IZVORNI ZNANSTVENI RAD / ORIGINAL SCIENTIFIC PAPER

Analysis of aroma of white wine (Vitis vinifera L. Pošip) by gas chromatography-mass spectrometry

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Abstract

Aroma of wine is influenced by many factors such as grape variety, viticulture practices and especially applied enological treatments during wine production and aging. Most of the aroma compounds are synthesized during the alcoholic fermentation, as secondary yeast metabolites. Different yeast strains contribute differently to the complexity of wine aroma. Also, final wine aroma is influenced by chemical reactions that occur during aging of wine, primarily oxidation and hydrolysis. The objective of this study was to determine the influence of yeast strain along with antioxidant addition (sulfur dioxide and glutathione) on aroma and sensory properties of Pošip wine. Wines were produced either by spontaneous alcoholic fermentation (indigenous yeasts) or by inoculation of commercial Saccharomyces cerevisiae yeast strain. Sulfur dioxide and glutathione were added to wines at the bottling, and analyses were carried out after 12 months of aging. Esters, higher alcohols, fatty acids, terpenes and norisoprenoid were analyzed by solid-phase microextraction coupled with gas chromatography mass spectrometry (SPME-GC/MS), while varietal thiols were first selectively extracted and concentrated by p-hydroxymercurybenzoate and strong anion exchange column prior to GC/MS analysis. The results showed that spontaneous alcoholic fermentation resulted in higher concentrations of some esters (i-amyl acetate, ethyl octanoate and ethyl decanoate), alcohol (phenylethyl alcohol) and terpene (α-terpienol) while commercial yeast strain influenced on higher concentrations of ethyl-2-methylbutyrate, C6 alcohols and varietal thiols. Wine storage with higher concentrations of sulfur dioxide and glutathione after 12 months resulted in higher concentrations of most of analyzed aroma compounds. The sensory analysis results showed similar trend to those obtained by GC/MS: higher varietal thiols intensities were determined in wines fermented by commercial yeast strain and bottled with addition of sulfur dioxide and glutathione.

Keywords: *wine aroma, yeast strain, antioxidants, Pošip, sensory analysis*

Sažetak

Na aromu vina utječu brojni faktori kao što su sorta grožđa, vinogradarska praksa te, posebno, primjenjeni enološki postupci tijekom proizvodnje i starenja vina. Veliki dio spojeva arome sintetizira se tijekom alkoholne fermentacije, kao sekundarni kvaščevi metaboliti. Različiti sojevi kvasaca različito doprinose kompleksnosti arome vina. Nadalje, konačan aromatski profil rezultat je kemijskih reakcija koje se odvijaju tijekom starenja vina, prvenstveno oksidacije i hidrolize. Cilj ovog rada bio je utvrditi utjecaj soja kvasaca uz dodatak antioksidansa (sumporov dioksid i glutation) na aromu i senzorske karakteristike vina Pošip. Vina su proizvedena spontanom alkoholnom fermentacijom (autohtoni kvasci) te inokulacijom komercijalnih Saccharomyces cerevisiae kvasaca. Sumporov dioksid i glutation dodani su u vino pri punjenu u boce, a analize su provedene nakon 12 mjeseci starenja vina u bocama. Esteri, viši alkoholi, hlapive masne kiseline, terpeni i norizoprenoid određeni su primjenom plinske kormatografije s masenom detekcijom uz prethodnu mikroekstrakciju na čvrstoj fazi (SPME-GC/MS), dok se analiza sortnih tiola sastojala od selektivne ekstrakcije i koncentriranja tiola primjenom p-hidroksimerkuribenzoata i jakog anionskog izmjenjivača te GC/MS analize. Rezultati su pokazali da su spontanom alkoholnom fermentacijom nastale više koncentracije nekih estera (i-amil acetat, etil oktanoat i etil heksanoat), zatim feniletanola te α-terpienola, dok je primjena komercijalnih kvasaca rezultirala višim koncentracijama etil-2-metilbutirata, C6 alkohola i sortnih tiola. Starenje vina uz kombinaciju visoke koncentracije sumporovog dioksida i glutationa rezultiralo je i višim koncentracijama većine analiziranih spojeva. Senzorska analiza pokazala je sličan trend kao i kromatografska analiza: viši intenziteti mirisa sortnih tiola određeni su u vinima proizvedenim primjenom komercijalnih kvasaca te punjeni u boce uz dodatak sumporovog dioksida i glutationa.

Ključne riječi: *aroma vina, soj kvasca, antioksidansi, Pošip, senzorska analiza*

Introduction

The aroma is one of the main characteristics that define the differences among the wines and it is affected by the numerous possible variations in wine's production, both in viticulture and in winemaking (Swiegers et al, 2005). Compounds derived from grapes are responsible for so-called varietal aroma, whereas monoterpenes, norisoprenoids, C6 compounds and varietal thiols are main representatives. Varietal thiols, namely 4-mercapto-4-methyl-pentan-2-one (4MMP),

3-mercaptohexanol (3MH) and 3-mercaptohexylacetate (3MHA) are highly aroma active compounds, responsible for the tropical flavor of wines, and their positive contribution to the aroma of wines made from several grape varieties has already been proven (Tominaga et al, 1998, Murat et al, 2001; Álvarez-Pérez et al, 2012). These compounds belong to the class of varietal aromas because, despite they result from the cleavage of odorless precursors present in grapes or musts by yeast enzymes during alcoholic fermentation they are not product of yeast metabolism (Roland et al, 2011).

In wine, aroma compounds are usually dominated by fermentation products, given that these compounds are present in the highest concentrations (Romano et al, 2003). Alcoholic fermentation increases the chemical and aroma complexity of wine by assisting in the extraction of compounds from solid parts present in grape musts, modifying some grape derived compounds and producing a large amount of yeast metabolites, some of which are aroma compounds (Lambrecht and Pretorius, 2000). Consequently, different yeast populations or strains used for alcoholic fermentation may result with different aroma characteristics of produced wines. Grape juice fermentation can either be natural, conducted by the microflora present in the grapes and in the winery, or inoculated with commercial *Saccharomyces cerevisiae* strain (Lambrecht and Pretorius, 2000). Inoculation minimizes the influence of wild yeast on wine quality and produces wines with consisted quality with predictable and desired characteristics. On the other hand, spontaneous fermentation may result in a significant and favorable effect on aroma development and in a new wine styles production.

Final aroma of wine is influenced by the chemical reactions that occur during wine aging and storage. During that period, oxidative processes lead to the loss of some characteristic aroma compounds and the appearance of new and distinctive aromas of older wines and/or atypical ones associated with wine deterioration (Escudero et al, 2002; Lambropoulos and Roussis, 2007). In order to preserve wine against oxidative degradation, the addition of antioxidants prior to bottling is widespread practice; whereas addition of glutathione (GSH) has been suggested since GSH exhibited increased protection toward important aroma compounds (Ugliano et al, 2011).

Along with the analytical assessment of wine aroma compounds, sensory evaluation has become a very popular research tool in wine and beverage food industry. Quantitative Descriptive Analysis (QDA) is one of the descriptive analysis methods which includes the detection and description of both qualitative and quantitative sensory components by the selected and trained sensory panel (Lawless et al, 2010). Panelists, by developed and adopted common language describe the characteristics of the wine and contribute to the understanding of the differences between wine samples**.**

The aim of this study was the characterization of aroma of Pošip wine by the GC/MS analyses, in order to examine the effect of applied enological treatments (alcoholic fermentation with indigenous and commercial yeast strain and antioxidant addition during wine bottling) on aroma composition of Pošip wine. Also, application of QDA method in assessing of the varietal thiols intensity (boxwood, grapefruit and passion fruit aromas) in Pošip wines will be conducted.

Materials and methods

Pošip wines

Wines were produced from healthy ripe grapes of *Vitis vinifera* cv. Pošip, grown in Dalmatia, Croatia, in September 2013. After grapes were destemmed and crushed reductive pressing with the addition of enzymes (3 g/L of Lafazym extract and Lafazym press, Laffort, France) and bisulfite solution (150 g/L) at concentration of 10 mg/L of total SO2 was conducted. Clarified must was decanted and separated in two inox tanks. First was inoculated with *Saccharomyces cerevisiae* yeast strain Zymaflore X5 (Laffort, France) (treatment *Com)* and kept with fermentation temperature under 16 °C and the second tank was subjected to spontaneous alcoholic fermentation with indigenous yeasts also with fermentation temperature kept under 16 °C (treatment *Ind*).

After alcoholic fermentation was finished wines were decanted and sulfur dioxide was set at 35 mg/L of free SO2, while temperature was kept at 12 °C. One month after alcoholic fermentation wines were stored in corked bottles and dark, each under three different conditions: (i) 35 mg/L of free sulfur dioxide (treatment C), (ii) higher sulfur content- 50 mg/L of free sulfur dioxide (treatment S) and (iii) 50 mg/L of free SO2 combined with 20 mg/L of glutathione (treatment SG). Wines were subjected to analytical and sensory evaluation after 12 months of storage.

Chemicals

Ethanol was HPLC grade and purchased from J. T. Baker (Deventer, Netherlands), sodium chloride p.a., hydrochloric acid (37 %) and sodium sulfate anhydrous were purchased from Carlo Erba (Val de Reuil, Spain), sodium acetate trihydrate from Gram-mol d.o.o. (Zagreb, Croatia), acetic acid from Alkaloid (Skopje, Macedonia), cystein hydrochloride hydrate, dichloromethane, *p*-hydroxymercurybenzoate (*p*-HMB), 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) and Dowex strong anion exchange resin, chloride form were purchased from Sigma Aldrich (St. Louis, USA). The aroma reference standards were also purchased from Sigma Aldrich, except 4-mercapto-4-methylpentan-2-on and 3-mercaptohexyl acetate which are purchased from Alfa Aesar (Ward Hill, USA) and 3-mercaptohexanol and 4-methoxy-2-methyl-2-mercaptobutane purchased from Endeavour Chemicals (Northamtonshire, UK).

Methods

GC/MS analysis of aroma compounds

Prior to GC/MS analysis volatile compounds were extracted from wine by two different procedures. First was extraction of aroma compounds by headspace solid-phase microextraction (HS-SPME) using 100 μm PDMS fiber (Supelco, Bellefonte, USA). 10 mL of wine sample, containing 20 mg/L internal standard, was placed into a 20 mL headspace vial containing solid NaCl p.a. (2 g) and capped with a crimp cap and silicone-PTFE septum. The fiber was exposed to the wine headspace for 30 minutes at 40 °C with constant shaking. Thermal desorption followed for 5 minutes in the injector (splitless mode) at 250 °C. The second procedure was extraction

of thiols, namely 3-mercaptohexanol, 3-mercaptohexylacetate and 4-mercapto-4-methyl-pentan-2-one, which were extracted using a procedure based on the method developed by Tominaga et al (1998). The original protocol was slightly changed with the use of an initial sample volume of 100 mL. As internal standard analyzed volatile thiols, 4- methoxy-2-methyl-2-mercaptobutane (4M2M2MB) was used.

The wine volatiles were analyzed using an Agilent Gas Chromatograph 6890 series coupled with an Agilent 5973 *Inert* mass-selective detector (Agilent Technologies, Santa Clara, CA, USA). The column used was a BP20 capillary column (SGE Analytical Science, Victoria, Australia) with dimensions 50 m x 220 μm x 0.25 μm. The interface temperature of the detector was kept at 250 °C and the ion source working in EI mode at 70 eV was held at 280 °C. Helium 5.0 was vector gas used (Messer Croatia Plin d.o.o., Zagreb, Croatia). Temperature program for aroma analysis extracted by HS-SPME procedure was: the initial temperature of 40 °C was held for 5 minutes and then raised to 200 °C at 3°/min, and finally raised to 240 °C at 30 °C/min, while temperature program for thiol analysis was: 35 °C was held for 10 minutes and then raised to 230 °C at 3°C/min. The three thiols and their internal standards were detected in single ion mode (SIM) with ions selected for their identification and quantification as follows: 4MMP *m/z 75, 132, 99*; 3MHA *m/z 116, 101*; 3MH *m/z 134, 100, 101*; 4M2M2MB (internal standard) *m/z 134, 75*, while the other aroma compounds were acquisited in scan mode. All analyses were performed in duplicate.

Volatile compounds were identified by GC/MS using the Enhanced Chemstation software (Agilent Technologies, Santa Clara, CA, USA). Aroma compounds were identified by comparing the peak retention times against those of authentic standards and matching the mass spectra against Nist05 mass library (Wiley & Sons, Hoboken, NJ, USA). For quantification, calibration curves for each compound were prepared and analyzed by GC/MS by the same extraction and chromatographic methods as wine samples.

Panel training and sample assessment

Panelist recruitment was done on the base of motivation, availability and voluntary participation. The selection, screening and training of judges was carried out during 10 consecutive weeks. First, the ability of candidates to recognize and distinguish each thiol in concentrations above known thresholds was tested through discrimination tasks by triangle tests. Thus, the first testing was done with standard water solutions of each thiol (4MMP, 3MH, 3MHA) and the second triangle testing was carried out with standards added in 10% water solution of ethanol. These tests were conducted in order to familiarize each candidate with aroma descriptors. Afterwards, judges were trained to rank the intensity of each thiol perception in different concentrations of standard solutions by ranking tests. Solutions were first prepared as individual standards in 10% ethanol solution and after that in neutral white wine. During the first two ranking tests in accordance with candidates, the descriptors for each thiol were established: 4MMP- boxwood, 3MH-grapefruit and 3MHA- passion fruit and these descriptors were used during the whole process. Finally, thiol intensities were evaluated by the addition of standards in wine using an unstructured 10 cm horizontal visual scale (Parr et al, 2007), where the left-hand end represent 'absence' and the right-hand end 'very intensive' aroma. Analyzes were repeated in duplicate in order to determine the consistency of the panel and repeatability of judges between sessions.

The wine samples (50 mL) were evaluated in clear wine glasses, identified with three digit random codes and covered by petri dishes, at room temperature, with the presence of referent standard solutions which were assigned as the highest score on the intensity scale. The assessors were asked to indicate the each thiol intensity in Pošip wines samples using the 10 cm unstructured horizontal line scale.

Data analysis

Statistical analysis was carried out using analysis of variance (ANOVA) of Statistica V.10 software (Statsoft Inc., Tulsa, USA). Tukey's HSD test was used as comparison test when samples were significantly different after ANOVA ($p<0.05$). The principal component analysis (PCA) was used to examine any possible grouping of samples by different enological practices (yeast strain, antioxidant addition) applied during wine production and aging.

Results and discussion

The results obtained by GC/MS analyses are showed in Table 1, according which aroma of Pošip wine is characterized by several chemical groups: esters, higher alcohols, terpenes, acids, norisprenoids and varietal thiols. There were no qualitative differences between wines obtained by fermentation with indigenous and commercial yeast strains wines, but yeast strain influenced to the concentration of several individual volatile compounds, which is in accordance to Romano (1997) who also concluded that the differences in the composition of wines made from different yeast species appeared to be quantitative rather than qualitative.

Among esters, vinification with indigenous yeast resulted in higher concentrations of *i*-butyl acetate, *i*-amyl acetate, ethyl octanoate, ethyl decanoate and 2-phenylethyl acetate, while commercial yeast produced higher concentrations of ethyl-2-methylbutyrate. Ethyl esters of hexanoic, octanoic and decanoic acid along with *i*-amyl acetate are considered to be the most important esters in wine, and their concentrations in all analyzed Pošip wine samples were higher than their perception thresholds, which amounted 0.08, 0.58 and 0.5 and 0,26 mg/L, respectively (Lambrechts and Pretorius, 2000). Generally, in white wines the main role of acetate and ethyl esters is in the perception of tree fruit and tropical fruit notes. It has been demonstrated that the former notes are linked to ethyl esters, while the latter are linked mainly to acetates of higher alcohols (Ferreira et al, 1995). Scacco et al (2012) tested the ability of three different yeast strains isolated in wineries of Sicily to produce quality wines, in comparison to commercial yeast strains. The new selected yeasts were able to increase the pear notes (ethyl decaonate isomers) which are fundamental for the aroma of investigated wines and these authors confirmed that isolated indigenous yeast strains are not inferior to those obtainable with the best commercial strains selected in other geographical areas. Two higher alcohols, *i*-butanol and phenylethyl alcohol were higher

in wine fermented by spontaneous alcoholic fermentation, similar to results obtained by Mateo et al (2001), who showed that concentration of *i*-butanol, 1-heptanol were higher in wines produced by spontaneous alcoholic fermentation, but total higher alcohol content in wine fermented by spontaneous fermentation was lower. Among other compounds it is worth mentioning that spontaneous alcoholic fermentation resulted in higher concentration of α-terpineol, while commercial *Saccharomyces cerevisiae* yeast strain influenced on higher concentration of C6 alcohols and varietal thiols, which is expected since the Zymaflore X5 yeast strain is characterized by higher thiol-type aroma production (Laffort, 2016).

Considering antioxidant addition during wine bottling, higher sulfur dioxide and combination of higher sulfur dioxide and glutathione resulted in higher concentrations of most of the analyzed compounds. During aging, concentrations of most compounds is decreasing, especially esters and varietal aroma compounds (terpenes, thiols…) which leads to loss of characteristic aromas of wine and subsequently to the formation of new aromas characteristic of older wines (Roussis and Sergianitis, 2008). The effect of sulfur dioxide and mixture of sulfur dioxide and glutathione is also represented in Table 1. In all cases, both antioxidant additions resulted in higher concentrations of *i*-amyl acetate, ethyl hexanoate, octanoate and decanoate, as well as linalool and α-terpineol. Roussis and Sergianitis (2008) determined that SO_2 addition inhibited decrease of *i*-amyl acetate and ethyl hexanoate, but did not affected on linalool concentration. Also, concentrations of varietal thiols are higher in wines with added antioxidants which is in accordance with other researches (Makhotkina et al, 2013; Ugliano et al, 2011; Dubordieu and Lavigne, 2004).

Training and evaluation of sensory panel for QDA analysis of varietal thiols in Pošip wines resulted in selecting 11 panel judges, from the number of 27 candidates who started training. Judges were selected after their consistency and repeatability was established. QDA results will be presented later in text, as PCA analysis along with the analytical results.

Table 1. *Aroma compounds in analyzed Pošip wines after 12 months of storage*

	Commercial yeast (Com)			Indigenous yeast (Ind)		
	C	S	SG	C	S	SG
Esters (mg/L)						
ethyl acetate	89.34±1.39	79.96±0.57	94.63±0.37	93.48±1.66	84.23±1.39	83.67±3.75
i-butyl acetate	28.19±0.63	33.55±1.34	36.65±0.57	32.19±1.11	36.04±0.22	37,31±0.30
2-phenylethyl acetate	0.11±0.01	0.14±0.01	0.16±0.01	0.14±0.01	0.17±0.01	0.15±0.01
i-amyl acetate	1.57±0.11	2.21±0.05	2.37±0.06	2.27±0.40	2.95±0.26	2.57±0.08
hexyl acetate	0.06±0.01	0.11±0.01	0.12±0.01	0.09±0.01	0.11±0.01	0.10±0.01
ethyl-2-methylbutyrate	16.99±0.44	18.95±0.50	20.05±0.35	11.08±2.54	12.69±0.46	13.17±0.95
ethyl hexanoate	1.24±0.01	1.28±0.01	1.42±0.01	1.18±0.12	1.48±0.17	1.59±0.14
ethyl octanoate	1.37±0.01	1.47±0.01	1.75±0.02	1.60±0.07	1.72±0.06	1.82±0.10
ethyl decanoate	0.77±0.06	0.86±0.02	0.96±0.11	1.14±0.01	1.37±0.13	1.42±0.07
diethyl succinate	4.51±0.40	3.99±0.30	3.80±0.13	4.99±0.21	4.70±0.12	4.55±0.14
Alcohols (mg/L)						
i-butanol	8.47±0.21	8.87±0.46	8.98±0.76	10.51±0.28	10.69±0.23	11.20±0.62
i-amyl alcohol	122.94±1.37	122.04±0.04	121.82±1.44	117.62±4.67	116.73±0.14	115.64±2.35
1-hexanol	1.26±0.01	1.27±0.02	1.30±0.01	1.08±0.06	1.36±0.07	1.35±0.08
cis-3-hexen-1-ol	0.310±0.00	0.315±0.02	0.325±0.01	0.213±0.02	0.278±0.01	0.270±0.00
phenylethyl alcohol	17.98±0.85	18.97±0.10	18.80±0.28	25.11±0.52	226.17±1.31	26.15±0.69
Acids (mg/L)						
hexanoic acid	4.83±1.16	5.73±0.59	6.99±3.18	5.33±0.53	5.82±0.21	5.97±0.47
Terpenes (μg/L)						
linalool	20.85±1.54	23.71±0.55	23.56±1.20	20.91±0.16	29.50±3.05	30.75±2.76
α-terpineol	6.16±0.09	9.73±0.62	10.28±0.37	10.13±0.09	12.62±0.85	12.18±0.52
Norisoprenoids (μg/L)						
β- damascenone	0.42±0.02	0.47±0.00	0.49±0.03	0.48±0.01	0.59±0.04	0.58±0.09
Volatile thiols (ng/L)						
4MMP	nd	nd	nd	nd	nd	nd
3MH	190.83±1.69	230.37±7.71	243.43±11.6	179.42±8.46	215.71±8.98	225.95±4.99
3MHA	8.26±0.10	11.40±0.02	12.53±0.45	6.83±0.19	9.83±0.23	9.77±0.14

nd, not detected, C- standard conditions (35 mg/L of free SO2), S- higher SO2 level (50 mg/L), SG- higher SO2 level (50 mg/L) with glutathione addition (20 mg/L)

In order to compare analyzed wine samples according to the aroma compounds and quantitative descriptive analysis, principal component analysis (PCA) was conducted. Applying PCA to the concentrations of variables (aroma compounds and QDA results) and 6 cases (wines), five factors were extracted with eigenvalues higher than 1, explaining 100 % of the total variance. First two factors (PC1 and PC2) accounted for 77.29 % of total variance.

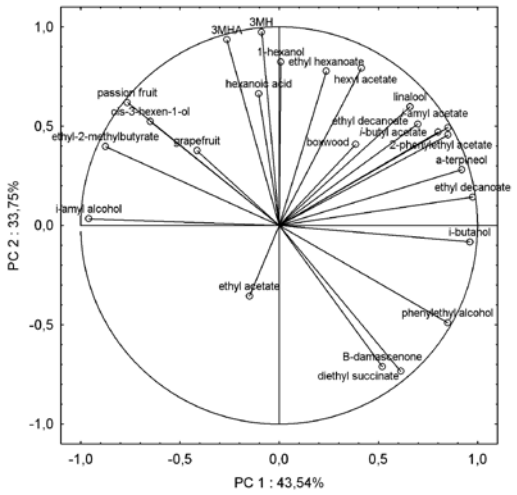
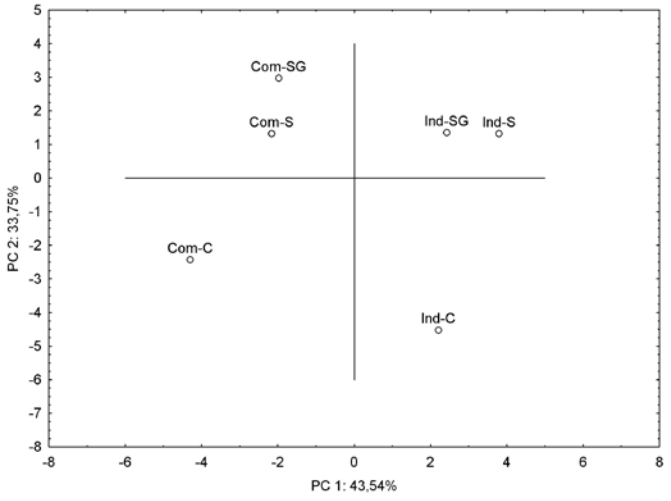


Figure 1 shows projection of variables in two dimensional coordinate system defined by first two variables. First variable, explaining 43.54 % of the total variance, was strongly negatively correlated with *i*-amyl alcohol (-0.96), ethyl-2-methylbutyrate (-0.87) and passion fruit (-0.76) and highly positively correlated with ethyl decanoate (0.98), *i*-butanol (0.96), α-terpineol (0.92), *i*-amyl acetate (0.85), 2-phenylethyl acetate (0.85), phenylethyl alcohol (0.85) and *i*-butyl acetate (0.80). The second principal component (33.75 % of the total variance) showed a high positive correlation with 3MH (0.97), 3MH (0.93), 1-hexanol (0.82), hexyl acetate (0.79) and ethyl hexanoate (0.78), while negative correlated with diethyl succinate (-0.73) and β-damascenone (-0.71).

Figure 2: *Tomasevic et al_Figure 2. Distribution of Pošip wines after 12 months aging in the two-dimensional system defined by PC1 i PC2*



Projection of the wine samples in two dimensional coordinate system defined by the previously mentioned factors is reported in Figure 2. It can be seen clear grouping of wines according to the first factorial plane: wines produced with spontaneous alcoholic fermentation were placed on the right side (and are characterized by higher concentrations of compounds that positively correlate with first factorial plane), while wines vinificated with commercial yeast strain were on the left side of this plane. Also, there is clear separation of wines aged with antioxidant addition in comparison to those aged in standard conditions (35 mg/L of free SO_2), where it can be seen that wines aged with antioxidant addition characterize higher amounts of most of compounds. Furthermore, QDA showed that the antioxidant addition during bottling achieved the higher thiols intensities, according to better preservation of these compounds during the wine aging and the higher intensities of thiols were determined in wines fermented by commercial yeast strain. Results showed as PCA are in correlation with analytical data.

Conclusions

Spontaneous alcoholic fermentation produced higher concentrations of some esters (*i*-amyl acetate, ethyl octanoate and ethyl decanoate), alcohol (phenylethyl alcohol) and terpene **(α-**terpienol) while commercial yeast strain produced higher concentrations of ethyl-2-methylbutyrate, C6 alcohols and varietal thiols. Higher concentrations of sulfur dioxide in combination with glutathione addition after 12 months' storage resulted in higher concentration of most of aroma compounds. Higher varietal thiols intensities, obtained by QDA analysis, were determined in wines produced by commercial yeast strain and bottled with addition of sulfur dioxide and glutathione.

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