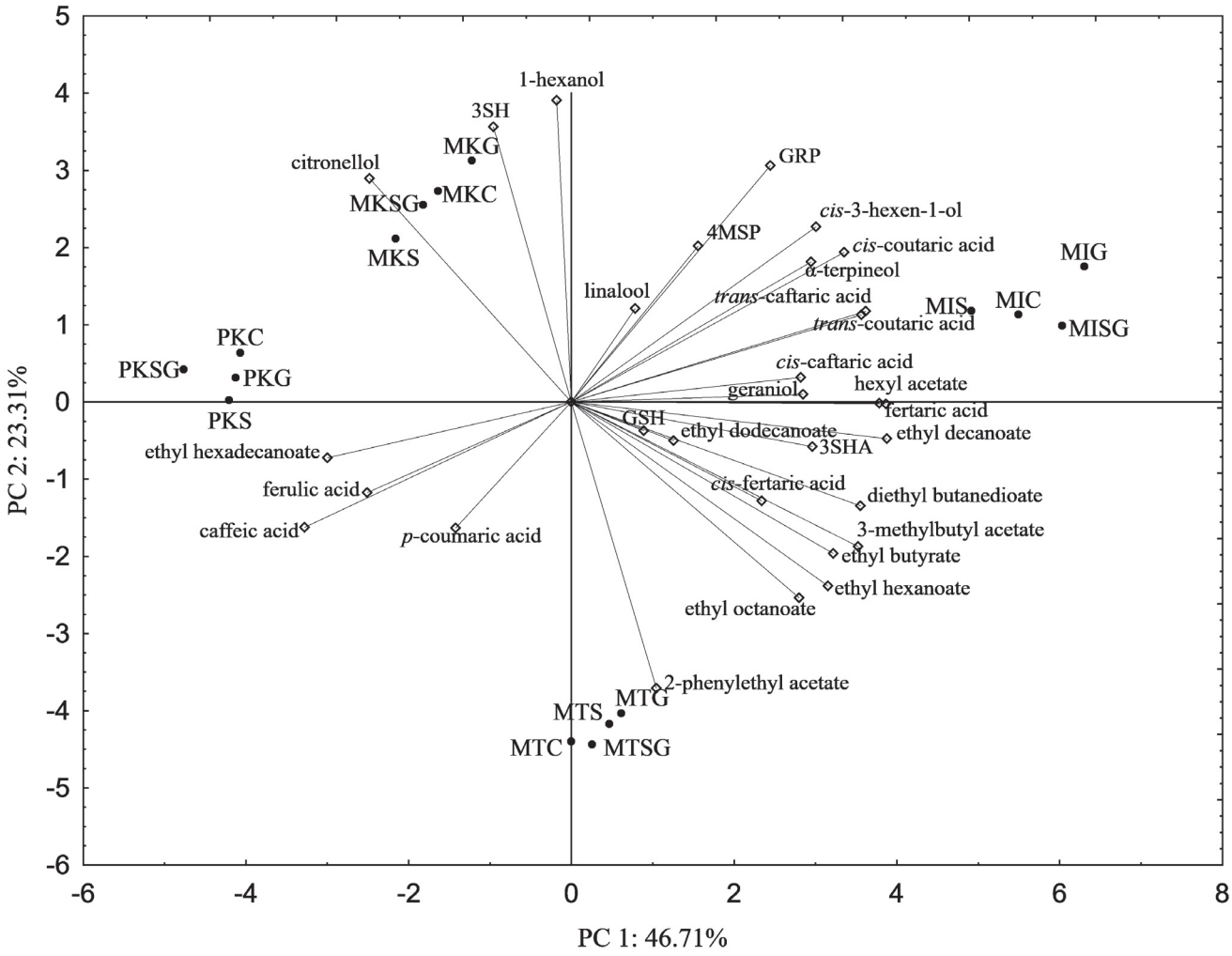

FIGURE 3. Distribution of the wine samples in two dimensional coordinate system defined by the first two principal components (PC1 and PC2) according to the applied practices and concentrations of aroma and phenolic compounds.

P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *T. delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione.

acid are present in grape skin and pulp and, during alcoholic fermentation and especially during wine maturation and aging, they undergo hydrolysis reaction which results in increased concentrations of their free forms, namely caffeic, coumaric, and ferulic acids. Also, it is known that a yeast strain could affect this hydrolytic activity and result in higher concentrations of their free forms [Monagas *et al.*, 2007], which could be the possible explanation for the obtained results. Beside the *trans*-caftaric acid, in wines produced by indigenous yeasts the highest concentration was also determined for GRP, followed by macerated wine fermented by commercial yeast strain (MK). Higher GRP concentrations potentially implicate that oxidation process occurred in previously mentioned wines. Furthermore, this thesis is additionally supported by lower concentrations of GSH in those wines (Figure 2). Besides oxidation reactions, GSH can possibly be taken up and employed by the yeast or react with other compounds present or formed during fermentation [Sonni *et al.*, 2011]. Similarly to the previously mentioned aroma compounds, the most significant changes in HCA composition were related to the yeast species used. Besides yeast species, the applied pre-fermentative maceration influenced higher concentrations of *cis*-caftaric, *trans*-caftaric, and *cis*-coutaric, as well as lower concentration of ferulic acid. Generally, maceration process influences increase in concentrations of these compounds due to their localization in the grape skin, and the previous investigations also demonstrated their higher concentrations after applied maceration [Di Lecce *et al.*, 2013], but these differences were pronounced to a greater extent than in our research. Furthermore, slightly higher concentrations of these compounds were determined in the case of antioxidants additions but, as in the case of aroma compounds, no differences could be observed among variants of antioxidants additions.

Statistical analyses

In order to evaluate the influence of the applied enological practices on the overall aroma and phenolic compounds in the analyzed Pošip samples, the ANOVA and principal component analysis (PCA) were conducted. The results of ANOVA are presented in Table 6, where the significance

was tested at three levels: p ≤0.001, p ≤0.01, and p ≤0.05. These results confirm previously described trends for individual compounds. The most significant effect showed to be caused by yeast species, since concentrations of all analyzed compounds differed significantly between yeast species variants. The second important factor was pre-fermentative maceration that influenced changes in almost all analyzed compounds (except 3SH, 1-hexanol, ethyl dodecanoate, and *cis*-fertaric acid). The least important factor was antioxidant addition; it caused statistically different differences in concentrations of only few compounds: α-terpineol, citronellol, diethyl butanedionate, 2-phenyl-ethyl acetate, *cis*-fertaric acid, and *p*-coumaric acid.

The results of PCA are shown in Figure 3, where it can be seen that the first two components explained 70.02% of the total variance. First variable (PC 1) showed a strong positive correlation with the content of ethyl decanoate (factor loading: 0.969), fertaric acid (0.966), hexyl acetate (0.946), caftaric acid (0.904); and a highly positive correlation with contents of coutaric acid (0.891), diethyl butanedioate (0.888), 3-methylbutyl acetate (0.880), *cis*-coutaric acid (0.838), ethyl butanoate (0.804), ethyl hexanoate (0.788), *cis*-3-hexen-1-ol (0.751), α-terpineol (0.736), geraniol (0.711), *cis*-caftaric acid (0.705), and ethyl octanoate (0.699); while caffeic acid (-0.820) and ethyl hexadecanoate (-0.749) were highly negatively correlated with this principal component. On the other hand, the second principal component showed a strong positive correlation with 1-hexanol (0.977) and a highly positive correlation with 3SH (0.891), GRP (0.765) and citronellol (0.724), as well as a strong negative correlation with 2-phenylethyl acetate (-0.927). Regarding the distribution of Pošip wines in a two-dimensional coordinate system, a clear separation of the analyzed samples can be observed. Herein, the samples were separated according the yeast species used for their production. As can be seen in Figure 2, wines produced by alcoholic fermentation with commercial yeast strain (both, directly pressed and macerated wines) were placed on the left from the PC1, the ones fermented by indigenous yeast on the right side of the same factorial plane, while wines produced by sequential fermentation of *T. delbrueckii* with *S. cerevisiae* were placed below the second factorial plane. Hence, alcoholic fermentation with commercial yeast strain was resulted in higher concentrations of caffeic acid and ethyl hexadecanoate (directly pressed ones), as well as of 3SH and citronellol (macerated samples). Furthermore, higher concentrations of 2-phenylethyl acetate and ethyl octanoate resulted from the sequential fermentation with *T. delbrueckii.* Also, these wines were characterized with higher concentrations of ethyl hexanoate, ethyl butanoate, 3-methylbutyl acetate, diethyl butanedioate, as well as *cis*-caftaric, *cis*-fertaric, caffeic, and *p*-coumaric acid. Finally, wines produced by indigenous yeast strain were characterized by higher concentrations of the majority of analyzed compounds, where the most significant ones were 3SHA, *cis*-3-hexen-1-ol, ethyl decanoate, diethyl butanedioate, 3-methylbutyl acetate, and hexyl acetate, compounds placed on the right side of the first factorial plane. In addition, these yeast also affected higher concentrations of most of the analyzed hydroxycinnamic acids (*cis*-caftaric, *trans*-caftaric, *cis*-coutaric, coutaric, *cis*-fertaric, and fertaric acids). Besides the yeast species, the influence of pre-fermentative maceration could also be observed, but in a lesser extent. It can be seen that the non-macerated wine samples (wines PK) where placed on the left side of the coordinate system and left of the other, macerated ones. Furthermore, the differences among macerated wines could be also observed where wine produced by commercial yeas strain (MK) showed to be more similar to the control wine (PK) than the other two macerated wines. These results imply the limited influence of this technique and, consequently, do not allow to draw a uniform conclusion of its influence on the analyzed aroma compounds. As stated previously for individual groups of compounds, the influence of antioxidant addition could not be observed.

CONCLUSIONS

Pošip grapes are characterized by higher contents of glutathionylated thiol precursors, especially Glut-3SH, while the applied enological practices resulted in differences in the produced wines. The most significant differences were caused by the yeast species used for alcoholic fermentation, where the indigenous yeast affected higher concentrations of most of the analyzed compounds, primarily 3SHA, α-terpineol, geraniol, *cis*-3-hexen-1-ol, ethyl hexanoate, diethyl butanedioate, 3-methylbutyl acetate, and hexyl acetate, as aroma representatives and most of the analyzed hydroxycinnamic acids. Furthermore, the pre-fermentative maceration found to be very limited since only higher concentrations of C6 compounds and lower concentrations of thiols 3SHA were found in macerated wines. Finally, the initial hypothesis regarding the protective effect of antioxidants during aging was not confirmed by this research, since only slightly higher concentrations of volatile thiols were found in wines bottled with higher SO_2 and glutathione (variant SG). This paper provides a new insight into Pošip grapes composition regarding the aroma precursors, as well as confirmation of a great prospect of indigenous yeast fermentation and sequential fermentation with *T. delbrueckii* yeasts for the potential production of more complex, aromatic wines.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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