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THE INFLUENCE OF DIFFERENT YEAST STRAINS AND YEAST NUTRIENTS ON THE AROMA OF KRSTAČ AND ŽIŽAK WINES

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Krstač and Žižak are autochthonous grape varieties grown in Montenegro. Although international varieties are more popular, the autochthonous varieties are very important, especially for countries developing tourism. The fermentation aromas produced during alcoholic fermentation contribute significantly to wine quality. The effects of yeasts (*Saccharomyces cerevisiae* and *Saccharomyces bayanus*) and yeast nutrients (Fermaid E and Fermaid O) on aromatic compounds in wines were investigated. Using GC/FID-MS analysis, aroma compounds in Krstač and Žižak wines were characterized and quantified. Wines produced with the addition of yeast and yeast nutrients had mostly lower total alcohol content than wines obtained by spontaneous fermentation of Krstač and Žižak varieties. The results of this study showed that the concentration of compounds depends on the yeast strains. The yeast *S. cerevisiae* (ICV) provided a higher content of higher alcohols, while *S. bayanus* produced a higher concentration of esters and (medium chain) fatty acids. Total ester content ranged from 3.34 to 11.97 mg/l for Krstač wines and 8.51 to 13.68 mg/l for Žižak wines. Among all wines, Krstač and Žižak wines produced with *S. bayanus* and Fermaid E addition had the highest concentration of total esters. The yeast nutrients Fermaid E and O emphasized different characteristics of the yeasts. They had a statistically significant effect on the content of ethyl and acetate esters. The highest overall scores were obtained for ICVE and BayE Krstač wines (18.1 out of 20 points) and Žižak ICVE wine (18.2 out of 20 points).

Keywords: aromatic compounds; yeasts and yeast nutrients; autochthonous grape varieties; GC/FID-MS analysis; sensory evaluation

ВЛИЈАНИЕ НА РАЗЛИЧНИ СОЕВИ ВИНСКИ КВАСЦИ И ХРАНЛИВИ МАТЕРИИ НА КВАСЕЦОТ ВРЗ АРОМАТА НА ВИНАТА КРСТАЧ И ЖИЖАК

Крстач и *жижак* се автохтони сорти грозје што се одгледуваат во Црна Гора. Иако меѓународните сорти се попопуларни, автохтоните сорти се многу важни, особено за земјите што го развиваат туризмот. Аромите на ферментација произведени за време на алкохолната ферментација значително придонесуваат за квалитетот на виното. Испитани беа ефектите на квасецот (*Saccharomyces cerevisiae* и *Saccharomyces bayanus*) и хранливите материи на квасецот (Fermaid E и Fermaid O) врз ароматичните соединенија во вината. Со помош на анализата GC/FID-MS, беа карактеризирани и квантифицирани ароматичните соединенија во вината *крстач* и *жижак*. Вината произведени со додавање на квасец и хранливи материи на квасец имаа главно помала вкупна содржина на алкохол од вината добиени со спонтана ферментација на сортите *крстач* и *жижак*. Резултатите од оваа студија покажаа дека концентрацијата на соединенијата зависи од соевите на квасецот. Квасецот *S. cerevisiae* (ICV) обезбеди поголема содржина на виши алкохоли, додека *S. bayanus* произведе поголема концентрација на естери и на масни киселини (со средна должина на низи). Вкупната содржина на естери се движеше од 3,34 до 11,97 mg/l за вината

крстач и од 8,51 до 13,68 mg/l за винаta *жижак*. Од сите вина, винаta *крстач* и *жижак* произведени со додаток *S. bayanus* и Fermaid E имаа најголема концентрација на вкупни естери. Хранливите материи од квасецот Fermaid E и O ги истакнаа различните карактеристики на квасецот. Тие имаа статистички значајно влијание врз содржината на етил и ацетатни естери. Највисоки вкупни оценки се добиени за винаta ICVE и BayE *крстач* (18,1 од 20 поени) и виното *жижак* ICVE (18,2 од 20 поени).

Клучни зборови: ароматични соединенија; квасци и хранливи материи на квасец; автохтони сорти грозје; анализа GC/FID-MS; сензорна евалуација

1. INTRODUCTION

Wine is a highly appreciated alcoholic beverage because of its specific aroma. Aroma is the most important indicator of whether a wine is accepted or rejected by consumers.[1] The quality and sensory characteristics of white wines depend largely on the aroma produced during alcoholic fermentation.[2]

Various aromatic compounds (ethyl esters, acetates, higher alcohols, and fatty acids) are synthesized by the metabolic activity of yeasts, transforming aromatic precursors present in the grape juice or producing new aromatic compounds.[3] Due to different compositions of the grape juice, different wines can be produced under the influence of the same yeast, while the quality of the wine is a result of the interaction between the composition of the grape juice and the yeast.[3] According to the literature, the yeast strain is an important factor that strongly influences the aroma and quality of wine.[2–9] The yeast *S. cerevisiae* is mainly used in the industry to perform alcoholic fermentation, while *S. bayanus* has a minor application in wine production. Data in the literature show that the yeasts used in the experiments significantly influenced the synthesis of various ethyl esters and acetates.[4,5] *S. bayanus* synthesized a higher content of acetates,[4,5] while *S. cerevisiae* produced ethyl esters of fatty acids.[7]

Alcoholic fermentation can be carried out with the epiphytic yeasts present on the grapes (spontaneous fermentation) or by adding commercial yeasts (directed fermentation). Some authors report good wine quality with spontaneous fermentation,[2] while others point to poorer quality than wines made with commercial yeasts.[10]

In addition to the yeast, the type of yeast nutrient (organic or inorganic) also influences the synthesis of fermentable aromatic compounds. The authors emphasize the importance of adding nitrogen-based preparations.[8,11–15] Studies have shown that fermentation stalls or slows down when there is not enough assimilable nitrogen in the must.[16,17] Yeasts differ greatly in their ability to assimilate nitrogen,[8,12] resulting in the production of wines with varying levels of volatile compounds.

In our previous research, data on the aromatic profile of the Krstač and Žižak varieties were published for the first time, and the influence of maceration and glycosidase enzyme preparations on the aromatic content of wine was studied.[18] The aim of this work was to investigate the influence of different commercial yeast strains *(S. cerevisiae* and *S. bayanus*) and yeast nutrients (Fermaid E and Fermaid O) on the concentration of aroma compounds and wine sensory characteristics. In addition, the effect of spontaneous alcoholic fermentation on the quality and sensory characteristics of Krstač and Žižak wines was investigated.

2. MATERIAL AND METODS

2.1. *Chemicals and plant material*

Methanol, anhydrous sodium sulfate, and 4-methyl-1-pentanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylene chloride was obtained from Merck (Darmstadt, Germany). Analytical grade solvents (methylene chloride and methanol) were used, and they were additionally purified by distillation and dried with anhydrous sodium sulfate.

Two autochthonous Montenegrin grape varieties, Krstač and Žižak, were studied in this research. Krstač is grown in the microsite "Dinoš", while Žižak in "Bunar 17" in Ćemovsko polje, 13 Jul Plantaže, Montenegro. The altitude of Krstač vineyards is 66 m and Žižak 33 m. The training system of Krstač and Žižak vines was a single Guyot. All vines were evenly pruned, leaving one shoot growth on a spur with two buds and an arc of nine buds long.[18]

2.2. *Winemaking*

The grapes of Krstač and Žižak varieties were harvested by hand at full ripeness. The phytosanitary state of grapes was healthy (determined visually). They were cooled and processed accord-

ing to the procedure for white wines. The grapes of both varieties were destemmed, crushed, sulfited with 10 g of $K_2S_2O_5$/100 kg of crushed grapes and pressed through a hydraulic press (Nuovo, Enopieve, Italy). The grape juice was clarified by static settling (48 hours at 5 °C) and racked.[18] The experiment was separated into five treatments: Ctrl (control) – no addition of yeasts or nutrients for yeast; ICVE – with the addition of 20 g/hl Lalvin ICV D47, *S. cerevisiae* var. *cerevisiae* (Lallemand Inc., Montreal, Canada) and 15 g/hl nutrients for yeast, Fermaid E (Lallemand Inc., Montreal, Canada); ICVO – with addition of 20 g/hl Lalvin ICV D47, *S. cerevisiae* var. *cerevisiae* and 15 g/hl nutrients for yeast, Fermaid O (Lallemand Inc., Montreal, Canada); BayE – with addition of 20 g/hl ENARTIS FERM SB, *S. cerevisiae ex r.f. bayanus* (Enartis, San Martino, Italy) and 15 g/hl nutrients for yeast, Fermaid E; BayO – with the addition of ENARTIS FERM SB, *S. cerevisiae ex r.f. bayanus* and 15 g/hl nutrients for yeast, Fermaid O.

Alcoholic fermentation was carried out by microvinification in glass vessels of 15 l at a temperature of 15 °C. For ICVE Krstač and Žižak wines, the duration times of alcoholic fermentation were 14 and 16 days, and for ICVO Krstač and Žižak wines 15 and 17 days. For Krstač and Žižak wines produced with *S. bayanus* yeast and Fermaid E (BayE), alcoholic fermentation lasted 11 and 12 days, while for BayO Krstač and Žižak wines, it lasted 11 and 14 days, respectively.

S. cerevisiae var. *cerevisiae* is sensitive to low temperatures, has average alcohol tolerance, and its fermentation rate is moderate. *S. cerevisiae ex r.f. bayanus* has good resistance to low temperatures and alcohol tolerance (≤15 % v/v). Its fermentation rate is rapid. In general, *S. bayanus* has the ability to complete fermentation in high-sugar musts.

The yeast nutrients Fermaid E and Fermaid O were added on the third day after the start of fermentation. Fermaid E is a nutrient called "complex" for its balanced levels of organic and inorganic nitrogen.[19] It supplements a series of important nutrients and bio-factors: di-ammonium phosphate, ammonium sulfate, inactive yeast hull products, and thiamine. Fermaid E reduces the occurrence of sluggish and stuck fermentations. Fermaid O is a 100 % organic nutrient comprised only of nitrogen in the form of amino acids.[19] It does not contain added ammonia salts (DAP) or micronutrients.

2.3. *Liquid-liquid extraction*

Liquid-liquid extraction was applied for sample preparation.[20] Twenty-five milliliters of wine and 5 ml of methylene chloride were used. Extraction was performed by mixing with a magnetic stirrer for 1 hour at 0 °C in an ice bath. After extraction, the obtained mixture was left in an ultrasonic bath for 5 minutes to "break" the emulsion. The organic phase was separated, dried with anhydrous sodium sulfate, and filtered. Afterward, 0.6 ml of the extracted wine was used for GC/FID-MS analysis.[18] All measurements were done in triplicate.

2.4. *GC/FID-MS analysis*

The GC/FID-MS system was used to analyze volatile compounds using the previously published method with some changes.[21] The analysis was performed using an Agilent 7890A gas chromatograph (GC) (Santa Clara, CA, USA). The instrument was equipped with an Agilent 19091N-113 HP-INNOWax fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). Injection was performed in a 3:1 split mode with helium as the carrier gas at a flow rate of 1.46 ml/min. The injection volume was 1 μl. The GC oven temperature was held at 40 °C for 5 min, then programmed to 220 °C at 10 °C min^{-1} and maintained at 220 °C for 4 min. The instrument had two detectors: a 5975C inert mass selective detector (MSD) XL EI /CI MSD and a flame ionization detector (FID) linked to makeup gas via a 2-way capillary splitter. The ion source of the MSD and the transfer line were maintained at 230 °C and 280 °C, respectively. The mass selective detector operated in positive ion electron impact (EI) mode. Electron impact spectra were collected in scan mode at 70 eV in the mass range from 35 to 500 *m*/*z*. The temperature of the FID detector was 300 °C.[18] The internal standard approach was used for quantitative evaluation. A known amount of 4-methyl-1-pentanol was used as an internal standard (IS). The (relative) percentages of the identified compounds were computed from the GC peak areas. The concentration of each volatile compound was determined using the peak area of the internal standard and reported as the relative concentration of each component in the analyzed sample. The components were identified based on comparison with reference spectra (Wiley and NIST databases).[18]

2.5. *Statistical analysis*

Statistical analysis was performed using R statistical software.[22] One-way analysis of variance (ANOVA) was carried out to compare the influ-

ence of using different yeasts (*S. cerevisiae* and *S. bayanus*) and nutrients for yeasts (Fermaid E and Fermaid O) on each aromatic compound separately. The Tukey post-hoc test with a significance level of $p < 0.05$ was performed to compare the means. Pearson correlation coefficients were used to determine the relationship between some volatile compounds analyzed. PCA was applied to investigate the differences between wine samples according to the amounts of their volatile compounds. The Pearson correlation coefficients among the total alcohols (TAL), acids (TAC), and esters (TEST) and the taste and odor were calculated.

2.6. *Sensory analysis*

The sensory evaluation of the wine samples was performed according to the Buxbaum method.[23] The wine samples were evaluated by a tasting panel composed of three members highly ranked in sensory evaluation. Color, clarity, taste, and odor were evaluated, with the highest total score being 20 points.[18]

3. RESULTS AND DISCUSSION

3.1. *Effect of two different yeasts and yeast nutrients on the content of aromatic compounds in wines of Krstač and Žižak varieties*

Tables 1 and 2 show the concentration of aromatic compounds in wines obtained from Krstač and Žižak using two yeasts, *S. cerevisiae* (ICV) and *S. bayanus* (Bay), and two yeast nutrients, Fermaid E (ammonium + amino acids) and Fermaid O (amino acids). As a result of GC/FID-MS analysis, higher alcohols (aromatic, aliphatic, and C6), fatty acids, ethyl esters, and acetates were detected in wines of the Krstač and Žižak varieties.[1] The most important groups of aromatic compounds related to yeast metabolism are the higher alcohols, esters, and fatty acids.[24] The concentration of total aromatic compounds ranged from 97.18 to 217.07 mg/l in wines of the Krstač variety (Table 1). In wines of the Žižak variety, it ranged from 171.93 to 233.75 mg/l (Table 2). *S. cerevisiae* (ICV) provided a higher concentration of the higher alcohols, while *S. bayanus* (Bay) produced a higher concentration of esters and fatty acids, which is consistent with other studies.[4]

Alcohols

The content of the higher alcohols is very important for the quality of white wine.[2] The wines of Krstač and Žižak varieties have higher alcohol concentrations below 300 mg/l, which contributes to the complexity and pleasant character of white wine.[24–26] In concentrations exceeding 400 mg/l, the higher alcohols are regarded as a negative quality factor.[24–26] Alcoholic fermentation performed at lower temperatures leads to a lower content of higher alcohols in wines, which could affect wine quality.[16] Yeast *S. cerevisiae* provided a higher content of isobutyl, isoamyl ($p < 0.05$), and higher alcohols overall than *S. bayanus* in Krstač wines. Krstač and Žižak wines obtained after spontaneous alcoholic fermentation had the highest concentrations of total higher alcohols, while wines treated with yeast and yeast nutrients had the lowest contents (except Z_ICVO). One-way ANOVA revealed that Krstač (ICVO, BayE, BayO) and Žižak (BayE, BayO) had statistically lower concentrations of isoamyl alcohol (except Z_BayE) and isobutyl alcohol compared to wines produced by spontaneous fermentation (Ctrl). These results are confirmed by literature data.[2,6,8]

Among the higher alcohols, isoamyl alcohol and 2-phenylethyl alcohol were most abundant in the wines from Krstač and Žižak, which is consistent with the data found in the literature.[6] 2-Phenylethyl alcohol is a very important aromatic alcohol responsible for pleasant floral notes reminiscent of roses.[27,28] The concentration of 2-phenylethyl alcohol ranged from 24.47 to 66.47 mg/l in Krstač wines (Table 1) and from 32.24 to 55.57 mg/l for Žižak wines (Table 2). Wine of the Krstač variety produced by spontaneous fermentation (K_Ctrl) had a statistically significantly higher concentration of 2-phenylethyl alcohol ($p < 0.05$) than wine K_ICVE. In contrast, wine Žižak ICVO (produced with *S. cerevisiae* addition) had a higher content of 2-phenylethyl alcohol. Literature data indicate that spontaneously fermented wines have the highest content of 2-phenylethyl alcohol.[2]

There was a statistical difference in the content of 1-hexanol between K_Ctrl and K_ICVO, K_BayE, and K_BayO. Krstač wine obtained by spontaneous fermentation (K_Ctrl) had the highest content of 1-hexanol (0.76 mg/l), consistent with literature data.[27] Among Žižak wines, Z_BayO had the highest value (0.57 mg/l). 1-Hexanol was usually formed in the prefermentative phase when skin contact provided more lipoxygenase enzymes and fatty acids.[18,29] Literature data suggest that the yeast can slightly influence the formation of 1-hexanol.[4]

The use of the different nitrogen additions, Fermaid E (ammonium+amino acid) and Fermaid O (amino acid), had an effect on the content of

aroma compounds. Fermaid E affected the production of higher alcohol content in Krstač wines than Fermaid O for both yeasts (*S. cerevisiae* and *S. bayanus*). In Žižak wines, Z_ICVO had the highest content of higher alcohols, while when *S. bayanus* yeast was used, Z_BayE had the highest higher alcohol content (Table 2).

T a b l e 1

The content of aromatic compounds in Krstač wines using yeasts (S. cerevisiae and S. bayanus) and yeast nutrients (Fermaid and Fermaid O) with results of the one-way ANOVA along with the Tukey post-hoc test.

	Sample (mg/l)					*F*	*p*
Compounds	K_Ctrl	K_ICVE	K_ICVO	K_BayE	K_BayO		
1-Hexanol	0.76 ± 0.07 a	0.68 ± 0.07 a	0.50 ± 0.06 b	0.51 ± 0.04 b	0.26 ± 0.03 c	36	0.000
Isobutyl alcohol	5.02 ± 0.14 a	5.36 ± 0.18 a	2.83 ± 0.11 c	3.66 ± 0.38 b	2.22 ± 0.05 d	129	0.000
Isoamyl alcohol	120.08 ± 3.59 a	114.63 ± 1.01 a	71.56 ± 8.60 bc	91.05 ± 7.33 b	55.02 ± 12.41 c	39	0.000
4-Methyl-1-pentanol	8.13	8.13	8.13	8.13	8.13		
3-Ethoxy-1-propanol	t	t	0.30 ± 0.04 b	0.49 ± 0.03 a	t	45	0.003
2,3 Butanediol	t	0.21 ± 0.01 d	1.20 ± 0.01 b	1.53 ± 0.02 a	0.72 ± 0.02 c	4856	0.000
3-(Methylthio)-1-propanol	0.38 ± 0.02 a	0.19 ± 0.01 b	t	t	0.12 ± 0.01 c	314	0.000
2-Phenylethyl alcohol	66.47 ± 2.96 a	39.71 ± 1.74 b	30.58 ± 7.30 bc	28.48 ± 2.16 c	24.77 ± 3.02 c	54	0.000
Total higher alcohols	**200.84**	**168.91**	**115.10**	**133.85**	**91.24**		
Hexanoic acid	2.13 ± 0.09 c	3.08 ± 0.05 b	4.24 ± 0.04 a	4.40 ± 0.07 a	t	803	0.000
Octanoic acid	4.05 ± 0.29 c	5.18 ± 0.37 b	9.96 ± 0.23 a	10.24 ± 0.32 a	2.60 ±0.25 d	416	0.000
Decanoic acid	0.56 ± 0.00 d	0.67 ± 0.00 c	1.98 ± 0.02 b	2.37 ± 0.01 a	t	14906	0.000
Isobutyric acid	t	0.24 ± 0.03 a	0.22 ± 0.02 a	0.26 ± 0.01 a	t	2.28	0.183
9-Decenoic acid	0.92 ± 0.01 a	0.25 ± 0.02 c	0.31 ± 0.01 b	nd	nd	2508	0.000
Total fatty acids	**7.66**	**9.42**	**16.71**	**17.27**	**2.60**		
Ethyl butyrate	1.02 ± 0.01 b	2.37 ± 0.01 a	t	t	t	22151	0.000
Ethyl hexanoate	0.25 ± 0.02 e	0.40 ± 0.01 c	0.97 ± 0.00 b	1.10 ± 0.01 a	0.33 ± 0.01 d	3207	0.000
Ethyl (*S*)-(-) lactate	0.97 ± 0.02 a	1.05 ± 0.00 a	0.43 ± 0.03 c	0.67 ± 0.06 b	0.29 ± 0.01 d	287	0.000
Ethyl octanoate	0.15 ± 0.02 e	0.24 ± 0.01 d	1.66 ± 0.00 b	1.76 ± 0.02 a	0.68 ± 0.01 c	8540	0.000
Ethyl decanoate	0.13 ± 0.02 c	0.21 ± 0.01 b	0.35 ± 0.02 a	0.36 ± 0.02 a	0.18 ± 0.00 bc	107	0.000
Diethyl succinate	0.83 ± 0.03 a	0.72 ± 0.01 b	0.09 ± 0.02 c	0.11 ± 0.01 c	0.12 ± 0.01 c	1538	0.000
Ethyl 9-decenoate	t	t	0.22 ± 0.02 a	0.20 ± 0.02 a	t	1.06	0.361
Ethyl 4- hydroxybutanoate	0.96 ± 0.02 b	0.57 ± 0.08 c	1.21 ± 0.02 a	1.05 ± 0.01 b	t	112	0.000
Diethyl hydroxybutanedioate	0.27 ± 0.01 a	0.10 ± 0.01 c	0.10 ± 0.01 c	0.17 ± 0.01 b	t	259	0.000
Ethyl ester 4-ethoxy benzoic acid	nd	t	0.23	t	nd		
Ethyl hydrogen succinate	1.47 ± 0.01 a	1.34 ± 0.06 b	t	t	t	13	0.022
Isoamyl acetate	0.70 ± 0.02 e	2.13 ± 0.00 c	2.46 ± 0.03 b	3.91 ± 0.02 a	0.80 ± 0.01 d	14295	0.000
Hexyl acetate	t	nd	t	0.18	nd		
1,3-Propanediol diacetate	0.20 ± 0.01 d	0.27 ± 0.02 c	0.31 ± 0.02 b	0.35 ± 0.01 a	0.19 ± 0.01 d	87	0.000
2-Phenylethyl acetate	0.37 ± 0.02 d	0.49 ± 0.00 c	0.58 ± 0.02 b	0.70 ± 0.02 a	0.32 ± 0.01 e	307	0.000
γ-Butyrolactone	1.25 ± 0.02 b	0.88 ± 0.00 c	1.21 ± 0.02 b	1.41 ± 0.02 a	0.43 ± 0.01 d	1510	0.000
Total ethyl esters, acetates and lactones	**8.57**	**10.77**	**9.82**	**11.97**	**3.34**		
Total aromatic compounds	**217.07**	**189.10**	**141.63**	**163.09**	**97.18**		

Values are presented as the mean (n = 3) ± standard deviation. Different lowercase letters (a, b, c, d, e) indicate significant differences between treatments at the 5 % level. t – trace (below limit of quantification = 0.01 mg/l). K_Ctrl–without addition of yeasts and nutrients for yeast, K_ICVE–with addition of *S. cerevisiae* and Fermaid E, K_ICVO–with addition of *S. cerevisiae* and Fermaid O, K_BayE–with addition of *S. bayanus* and Fermaid E, K_BayO–with addition of *S. bayanus* and Fermaid O.

T a b l e 2

The contents of aromatic compounds in Žižak wines using yeasts (S. cerevisiae and S. bayanus) and yeast nutrients (Fermaid E and Fermaid O) with results of the one-way ANOVA along with the Tukey post-hoc test.

	Sample (mg/l)					*F*	*p*
Compounds	Z_Ctrl	Z_ICVE	Z_ICVO	Z_BayE	Z_BayO		
1-Hexanol	0.37 ± 0.01 c	0.44 ± 0.01 b	0.46 ± 0.01 b	0.56 ± 0.05 a	0.57 ± 0.02 a	40.1	0.000
Isobutyl alcohol	6.27 ± 0.32 ab	6.15 ± 0.08 b	6.60 ± 0.16 a	4.72 ± 0.05 c	4.79 ± 0.06 c	84.1	0.000
Isoamyl alcohol	123.60 ± 9.46 ab	114.93 ± 4.78 abc	132.59 ± 12.02 a	108.18 ± 3.31 bc	98.99 ± 2.45 c	9.37	0.002
4-Methyl-1-pentanol	8.13	8.13	8.13	8.13	8.13		
3-Ethoxy-1-propanol	t	t	t	t	t		
2,3 Butanediol	2.21 ± 0.01 b	1.28 ± 0.00 d	2.63 ± 0.01 a	1.93 ± 0.01 c	1.11 ± 0.02 e	8798	0.000
3-(Methylthio)-1-propanol	0.14 ± 0.02 b	0.14 ± 0.02 b	0.21 ± 0.00 a	0.20 ± 0.00 a	0.16 ± 0.01 b	20.3	0.000
2-Phenylethyl alcohol	42.31 ± 1.80 b	43.17 ± 4.99 b	55.57 ± 5.02 a	36.87 ± 1.73 bc	32.24 ± 3.09 c	17.5	0.000
Total higher alcohols	**183.03**	**174.24**	**206.19**	**160.59**	**145.99**		
Hexanoic acid	3.48 ± 0.05 d	4.07 ± 0.09 c	4.16 ± 0.12 c	4.77 ± 0.06 a	4.42 ± 0.10 b	93.5	0.000
Octanoic acid	6.15 ± 0.25 d	8.07 ± 0.16 c	8.53 ± 0.34 bc	11.48 ± 0.18 a	8.76 ± 0.05 b	228	0.000
Decanoic acid	0.87 ± 0.01 e	1.14 ± 0.02 d	2.83 ± 0.02 b	2.94 ± 0.01 a	1.38 ± 0.02 c	8925	0.000
Isobutyric acid	0.66 ± 0.01 a	0.58 ± 0.01 b	0.37 ± 0.02 c	0.34 ± 0.02 c	0.35 ± 0.01 c	427	0.000
9-Decenoic acid	0.83 ± 0.02 c	1.54 ± 0.01 b	2.14 ± 0.02 a	0.56 ± 0.02 d	0.44 ± 0.03 e	4100	0.000
Total fatty acids	**11.99**	**15.40**	**18.03**	**20.09**	**15.35**		
Ethyl butyrate	1.67 ± 0.01 b	2.30 ± 0.02 a	t	t	1.43 ± 0.02 c	3149	0.000
Ethyl hexanoate	0.50 ± 0.01 e	0.54 ± 0.01 d	0.96 ± 0.01 b	1.13 ± 0.01 a	0.65 ± 0.01 c	2383	0.000
Ethyl (S)-(-) lactate	0.20 ± 0.03 c	0.34 ± 0.07 a	0.24 ± 0.01 bc	0.37 ± 0.02 a	0.32 ± 0.01 ab	12.2	0.001
Ethyl octanoate	0.25 ± 0.01 d	0.26 ± 0.01 d	1.70 ± 0.02 b	2.24 ± 0.03 a	0.32 ± 0.01 c	8482	0.000
Ethyl decanoate	0.23 ± 0.01 d	0.24 ± 0.01 d	0.45 ± 0.01 b	0.75 ± 0.00 a	0.29 ± 0.01 c	1500	0.000
Diethyl succinate	0.84 ± 0.02 a	0.82 ± 0.02 a	t	0.10 ± 0.02 c	0.49 ± 0.02 b	929	0.000
Ethyl 9-decenoate	t	t	0.24 ± 0.02 b	0.41 ± 0.02 a	t	108	0.000
Ethyl 4-hydroxybutanoate	1.45 ± 0.00 d	1.37 ± 0.02 e	1.78 ± 0.02 c	3.57 ± 0.06 a	2.58 ± 0.01 b	2982	0.000
Diethyl hydroxybutanedioate	0.05 ± 0.02 c	0.10 ± 0.01 b	0.19 ± 0.01 a	0.18 ± 0.01 a	0.04 ± 0.03 c	43.8	0.000
Ethyl ester 4-ethoxy benzoic acid	t	t	t	nd	t		
Ethyl hydrogen succinate	0.76 ± 0.04 c	2.55 ± 0.02 a	t	t	1.24 ± 0.03 b	3351	0.000
Isoamyl acetate	1.32 ± 0.01 e	1.76 ± 0.00 c	1.98 ± 0.01 b	2.47 ± 0.01 a	1.51 ± 0.01 d	8688	0.000
Hexyl acetate	t	t	0.20	t	t		
1,3-Propanediol diacetate	0.22 ± 0.02 c	0.26 ± 0.01 b	0.31 ± 0.01 a	0.26 ± 0.01 b	0.21 ± 0.01 c	32	0.000
2-Phenylethyl acetate	0.22 ± 0.00 d	0.38 ± 0.02 b	0.49 ± 0.03 a	0.36 ± 0.01 b	0.28 ± 0.02 c	116	0.000
γ-Butyrolactone	0.80 ± 0.00 e	0.84 ± 0.01 d	0.99 ± 0.01 c	1.84 ± 0.02 a	1.23 ± 0.01 b	4099	0.000
Total ethyl esters, acetates and lactones	**8.51**	**11.76**	**9.53**	**13.68**	**10.59**		
Total aromatic compounds	**203.53**	**201.40**	**233.75**	**194.36**	**171.93**		

Values are presented as the mean (n = 3) ± standard deviation. Different lowercase letters (a, b, c, d, e) indicate significant differences between treatments at the 5 % level. t – trace (below limit of quantification = 0.01 mg/l). Z_Ctrl–without addition of yeasts and nutrients for yeast, Z_ICVE–with addition of *S. cerevisiae* and Fermaid E, Z_ICVO–with addition of *S. cerevisiae* and Fermaid O, Z_BayE–with addition of *S. bayanus* and Fermaid E, Z_BayO–with addition of *S. bayanus* and Fermaid O.

Krstač wines made with the addition of Fermaid E and yeasts had statistically higher levels of isobutyl alcohol, isoamyl alcohol, and 1-hexanol than wines made with Fermaid O and the same yeast. Based on all these results, we can confirm that the yeast nutrient Fermaid E had a greater ef-

fect on the production of higher alcohols in Krstač wines than the yeast nutrient Fermaid O.[1] The effect of ammonium-based supplements was to increase the ability of the yeast to convert α-keto acids, while the addition of amino acids led to an increase in catabolic products.[8]

Fatty acids

Krstač and Žižak wines made with *S. bayanus* (Bay) yeast and Fermaid E yeast nutrient had the highest total fatty acids concentration, while Krstač and Žižak BayO wines had the lowest concentration (Tables 1 and 2). Statistical analysis using Tukey's test showed statistically significantly higher content of hexanoic, octanoic, and decanoic acids in all wines from Krstač and Žižak (except K_BayO) in comparison with wines produced by spontaneous fermentation (K_Ctrl). Our results are consistent with other research.[2]

The results of this study show that the concentration of total fatty acids is highly variable and depends on the yeast strain used. *S. bayanus* leads to statistically significantly higher hexanoic, octanoic, and decanoic acid content in all Krstač and Žižak wines (except K_ICVO) compared to *S. cerevisiae*.

During alcoholic fermentation at low temperatures, *S. bayanus* produced a higher content of medium-chain fatty acids (hexanoic, octanoic, and decanoic acids).[7] In the literature, it was found that medium-chain fatty acids (MCFA) may have a negative influence on yeast growth and metabolism.[7] By adding Fermaid O during alcoholic fermentation, *S. cerevisiae* produced a statistically significantly higher concentration of hexanoic, octanoic, and decanoic acids than Fermaid E in Krstač wines. In Žižak wines, Fermaid E produced statistically significantly higher levels of these compounds during alcoholic fermentation with *S. cerevisiae* compared to Fermaid O. Our results are consistent with other studies[30] in which the addition of ammonium increased the concentration of medium-chain fatty acids.

Ethyl esters, acetates, and lactones

Esters are a group of aromatic compounds in wine that generally have a pleasant fruity and floral odor.[24,25] Acetates of higher alcohols and fatty acid ethyl esters have an interesting aroma. Isoamyl acetate is responsible for the banana-like aroma, 2-phenylethyl acetate for the rose-like aroma, ethyl hexanoate for apple and banana, and ethyl octanoate for pear.[24,25]

The concentration of total esters ranged widely, from 3.34 to 11.97 mg/l in Krstač wines (Table 1) and from 8.51 to 13.68 mg/l in Žižak wines (Table 2). Wines of Krstač and Žižak, produced with the addition of *S. bayanus* and nutrient Fermaid E, had the highest concentration of total esters. *S. bayanus* produced a higher content of total esters than *S. cerevisiae* (except K_BayO).

Compared with wines obtained by spontaneous fermentation, wines treated with Krstač and Žižak (ICVE, ICVO, BayE) had statistically significantly higher levels of isoamyl acetate, 2-phenylethyl acetate, and 1,3-propanediol diacetate, which is consistent with other literature data examining other grape varieties.[6,27] *S. bayanus* with Fermaid E and *S. cerevisiae* with Fermaid O produced statistically significantly higher levels of isoamyl acetate, 2-phenylethyl acetate, and 1,3-propanediol diacetate in Krstač wines and isoamyl acetate in Žižak wine. *S. cerevisiae* with Fermaid O (ICVO) produced statistically significantly higher levels of 2-phenylethyl acetate and 1,3-propanediol diacetate. The yeast *S. bayanus* is an important producer of aroma compounds.[4]

The wines of Krstač and Žižak varieties, which had the highest acetate content, also contained the highest concentration of ethyl esters of medium-chain fatty acids (ethyl hexanoate, ethyl octanoate, and ethyl decanoate). These results are confirmed by literature data examining other grape varieties that were studied.[4] Vinifications with yeasts and adding yeast nutrients resulted in statistically significantly higher levels of medium-chain fatty acids (ethyl esters) in almost all wines of both varieties compared to spontaneous vinification. The yeast *S. bayanus* with Fermaid E (BayE) produced a higher concentration of medium-chain ethyl ester fatty acids ($p < 0.05$) than ICVE. However, *S. cerevisiae* with Fermaid O (ICVO) produced a higher concentration of medium-chain ethyl ester fatty acids ($p < 0.05$) than BayO. It should be noted that each yeast nutrient (Fermaid O, Fermaid E) emphasized different properties of the yeasts. Our results are confirmed by literature data.[8]

A Pearson correlation coefficient calculation was performed to evaluate the relationship between ethyl esters of medium-chain fatty acids and the corresponding fatty acids. The correlations between ethyl hexanoate and hexanoic acid for the wine from Žižak ($r = 0.973$, $p < 0.05$) and ethyl decanoate and decanoic acid for the wines from Krstač and Žižak ($r = 0.970$; $r = 0.986$, $p < 0.05$) were statistically significant. The correlation between acetates and their corresponding higher alcohols was not statistically significant.

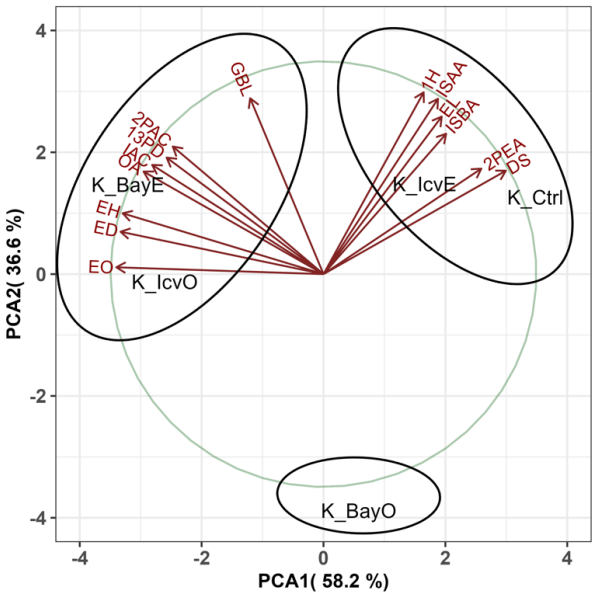
The most abundant ethyl esters of organic acids in Krstač and Žižak wines were ethyl lactate, diethyl succinate, and ethyl hydrogen succinate. Ethyl lactate and diethyl succinate are normally formed during malolactic fermentation. In addition, ethyl lactate can be formed by yeasts during alcoholic fermentation.[4,6] The content of ethyl lactate increased in treated Žižak wines, and the lowest concentration was found in wines obtained by spontaneous fermentation (Z_Ctrl), consistent with other literature data.[6] Based on the Tukey post-hoc test, a statistically significant difference in ethyl lactate and diethyl succinate content was found between wines from Krstač and Žižak Ctrl and BayE, BayO. The yeast *S. bayanus* is a major producer of ethyl lactate.[4]

The yeast *S. bayanus* with Fermaid E provided statistically significantly higher content of ethyl 4-hydroxybutanoate and diethyl hydroxybutanedioate than *S. cerevisiae*. γ-Butyrolactone was the only lactone detected in Krstač and Žižak wines. Krstač and Žižak BayE had the highest concentrations of γ-butyrolactone, 1.41 mg/l and 1.84 mg/l, respectively. The correlation between ethyl 4-hydroxybutanoate and γ-butyrolactone is statistically significant ($r = 0.971$, $p < 0.05$). These compounds are derived from a glutamic acid precursor via 4-hydroxybutanoic acid.[31]

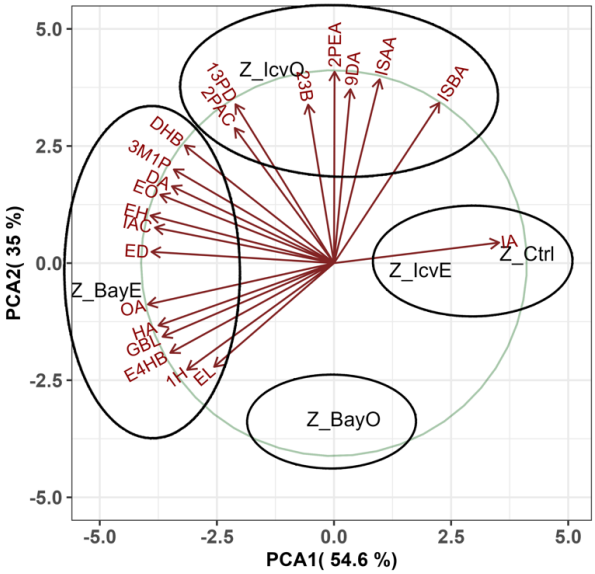
The addition of yeast nutrients E and O had a statistically significant effect on the content of ethyl esters and acetates. The yeast nutrient Fermaid O resulted in statistically significantly higher concentrations of ethyl esters (ethyl hexanoate, ethyl octanoate, ethyl decanoate) and acetates (isoamyl acetate, 1,3-propanediol diacetate, and 2-phenylethyl acetate) in the Krstač and Žižak wines during alcoholic fermentation with *S. cerevisiae* compared to Fermaid E. During alcoholic fermentation with *S. bayanus*, Fermaid E resulted in statistically significantly higher content of ethyl esters and acetates. This different behavior of the yeasts can be explained by their different metabolism in the presence of different nitrogen sources. Yeast nutrients with amino acids were greatly dependent on the yeast strain.[32]

Principal component analysis

Principal component analysis (PCA) was applied to evaluate which higher alcohols, esters, and fatty acids best differentiated wines produced from Krstač and Žižak varieties by yeast strain and yeast nutrient (Fig. 1a for Krstač wines and Fig. 1b for Žižak wines).



(a)



(b)

Fig. 1. Results of the PCA analysis performed on the volatile compound data: (a) Krstač wines, (b) Žižak wines–1H: 1-hexanol; ISBA: isobutyl alcohol; ISAA: isoamyl alcohol; 23B: 2,3 butanediol; 3M1P: 3-(methylthio)-1-propanol; 2PEA: 2-phenylethyl alcohol; HA: hexanoic acid; OA: octanoic acid; DA: decanoic acid; IA: isobutyric acid; 9DA: 9-decenoic acid; EH: ethyl hexanoate; EL: ethyl lactate; EO: ethyl octanoate; ED: ethyl decanoate; DS: diethyl succinate; E4HB: ethyl 4-hydroxybutanoate, DHB: diethyl hydroxybutanedioate; IAC: isomyl acetate; 13PD: 1,3-propanediol diacetate; 2PAC: 2- phenylethyl acetate; GBL: γ-butyrolactone.

In the Krstač wine samples (Figure 1a), the first two principal components had eigenvalues above 1. The first component (PC1) explained 58.2 % of the total variance, and the second component (PC2) explained 36.6 % of the total variance. To-

gether they explained 94.8 % of the total variability. The first component (PC1) was negatively related to EH, EO, ED, IAC, 13PD, and OA, and positively related to 2PEA and DS. The second component (PC2) was positively loaded with 1H, ISBA, ISAA, EL, 2PAC, and GBL (Fig. 1a).

Figure 1a shows that the wines obtained from the Krstač variety were clearly divided into three groups. The Krstač wines (K_ICVE) and K_Ctrl were located in the upper right quadrant and were characterized by high alcohol content (1-hexanol, isobutyl alcohol, and isoamyl alcohol), ethyl lactate, 2-phenylethyl alcohol, and diethyl succinate. The second group of wines was located on the left side of the PCA plot. The K_BayE and K_ICVO wines were rich in ethyl esters of medium-chain fatty acids (ethyl hexanoate, ethyl octanoate, and ethyl decanoate), acetates (1,3-propanediol diacetate, isoamyl acetate, and 2-phenylethyl acetate), octanoic acid, and γ-butyrolactone.

In the Žižak wine samples (Fig. 1b), the first two principal components had eigenvalues greater than 1 and accounted for 54.6 % and 35.0 % of the total variance, respectively. Compounds 1H, 3M1P, HA, DA, EH, EO, ED, E4HB, DHB, IAC, GBL, OA, and IAC had negative loadings, and IA had positive loadings in the first component (PC1). The second component (PC2) was positively associated with ISBA, ISAA, 23B, 2PEA, 9DA, 13PD, and 2PAC (Fig. 1b).

The wines obtained from the Žižak variety were well separated from each other (Fig. 1b). Z_BayE was richest in volatile compounds and was characterized by alcohols [1-hexanol, 3-(methylthio)-1-propanol)], esters (ethyl hexanoate, ethyl octanoate, and ethyl decanoate, ethyl 4-hydroxybutanoate, diethyl hydroxybutanedioate, and isoamyl acetate), MCFA (hexanoic acid and octanoic acid), and γ-butyrolactone. Z_BayE was on the negative side of the PC1 component. Z_ICVO wine was richest in acetates (1,3-propanediol diacetate and 2-phenylethyl acetate), higher alcohols (isoamyl alcohol, isobutyl alcohol, 2,3-butanediol, and 2-phenylethyl alcohol) and 9-decenoic acid, which have positive loadings in the second component.

When comparing the influence of different yeasts on the content of volatile compounds, *S. bayanus* with the yeast nutrient Fermaid E (BayE) produced more ethyl esters and acetates in wines from Krstač and Žižak, while *S. cerevisiae* (ICVE) produced a higher content of alcohols in Krstač wine. Krstač and Žižak wines produced with *S. cerevisiae* and Fermaid O (ICVO) had a higher content of esters and acetates, while BayO had the lowest content of all aromatic compounds. The difference between these wine samples is evidenced by the fact that they are located on opposite sides of the PCA plot. The different behavior of the yeasts could be explained by their different metabolic activity.[24,32] Yeasts can produce high or low amounts of higher alcohols, which depends on the individual characteristics of the yeast.[24,33]

Krstač and Žižak wines obtained after alcoholic fermentation using different yeast nutrients, Fermaid E (ammonium + amino acids) and Fermaid O (amino acids), with the same yeast strain differed greatly in the content of aromatic compounds. Figures 1a and 1b show a separation of BayE and BayO wines, where using *S. bayanus* with Fermaid E resulted in higher synthesis of aromatic compounds than BayO. When comparing ICVE and ICVO wine samples, the PCA plot clearly shows their separation. In wines where the yeast nutrient Fermaid O (amino acids) was used, high concentrations of 2-phenylethyl acetate, 1,3-propanediol diacetate, and ethyl esters of MCFAs were characterized for Krstač wine, while Z_ICVO had higher alcohols and acetates, which is consistent with the research.[32] Wines with Fermaid E (ammonium+amino acids) had a higher content of isoamyl alcohol, ethyl lactate, hexanoic acid, octanoic acid, and diethyl succinate, which can be explained by the different nitrogen metabolism of yeast in the presence of different nitrogen sources (inorganic or organic), which has been confirmed in other research.[8,32]

3.2. *Sensory evaluation wines of Krstač and Žižak*

The Krstač wine samples, using two different yeasts and yeast nutrients, had a light yellow color. The odor of K_BayE was more intense (3.5 out of 4.0 points), with fewer apple notes than K_BayO (3.3 out of 4.0 points) (Fig. 2b). K_ICVE had a long-lasting aroma and seemed more full-bodied (10.6 out of 12.0 points). K_BayO had lower aroma quality and persistence and was lighter in body (10.2 out of 12.0 points) (Fig. 2a) compared to the previous treatment. Total acids (TAC) and total esters (TEST) showed a positive statistically significant correlation between each other and both sensory properties (taste and odor). The strongest correlation was observed between TEST and odor ($r = 0.738$, $p < 0.01$), followed by the correlation between TAC and taste ($r = 0.599$, $p < 0.01$) (Table 3).

The wines produced after vinification with Fermaid E had a higher overall score (K_ICVE, K_BayE) (18.1 out of 20.0 points) than the wine samples to which Fermaid O was added (K_ICVO, K_BayO). The lowest overall score was obtained for the control wine K_Ctrl (17.4 out of 20.0 points).

The color was light yellow and similar in all samples of Žižak wine. The most intense odor with the greatest fruitiness and elegance was exhibited by the Z_ICVE wine sample (3.6 out of 4.0 points), while the least intensity was observed in the control and Z_BayO (3.3 out of 4.0 points) (Fig. 2b). The wine samples with Fermaid E (Z_ICVE, Z_BayE) showed the most intense aroma, and the least intensity was obtained in the wines with Fermaid O (Z_ICVO, Z_BayO). The Z_ICVE wine was the smoothest and had the best flavor characteristics (10.6 out of 12.0 points) (Fig. 2a). The control showed an astringent and harsh taste (10.0 out of 12.0 points). Total acids (TAC) and total esters (TEST) showed a positive statistically significant correlation between themselves and the taste. The strongest correlation was observed between TEST and TAC and taste ($r = 0.671$, $p < 0.05$), followed by the correlation between TEST and odor ($r = 0.612$, $p < 0.05$) (Table 4).

In general, the Žižak wines had a more pronounced varietal aroma, pleasant flavor, moderate richness, and acidity. When considering all samples of the Žižak variety, the highest overall score was obtained for the wine where *S. cerevisiae* and the yeast nutrient Fermaid E (ICVE) were used for alcoholic fermentation (18.2 out of 20.0 points). The lowest overall score was obtained for the control wine Z_Ctrl (17.3 points).

T a b l e 3

Correlation between the results of instrumental methods and the sensory evaluation (taste and odor) for Krstač wines

	TAL	TAC	TEST	Taste	Odor
TAL	1				
TAC	–0.014	1	**	*	*
TEST	0.447	0.830	1	*	**
Taste	–0.118	0.599	0.593	1	**
Odor	0.060	0.600	0.738	0.770	1

The stars represent statistically significant correlations at $p < 0.05$ (*) and $p < 0.01$ (**).
(TAL – total alcohols, TAC – total acids,TEST – total esters)

T a b l e 4

Correlation between the results of instrumental methods and the sensory evaluation (taste and odor) for Žižak wines

	TAL	TAC	TEST	Taste	Odor
TAL	1				
TAC	–0.020	1	*	*	
TEST	–0.486	0.725	1	*	*
Taste	–0.210	0.671	0.671	1	
Odor	–0.156	0.286	0.612	0.271	1

The stars represent statistically significant correlations at $p < 0.05$ (*) and $p < 0.01$ (**).
(TAL – total alcohols, TAC – total acids, TEST – total esters)

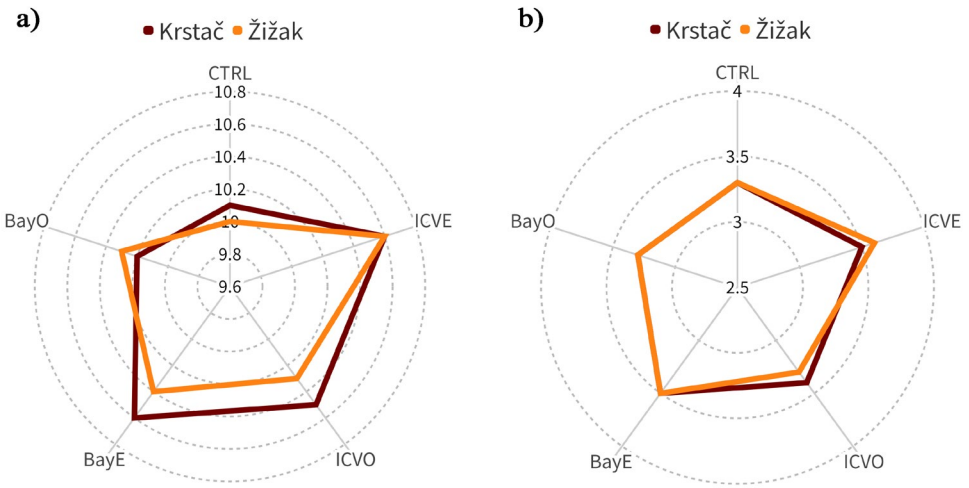


Fig. 2. Sensory evaluation for (a) taste and (b) odor for Krstač and Žižak wines

4. CONCLUSION

Krstač and Žižak wines obtained after spontaneous alcoholic fermentation had the highest concentrations of higher alcohols compared to treated wines (except Z_ICVO). In addition, the yeast nutrient Fermaid E had a greater influence on the production of higher alcohols. The concentration of hexanoic acid, octanoic acid, and decanoic acid was statistically significantly higher in the treated wines from Krstač and Žižak than in the spontaneously fermented wines. In addition, *S. bayanus* produced statistically significantly higher levels of hexanoic and octanoic acid than *S. cerevisiae* in almost all Krstač and Žižak wines. Yeasts and yeast nutrients significantly increased the content of fatty acid ethyl esters (ethyl hexanoate, ethyl octanoate, and ethyl decanoate) and acetates (2-phenylethyl acetate, isoamyl acetate, and 1,3-propanediol diacetate) in almost all wines produced from both varieties. When comparing the influence of different yeasts on the content of volatile compounds, *S. bayanus* with the yeast nutrient Fermaid E (BayE) produced more ethyl esters and acetates in Krstač and Žižak wines, while *S. cerevisiae* (ICVE) produced a higher content of alcohols in Krstač wine.

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