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The impact of maceration and fermentation protocol on the composition and sensory properties of cv. Trnjak (*Vitis vinifera* L.) wine

Utjecaj maceracije i fermentacijskog protokola na sastav i senzorna svojstva vina 'Trnjak'

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ABSTRACT

The present study investigates the impact of sequential fermentation with *Lachancea thermotolerans* and *Saccharomyces cerevisiae*, compared to *S. cerevisiae* alone and spontaneous fermentation, on the sensory and chemical properties of cv. Trnjak wine. Red grapes from the Dubrave position (Mostar wine-growing region, Bosnia and Herzegovina) were subjected to three fermentation treatments with two maceration lengths (8 and 12 days). The research examines how these factors influence aroma compounds and overall sensory characteristics. *Lachancea thermotolerans* is a valuable non-*Saccharomyces* yeast known for its ability to acidify wine, improve aroma complexity, and stabilize color. Its role is particularly important in warm-climate regions, where accelerated grape ripening reduces natural acidity, posing challenges for wine stability. This is particularly significant for Trnjak, a variety from the warm climates of Dalmatia and Herzegovina, where lower acidity and higher pH can affect wine quality. HS-SPME-Arrow-GC-MS analysis shows that sequential fermentation enhanced fruity, floral and lactic aromatic compounds (e.g., *β*-damascenone, linalool, ethyl lactate) while reducing higher alcohols and fatty acids. *L. thermotolerans* contributed to a refined volatile profile that improved sensory properties. The results show that *L. thermotolerans* and prolonged maceration contribute to a greater aromatic complexity and a more structured body of Trnjak red wine, which ultimately improves its balance and overall quality.

Keywords: descriptive sensory evaluation, GC-MS, *Lachancea thermotolerans*, *Saccharomyces cerevisiae*, spontaneous fermentation

SAŽETAK

Ovim istraživanjem ispituje se utjecaj sekvencijalne fermentacije s *Lachancea thermotolerans* i *Saccharomyces cerevisiae* kao i fermentacije sa *S. cerevisiae* u usporedbi sa spontanom fermentacijom, na senzorna i kemijska svojstva vina sorte ´Trnjak´. Crno grožđe s položaja Dubrave (vinogorje Mostar, Bosna i Hercegovina) podvrgnuto je trima fermentacijskim tretmanima s dvije duljine maceracije (8 i 12 dana). Istraživanje ispituje kako ti čimbenici utječu na aromatske spojeve i senzorne karakteristike. *Lachancea thermotolerans* je vrlo značajan ne-*Saccharomyces* kvasac, poznat po svojoj sposobnosti zakiseljavanja vina, poboljšanja složenosti arome i stabilizacije boje. Njegova uloga je posebno

važna u toplim klimatskim regijama, gdje ubrzano dozrijevanje grožđa smanjuje prirodnu kiselost, što predstavlja izazov za stabilnost vina. To je posebno značajno za ´Trnjak´, sortu iz toplih klimata Dalmacije i Hercegovine, gdje niža kiselost i viši pH mogu utjecati na kvalitetu vina. HS-SPME-Arrow-GC-MS analiza pokazuje kako je sekvencijalna fermentacija naglasila voćne, cvjetne i mliječne aromatske spojeve (npr. β-damaskenon, linalol, etil-laktat), a istovremeno smanjila koncentraciju viših alkohola i masnih kiselina. *L. thermotolerans* doprinijela je profinjenom aromatskom profilu koji je poboljšao senzorna svojstva. Rezultati pokazuju kako *L. thermotolerans* i produljena maceracija doprinose većoj aromatskoj složenosti i strukturi crnog vina ´Trnjak´, što u konačnici poboljšava njegovu ravnotežu i ukupnu kvalitetu.

Ključne riječi: deskriptivna senzorna analiza, GC-MS, *Lachancea thermotolerans*, *Saccharomyces cerevisiae*, spontana fermentacija

INTRODUCTION

Alcoholic fermentation is primarily carried out by *Saccharomyces cerevisiae* (*Sc*), but recent advances in enological biotechnology have highlighted the benefits of co-inoculation or sequential inoculation with non-*Saccharomyces* (non-*Sc*) yeasts (Pretorius, 2016). These innovative fermentation strategies have shown the potential to improve the freshness and typicity of wine compared to conventional single-strain fermentations. Although *Sc* remains the dominant species for alcoholic fermentation, non-*Sc* yeasts have gained attention in current research (Morata et al., 2019).

Over the past decade, the enological relevance of non-*Sc* yeasts has been re-evaluated in numerous studies (Ciani et al., 2016; Padilla et al., 2016; Binatti et al.; Jagatić Korenika et al., 2021) and their ability to improve wine quality through metabolic pathways that are not present in *Sc*. Among them, *Lachancea thermotolerans* (*Lt*) is particularly appreciated for its positive contribution to sensory characteristics (Capece and Romano, 2019).

One of the most remarkable properties of *Lt* is its ability to acidify must from grapes grown in warm wine-growing regions, where higher pH values can affect the stability of the wine. The primary metabolic contribution of *Lt* strains is the biosynthesis of lactic acid during alcoholic fermentation. Sequential fermentations with *Lt*+*Sc* lead to a decrease in pH and an increase in total acidity due to L-lactic acid synthesis (Jagatić Korenika et al., 2021). In addition, *Lt* contributes to a slight decrease in alcohol content (~0.7 % *v/v*) (Ciani et al., 2016; Blanco et al., 2020), making it valuable for moderating ethanol content in warmer climates (Comitini et al., 2011). The species proves to be resilient under stress, maintaining its viability for several days at 9 % *v/v* (Kapsopoulou et al., 2007; Gobbi et al., 2013) and even surviving in fermentations dominated by *Sc* (Mills et al., 2002). These properties make it important in the face of global climate change, which has led to a significant decrease in the acidity of wines (Comitini et al., 2011). The alcoholic strength of wine has become an increasingly important issue, as rising global temperatures accelerate sugar accumulation in grapes, which results in higher ethanol levels in finished wines (Jones et al., 2005; Mira de Orduña, 2010). Lower alcohol wines, however, are more marketable due to reduced taxation and better alignment with legal requirements (Mueller et al., 2011). At the same time, growing health awareness and shifting consumer preferences, particularly among younger demographics, are driving demand for wines with reduced alcohol (Saliba et al., 2013). This trend is reinforced by “value perception shift” in the global market, where authentic, high-quality wines with balanced profiles are preferred (Wine Australia. 2017). To meet these demands, winemakers are exploring new practices that can lower alcohol content without compromising quality (Varela et al., 2015; Gonzalez et al., 2021). Within this framework, our study on sequential fermentation with *Lachancea thermotolerans* in the indigenous variety wines highlights its potential to reduce ethanol levels while enhancing freshness, competitiveness, and consumer acceptance.

Yeasts also influence wine color stability by lowering the pH and promoting the formation of stable pigment derivates, such as pyranoanthocyanins (Morata et al., 2006; 2007) and polymeric pigments (Escott, 2018). The lactic acid produced during fermentation can be lowered by 0.3-0.5 units, which further stabilizes the wine color (Morata et al., 2019).

Non-*Sc* yeasts are also valuable for their ability to enhance wine aroma. They produce a variety of fermentation-derived compounds, such as higher alcohols, esters, and fatty acids, in different proportions than *Sc* (Vejarano and Gil-Calderón, 2021). Sequential fermentation with *Lt* has been shown to increase concentrations of fruity and floral volatile compounds, including terpenes, norisoprenoids, and esters, and contribute to greater aromatic complexity (Tufariello et al., 2021).

Maceration time represents another critical factor influencing red wine composition and sensory properties. Maceration parameters, such as temperature and duration, have a significant influence on the extraction of polyphenols, astringency, and aroma development (Budić-Leto et al., 2008).

Trnjak (syn. Trnak, Rudežuša), an autochthonous grape variety originating from the Imotski and western Herzegovina regions, was classified as "near threatened" in 2018. Nevertheless, it has experienced a resurgence, with over 50,000 vines planted by 2019. Trnjak is characterized by low yields, a high sugar content (20–26%), moderate acidity (4.5–6 g/L), and high extract and polyphenols concentrations, producing full-bodied wines with a deep ruby red color and ripe black fruit aromas (Mirošević and Turković, 2003; Sokolić, 2006; Maletić et al., 2015). Despite its high enological potential, Trnjak is still little explored in scientific research.

The objective of this study is to evaluate the impact of *Lt* in sequential fermentation with *Sc* on the physicochemical, aromatic, and sensory properties of Trnjak wine. The study further investigates how different fermentation strategies and maceration durations influence key enological parameters, volatile composition, and overall wine quality.

MATERIALS AND METHODS

Sample preparation

Grapes of *cv.* Trnjak from the Dubrave site (Mostar wine region, Bosnia and Herzegovina), harvested in 2019, were used in this study. The Dubrava vineyard, located at an altitude of 200–210 m, spans 50 ha. Between 2006 and 2010, 13,400 vines were planted, which remain in production today. The soil is rigosol (vitisol subtype), derived from leached red soils of limestone calcarenites. The vines, grafted onto *Vitis riparia* × *Vitis rupestris* Schwarzmann rootstock, are planted at a spacing of 2.2×0.85 m.

A total of 300 kg of grapes were processed, homogenized, and divided into six 60 L plastic containers, then sulfited with 5% H_2SO_3. The sugar content in the must was measured at 98 °Oe, with a pH of 3.65 and a total acidity of 5.6 g/L. Maceration lasted eight days for three fermentations and 12 days for the other three, with regular punch-downs every 12 hours. The temperature of the environment was around 20 °C, without exposure to natural light. Treatment K (control) underwent spontaneous fermentation and was sulfited with 50 mL of 5% H_2SO_3. Treatment *Lt+Sc* was sulfited with 10 mL of 5% H_2SO_3 and inoculated with *Lt* yeast (15 g/50 L), followed by *S. cerevisiae* (15 g/50 L) after 24 hours. Treatment *Sc* was sulfited with 50 mL of 5% H_2SO_3 and inoculated with *Sc* (15 g/50 L). Alcoholic fermentation was subjected to natural fluctuations.

After eight days of maceration and alcoholic fermentation, the must from the three treatments was pressed using a hydraulic press, and the fermenting must was transferred into three 10 L glass containers per treatment (in duplicate) with airlocks, allowing fermentation to continue. The same procedure was repeated for the remaining three treatments after 12 days of maceration. Following fermentation, the wines were decanted, and sulfite levels were adjusted with 50 mL of 5% H_2SO_3. The second decantation of the wines took place after five months, when the samples were bottled in 750 mL bottles, with the SO_2 correction of 0.5 ml 5% - H_2SO_3 per bottle.

Table 1. Fermentation and maceration protocols in the present study

K	*Lt+Sc*	*Sc*
Epiphytic yeast (spontaneous fermentation); maceration 8 and 12 days	Sequential alcoholic fermentation with the yeast strains *Lachancea thermotolerans* (Laktia®, Lallemand, Montreal, QC,Canada) + *Saccharomyces cerevisiae* (Uvaferm BDX®, Lallemand,Montreal, QC, Canada); maceration 8 and 12 days	*Saccharomyces cerevisiae* (Fermol Premier Cru®, AEB, Brescia, Italy); maceration 8 and 12 days

Physicochemical analysis

Basic wine parameters, including alcohol content (%, *v/v*), pH value, dry and reduced extract, reducing sugars, ash, total and volatile acidity, were quantified applying methods recommended by the International Organization of Vine and Wine (OIV, 2016).

HS-SPME-Arrow-GC-MS analysis of volatile compounds

The analysis of volatile compounds in wine samples was determined as previously described by Tomaz et al. (2024), using the RSH Triplus autosampler (Thermo Fisher Scientific Inc., Brookfield, WI, USA). Sample analysis was conducted on a TRACE™ 1300 Series gas chromatographer coupled to an ISQ 7000 TriPlus quadrupole mass spectrometer (Thermo Fisher Scientific Inc., USA) equipped with a TG-WAXMS A capillary column (60 m × 0.25 mm × 0.25 µm film thickness; Thermo Fisher Scientific, USA).

Sensory Analysis

Sensory analysis of red wines was carried out by five experts (two females and three males), who are members of the Committee for Organoleptic Evaluation of Wine and Fruit Wines appointed by the Ministry of Agriculture. The panellists were experts in this field and had extensive experience with the evaluations in the Croatian Agency for Agriculture and Food, accredited according to the HRN EN ISO/IEC 17065 standard for the implementation of the procedure for placing PDO wines on the market. The evaluation was approved and carried out in a Laboratory for Sensory Analysis of Agricultural and Food Products, University of Zagreb Faculty of Agriculture, under standardized conditions. Thirty millilitres (30 mL) of wine samples were served at 15 °C in coded wine glasses. Blind tasting using Quantitative Descriptive Analysis (QDA) of the coded wines was performed randomly on three replicates, during three evaluation sessions. A total of 14 wine attributes for taste and odor (Figures 1 and 2) were selected by the research group and further developed and evaluated by the panellists. The panel evaluated five referent monovarietal Trnjak 2019 wines to achieve a consensus about the attributes describing the wine's sensory profiles. The additional training of the panel before the formal evaluation included assessing wine aroma using the aqueous solutions of different selected compounds and an "Aromaster" kit (Vinofil Co., Ltd., Hong Kong) that includes 88 typical wine aromas in vials. Quantification was performed using a six-point scale, on a paper sheet, as follows: 0–1 weak, 2–3 medium, and 4–5 strongly intensive attribute. Sample differences were graphically presented using radar charts.

Statistical Analysis

Chemical composition differences between the wine treatments (maceration x yeast) were tested using a two-way analysis of variance (ANOVA). Sensory properties differences were tested using a one-way analysis of variance (ANOVA). Comparison of mean values was performed by using Duncan's multiple range and Tukey *post-hoc* test with $P<0.05$. Principal component analysis (PCA) was used to analyze the overall variability of the content of aromatic compound groups, basic and sensory parameters in the analyzed wine samples. Statistical data processing was performed by using the computer program XLSTAT 2022.3.2. (Addinsoft - Lumivero, Paris, France).

RESULTS AND DISCUSSION

Physicochemical Parameters

The results of basic enological analysis of wines are presented in Table 2. This study revealed significant differences among treatments, especially after 12 days of maceration. Compared to K, the wines produced with *Lt+Sc* had a lower alcohol content, indicating the significant role of *Lt* in reducing ethanol levels. These findings are aligned with previous studies in which *Lt* strains were considered to have the greatest potential to reduce ethanol content (Binati et al., 2020). According to Gobbi et al. (2013), the use of *Lt* in sequential fermentation with *Sc* significantly reduced the ethanol concentration by 0.7 to 0.9% *v/v*. The lower alcohol content can be attributed to the synthesis of lactic acid, which is found in higher concentration in *Lt+Sc* wines, which is consistent with the results of Dutraive et al. (2019).

Lt also significantly influenced total acidity, with concentrations ranging from 5.10 g/L in the *Sc* treatment to 6.90 g/L in the *Lt+Sc* treatment, alongside a notable reduction in pH. These results are consistent with previous studies (Morata et al., 2019; Comitini et al., 2011; Ivić et al., 2024). This contributes to the stability of the wine, which is particularly important for wines from southern vineyards with lower total acidity and higher pH values. The research by Dutraive et al. (2019) has shown that inoculation of *Lt* with *Sc* results in a greater pH reduction compared to inoculation with other non-*Sc* yeasts. The total dry extract and ash content, as well as the almost equal amounts of reducing sugars, support the findings of Dutraive et al. (2019) that *Lt* inoculated with *Sc* can complete fermentation (Ciani et al., 2016; Kapsopoulou et al., 2005). It is noteworthy that the total dry extract concentrations were significantly higher in the *Lt+Sc* treatments, which is consistent with the results of Ivić et al. (2024).

Table 2. Physicochemical parameters of Trnjak red wines, vintage 2019

Compound	Maceration length (days)	K	*Lt+Sc*	*Sc*
Alcohol (% *v/v*)	8	11.80^{c}	11.70^{d}	11.24^{e}
	12	12.17^{B}	11.71^{D}	12.54^{A}
Extract (g/L)	8	23.73^{c}	29.10^{a}	25.60^{b}
	12	28.06^{B}	29.43^{A}	29.60^{A}
Reducing sugar (g/L)	8	1.03^{a}	1.03^{a}	1.10^{a}
	12	1.40^{A}	1.10^{C}	1.30^{B}
Ash (g/L)	8	3.41^{b}	3.88^{a}	3.82^{a}
	12	3.82^{B}	3.85^{B}	4.61^{A}
Total acidity, as tartaric acid (g/L)	8	4.82^{b}	6.90^{a}	4.25 c
	12	5.52^{C}	6.47^{A}	5.10^{CD}
Volatile acidity, as acetic acid (g/L)	8	0.37^{a}	0.35^{a}	0.32^{a}
	12	0.32^{A}	0.29^{A}	0.35^{A}
pH	8	3.86^{b}	3.76^{a}	4.02^{a}
	12	3.86^{b}	3.77^{C}	4.02^{A}

Concentrations are expressed as mean values (n = 3). Different letters in the rows indicate statistically significant differences between treatments at the $P < 0.05$ significance level, separately for two maceration lengths (two-way ANOVA and Duncan's multiple range test). Different letters in the columns represent statistically significant differences between macerations of the same treatment at the significance level of $P<0.05$. K-spontaneous fermentation, *Lt - Lachancea thermotolerans*, *Sc - Saccharomyces cerevisiae*

Maceration duration significantly impacted ethanol levels, with variations observed across treatments. In K and *Sc* treatments, extended maceration was associated with increased ethanol content. These findings are consistent with research by Jagatić Korenika (2023), where prolonged maceration led to higher alcohol content. However, the relationship between ethanol concentration and maceration time is not always linear. Casassa et al. (2013) reported that ethanol concentration differences of approximately 1.2% *v/v* had no significant effect on tannin and anthocyanin extraction, suggesting that maceration duration itself, rather than ethanol concentration alone, plays a more dominant role in shaping wine composition. Additionally, Rossi et al. (2024) found that Teran wines subjected to a 21-day maceration exhibited higher alcohol concentrations. Interestingly, this study demonstrated that *Lt+Sc* resulted in lower ethanol levels despite extended maceration. This result emphasizes the ability of *Lt* to modulate ethanol concentration by converting part of the fermentable sugars into lactic acid instead of ethanol, as previously reported by Binati et al. (2020) and Gobbi et al. (2013). These results suggest that while extended maceration typically increases ethanol concentration in traditional *Sc* fermentations, the presence of *Lt* can counteract this effect. This highlights its potential as a strategic tool to reduce alcohol content while maintaining the beneficial impact of prolonged skin contact on phenol extraction.

Volatile aromatic compounds in wine

The analysis of 90 volatile aroma compounds in Trnjak wines (Table 3) revealed significant differences in the volatile profiles between wines fermented solely with *Sc* and sequential fermentation. The impact of yeast selection and maceration length on volatile composition is well documented, as non-*Sc* yeasts enhance aromatic complexity by modulating the synthesis of key volatile compounds (Gobbi et al., 2013; Balikci et al., 2016; Ivić et al., 2024).

Higher alcohols are a diverse class of compounds derived from yeast metabolism that contribute both positive and negative sensory attributes to wine (Petropulos et al., 2014). In this study, total alcohol concentrations showed no statistically significant differences between treatments. This is consistent with the findings of Petropulos et al. (2014), who reported that the alcohol content in Vranac wines initially increased with maceration before stabilizing at the level of the control wines. A similar trend was observed in Chenin Blanc wines (Wang et al., 2016). The concentration of 2-ethyl-1-hexanol was significantly higher in extended maceration treatments, supporting the findings of Martínez-Moreno et al. (2024). In contrast, higher alcohols such as 1-decanol, 1-octanol, and 4-methyl-1-pentanol were significantly lower in the sequential fermentation treatments, supporting previous studies (Jagatić Korenika et al., 2021; Ivić et al., 2024). The concentration of isoamyl alcohol remained unchanged between treatments, which differs from previous findings (Jagatić Korenika et al., 2021). However, the content of 1-decanol was significantly reduced in *Lt* sequential fermentation, confirming previous reports of a moderate decrease in this compound (Gobbi et al., 2013; Ivić et al., 2024). Similarly, Martínez-Moreno et al. (2024) reported lower 1-hexanol concentrations with prolonged maceration, which was not observed in this study.

Esters contribute significantly to the fruity and floral aromas of wine. In this study, the total ester concentrations in control wines were significantly higher. Most abundant esters in the control treatment were isoamyl acetate and ethyl hexanoate, which contrasts with findings of Gobbi et al. (2013) and Jagatić Korenika et al. (2021), where commercial *Sc* strains produced these elevated levels. The exception was ester hexyl acetate, that was detected in *Sc* treatment, which agrees with previous studies. Previous research has shown that extended maceration can decrease ester concentrations (Petropulos et al., 2014; Frost et al., 2018; Lukić et al., 2015), while others observed varying trends (Prezioso et al., 2024; Wang et al., 2016). Our results indicate a decrease in ester concentrations in extended maceration wines.

Table 3. Volatile aromatic compounds in Trnjak red wines, vintage 2019

Compounds (µg/L)	Maceration length (days)	Treatment		
		K	*Lt+Sc*	*Sc*
2-Nonanol	8	13.19^{b}	21.04^{a}	16.72^{ab}
	12	17.26^{AB}	19.27^{AB}	15.97^{AB}
trans-2-Octen-1-ol	8	33.73^{c}	32.83^{c}	38.27^{b}
	12	39.00^{B}	33.35^{C}	45.09^{A}
2-Pentanol	8	933.94^{ab}	891.62^{b}	885.48^{b}
	12	985.14^{AB}	1013.75^{AB}	1099.80^{A}
3-Ethyl-4-methylpentan-1-ol	8	2617.75^{b}	2952.61^{ab}	3108.93^{ab}
	12	3624.55^{A}	3304.33^{AB}	3393.39^{AB}
trans-3-Hexen-1-ol	8	126.45^{d}	182.25^{cd}	302.95^{b}
	12	268.35^{BC}	448.40^{A}	460.09^{A}
cis-3-Hexen-1-ol	8	273.36^{d}	1323.51^{b}	1079.32^{c}
	12	1622.51^{A}	1136.09^{C}	1255.16^{BC}
3-Methylpentan-1-ol	8	4921.50^{b}	3522.22^{b}	3473.33^{b}
	12	7537.11^{A}	3183.31^{B}	5392.82^{B}
3-Octanol	8	33.19^{a}	34.43^{a}	32.07^{a}
	12	30.87^{A}	33.94^{A}	31.44^{A}
4-Vinylguaiacol	8	19.11^{a}	16.37^{a}	13.97^{a}
	12	20.31^{A}	15.01^{A}	17.45^{A}
Benzyl alcohol	8	288.85^{bcd}	313.51^{b}	513.21^{a}
	12	228.39^{CD}	306.47^{BC}	205.61^{D}
Eugenol	8	63.90 ab	60.48^{b}	72.48^{a}
	12	71.55^{A}	66.71^{AB}	66.17^{AB}
Isobutanol	8	2030.61^{c}	6430.62^{a}	3596.07^{b}
	12	2544.28^{C}	2130.09^{C}	2325.32^{C}
Phenylethyl alcohol	8	4198.28^{ab}	4264.35^{ab}	3938.90^{b}
	12	4404.76^{A}	4085.87^{AB}	3942.47^{B}
trans-6-Nonen-1-ol	8	11.41^{a}	10.25^{a}	11.31^{a}
	12	12.00^{A}	11.80^{A}	10.70^{A}
Isoamyl alcohol	8	56166.633^{a}	55604.90^{a}	38140.27^{a}
	12	58300.960^{A}	45520.24^{A}	54224.30^{A}

Continued. Table 3

Compounds (µg/L)	Maceration length (days)	Treatment		
		K	*Lt+Sc*	*Sc*
1-Decanol	8	4688.033^{ab}	3195.78^{b}	6931.26^{a}
	12	5725.46^{AB}	3512.94^{AB}	3535.80^{AB}
1-Hexanol	8	9237.546^{a}	11553.28^{a}	7987.74^{a}
	12	10060.38^{A}	10543.73^{A}	7973.04^{A}
2-Ethyl-1-hexanol	8	nd.	nd.	nd.
	12	38.71^{A}	36.71^{A}	37.48^{A}
1-Nonanol	8	24.07^{a}	23.34^{a}	23.22^{a}
	12	28.08^{A}	27.62^{A}	27.08^{A}
1-Octanol	8	82.82^{a}	46.17^{c}	89.63^{a}
	12	82.36^{A}	58.83^{C}	72.18^{B}
1-Octen-3-ol	8	43.26^{b}	42.71^{b}	43.73^{b}
	12	42.78^{B}	38.73^{B}	59.17^{A}
1-Pentanol	8	1392.01^{b}	2020.21^{a}	1725.40^{ab}
	12	1728.44^{AB}	1556.09^{AB}	1722.73^{AB}
4-Methyl-1-pentanol	8	293.71^{c}	157.91^{e}	231.35^{d}
	12	650.87^{A}	150.31^{E}	410.75^{B}
1-Propanol	8	50.07^{e}	240.77^{d}	443.17^{c}
	12	635.47^{B}	1776.19^{A}	335.68^{CD}
3-Ethoxy-1-propanol	8	1485.83^{c}	3291.85^{b}	1565.94^{c}
	12	976.46^{C}	8275.90^{A}	1658.64^{C}
2,3-Butanediol	8	328.88^{a}	296.24^{a}	424.86^{a}
	12	491.03^{A}	462.65^{A}	515.80^{A}
2-Heptanol	8	2.60^{c}	3.10^{c}	10.70^{a}
	12	5.33^{B}	4.74^{B}	6.11^{B}
∑ Higher alcohols	8	89360.80^{a}	96532.46^{a}	74700.34^{a}
	12	100171.38^{A}	87740.17^{A}	88840.34^{A}
Isoamyl acetate	8	1031.31^{a}	139.86^{b}	520.80^{b}
	12	1020.07^{A}	1279.04^{A}	1405.50^{A}
Diethyl malate	8	56.70^{b}	70.74^{a}	53.28^{b}
	12	54.05^{B}	55.51^{B}	51.24^{B}
2-Phenylethyl acetate	8	235.63^{a}	187^{b}	163.53^{b}
	12	219.50^{A}	156.84^{B}	161.18^{B}

Continued. Table 3

Compounds (µg/L)	Maceration length (days)	Treatment		
		K	*Lt+Sc*	*Sc*
Hexyl acetate	8	nd.	nd.	65.07^{b}
	12	72.66^{A}	nd.	69.64^{A}
Diethyl succinate	8	4321.50^{a}	2566.62^{b}	nd.
	12	nd.	nd.	nd.
Ethyl decanoate	8	15.05^{b}	7.50^{c}	19.83^{a}
	12	16.60^{AB}	9.21^{C}	6.33^{C}
Ethyl lactate	8	635.18^{b}	554.60^{bc}	466.93^{c}
	12	346.30^{D}	838.92^{A}	473.92^{C}
Ethyl 4-hydroxybutanoate	8	130.12^{c}	306.80^{b}	295.21^{b}
	12	243.05^{B}	394.95^{A}	254.87^{B}
Ethyl 9-decenoate	8	1236.81^{ab}	126.51^{b}	127.50^{a}
	12	127.35^{AB}	126.77^{AB}	126.80^{AB}
Ethyl 9-hexadecenoate	8	4.76^{a}	2.32^{b}	5.20^{a}
	12	5.98^{A}	2.07^{B}	2.81^{B}
Ethyl hydrogen succinate	8	801.05^{b}	431.36^{cd}	1668.88^{a}
	12	675.40^{BC}	555.88^{BCD}	350.60^{D}
Ethyl heptanoate	8	20.90^{b}	18.97^{b}	37.50^{a}
	12	25.97^{B}	16.73^{B}	18.74^{B}
Ethyl hexanoate	8	1215.91^{a}	193.80^{c}	738.76^{b}
	12	175.87^{C}	122.26^{CD}	30.60^{D}
Isoamyl lactate	8	nd.	430.30^{a}	317.11^{b}
	12	nd.	nd.	nd.
Ethyl nonanoate	8	nd.	nd.	nd.
	12	nd.	nd.	50^{A}
Methyl octanoate	8	nd.	nd.	nd.
	12	15.71^{A}	16.18^{A}	15.04^{A}
Octyl octanoate	8	1.27^{ab}	1.03^{b}	1.78^{a}
	12	1.05^{B}	0.92^{B}	1.30^{AB}
Isoamyl decanoate	8	103.90^{b}	91.98^{b}	152.36^{a}
	12	120.64^{AB}	79.53^{BC}	41.64^{C}

Continued. Table 3

Compounds (µg/L)	Maceration length (days)	Treatment		
		K	*Lt+Sc*	*Sc*
Ethyl butyl succinate	8	129.34^{a}	120.66^{a}	46.78^{b}
	12	126.42^{A}	140^{A}	157.70^{A}
∑ Esters	8	9463.74^{a}	5435.27^{b}	5340.38^{b}
	12	3818.24^{C}	4062.57^{C}	3595.50^{C}
3-Methylbutanoic acid	8	2055.27^{c}	1884.90^{c}	3918.09^{b}
	12	3053.84^{BC}	2057.93^{C}	6196.66^{A}
Butanoic acid	8	350.70^{d}	377.38^{d}	622.05^{bc}
	12	570.12^{C}	684.60^{AB}	777.90^{A}
Decanoic acid	8	204.47^{a}	60.61^{b}	80.48^{b}
	12	89.30^{B}	50.36^{B}	46.33^{B}
Dodecanoic acid	8	18.84^{a}	16.87^{abc}	16.95^{abc}
	12	18.17^{AB}	16.46^{BC}	15.75^{C}
Hexanoic acid	8	6454.26^{a}	2945.50^{c}	6431.22^{a}
	12	6509.77^{A}	4100.58^{BC}	5001.51^{AB}
2-Ethylhexanoic acid	8	216.66^{ab}	167.28^{bc}	203.75^{b}
	12	270.25^{A}	133.05^{C}	106.58^{C}
Nonanoic acid	8	20.36^{b}	20.58^{b}	31.24^{a}
	12	23.94^{B}	21.07^{B}	9.91^{C}
Octanoic acid	8	2603.45^{a}	657.32^{c}	1832.22^{b}
	12	1721.95^{B}	788.57^{C}	751.15^{C}
Isobutanoic acid	8	2627.00^{c}	2592.24^{c}	1300.96^{d}
	12	2674.63^{C}	6173.74^{A}	4592.56^{B}
trans-2-Undecenoic acid	8	12.07^{b}	13.50^{ab}	13.66^{ab}
	12	13.02^{AB}	20.82^{A}	15.19^{AB}
∑ Fatty acids	8	14563.11^{a}	8736.19^{b}	14452.64^{a}
	12	14944.02^{A}	14047.21^{A}	17513.57^{A}
Methionol	8	37.24^{ab}	44.75^{ab}	25.05^{b}
	12	34.52^{AB}	50.38^{A}	30.44^{AB}
trans-Allocimene	8	34.30^{b}	63.56^{a}	34.70^{b}
	12	24.97^{C}	35.00^{B}	12.27^{D}

Continued. Table 3

Compounds (µg/L)	Maceration length (days)	Treatment		
		K	*Lt+Sc*	*Sc*
Citronellol acetate	8	11.58^{b}	16.70^{a}	16.54^{a}
	12	13.13^{AB}	13.84^{AB}	12.22^{B}
cis-*α*-Bisabolene	8	5.31^{b}	6.07^{a}	5.58^{b}
	12	5.53^{B}	6.28^{A}	5.35^{B}
Citronellol	8	266.75^{d}	280.32^{cd}	535.17^{a}
	12	384.14^{B}	248.25^{D}	338.84^{BC}
Limonene	8	27.00^{b}	24.30^{b}	4.50^{c}
	12	38.94^{A}	37.22^{A}	ND.
Geranylacetone	8	8.70^{b}	6.75^{c}	10.67^{a}
	12	8.70^{B}	8.16^{B}	7.65^{BC}
Hotrienol	8	8.34^{b}	11.82^{ab}	9.95^{b}
	12	11.22^{AB}	10.72^{AB}	16.25^{A}
Linalool	8	5.74^{c}	19.01^{a}	11.87^{b}
	12	9.30^{BC}	14.31^{AB}	10.46^{BC}
Menthol	8	6.64^{ab}	5.80^{b}	8.17^{a}
	12	6.70^{AB}	6.52^{AB}	7.65^{AB}
p-Cymene	8	1.53^{bc}	3.50^{a}	1.94^{b}
	12	3.60^{A}	0.64^{C}	1.54^{BC}
trans-Linalool oxide (furanoid)	8	14.04^{bc}	13.52^{c}	13.18^{c}
	12	20.40^{AB}	26.03^{A}	7.10^{C}
α-Terpineol	8	8.81^{d}	8.47^{d}	9.51^{cd}
	12	11.20^{BC}	15.34^{A}	12.12^{B}
β-Myrcene	8	51.92^{ab}	49.71^{ab}	60.80^{a}
	12	47.55^{B}	54.16^{AB}	36.20^{C}
∑ Terpenes	8	487.95^{d}	554.33^{c}	746.65^{a}
	12	620.00^{D}	526.86^{CD}	498.13^{CD}
2,5,8-Trimethyl-1,2,3,4-tetrahydro-1-naphthol	8	6.53^{b}	8.30^{b}	12.11^{a}
	12	10.52^{A}	7.15^{B}	8.38^{B}
β-Damascenone	8	5.27^{b}	6.23^{a}	5.90^{ab}
	12	5.91^{AB}	6.20^{A}	5.77^{AB}

Continued. Table 3

Compounds (µg/L)	Maceration length (days)	Treatment		
		K	*Lt+Sc*	*Sc*
2,5,8-Trimethyl-1,2-dihydronaphthalene	8	5.70^{a}	6.08^{a}	nd.
	12	ND.	ND.	ND.
TDN	8	6.78^{a}	6.74 a	7.05^{a}
	12	6.48^{A}	6.72^{A}	6.81^{A}
TPB	8	6.31^{a}	5.62^{a}	5.67^{a}
	12	6.07^{A}	6.85^{A}	5.50^{A}
Vitispiran A	8	8.34^{bc}	15.58^{a}	8.13^{bc}
	12	9.33^{BC}	10.50^{B}	6.42^{C}
Vitispiran B	8	7.08^{c}	26.15^{a}	8.78^{c}
	12	8.52^{C}	15.32^{B}	6.40^{C}
∑ C13-Norisoprenoids	8	46.02^{bc}	74.71	47.65^{bc}
	12	46.85^{BC}	52.76^{B}	39.27^{C}
cis-Whiskey lactone	8	74.02^{cd}	84.65^{bc}	61.13^{cd}
	12	117.22^{B}	225.80^{A}	42.71^{D}
γ-Nonalactone	8	206.72^{c}	1498.93^{a}	179.26^{c}
	12	207.58^{C}	254.50^{B}	128.93^{C}
Butyrolactone	8	38.58^{b}	33.01^{b}	116.06^{b}
	12	371.46^{A}	427.08^{A}	409.32^{A}
∑ Lactones	8	319.32^{d}	1616.60^{a}	356.46^{d}
	12	696.26^{C}	1177.40^{B}	580.97^{CD}
Benzaldehyde	8	493.01^{b}	551.06^{a}	577.10^{a}
	12	437.54^{B}	440.10^{B}	429.75^{B}
Furfural	8	284.15^{a}	149.60^{b}	272.26^{a}
	12	201.34^{B}	139.90^{B}	303.78^{A}
∑ Other compounds	8	777.16^{ab}	700.65^{bc}	849.37^{a}
	12	638.88^{CD}	580.00^{D}	733.54^{BC}

Concentrations are expressed as mean values (n = 3). Different letters in the rows indicate statistically significant differences between treatments at the $P<0.05$ significance level, separately for two maceration lengths (two-way ANOVA and Duncan's multiple range test). Different letters in the columns represent statistically significant differences between macerations of the same treatment at the significance level of $P<0.05$. K – spontaneous fermentation, *Lt.* – *Lachancea thermotolerans*, *Sc.* – *Saccharomyces cerevisiae*; n.d. – not detected.

Furthermore, ethyl hexanoate and ethyl octanoate were significantly lower in shorter maceration treatments, a trend also observed in Monastrell wines (Martínez-Moreno et al., 2024). Sequential fermentation resulted in lower ester concentrations overall, aligning with previous findings by Jagatić Korenika et al. (2021).

However, ethyl lactate concentrations were significantly higher in *Lt+Sc* wines, consistent with previous studies (Ivić et al., 2023; Jagatić Korenika et al., 2021; Delač Salopek et al., 2022; Vaquero et al., 2020). According to Morata et al. (2019), ethyl lactate occurs in higher concentration in *Lt* fermentations. The higher ethyl lactate concentration is a direct consequence of the high lactic acid production of most *Lt* strains and agrees with previous investigations (Morata et al., 2019; Petitgonnet et al., 2019).

Fatty acid composition in wine is influenced by grape composition and fermentation conditions, with some fatty acids contributing undesirable sensory properties. In this study, *Lt+Sc* significantly reduced the hexanoic and octanoic acids concentrations, consistent with findings by Jagatić Korenika et al. (2021). Prolonged maceration also had a notable impact on the fatty acid content and resulted in significantly lower concentrations of octanoic and isobutyric acids, supporting the findings of Martínez-Moreno et al. (2024).

Terpenes and C13-norisoprenoids contribute to varietal aromas by imparting floral and fruity notes. Among the detected terpenes, linalool concentrations were significantly higher in *Lt+Sc*. Conversely, citronellol levels were lower in *Lt+Sc*, and most abundant in *Sc* treatments, which agrees with Jagatić-Korenika et al. (2021). Extended maceration did not significantly impact terpene concentrations overall, but certain compounds such as *trans*-aloocimen, citronellol, geranyl acetone, and linalool were significantly lower in extended maceration treatments, aligning with previous findings in *Verdicchio* wines (Prezioso et al., 2024) and previous reports of terpene reductions with prolonged maceration (Lukić et al., 2015). While Jagatić Korenika et al. (2021) reported that *Lt* had no effect on total C13-norisoprenoid concentrations across different grape varieties, our study found significantly higher levels in *Lt+Sc* wines. β-Damascenone, a key compound associated with fruity aromas, was significantly more abundant in *Lt* fermentations, in agreement with Ivić et al. (2024), Delač Salopek et al. (2022), and Jagatić Korenika et al. (2021).

Lactones contribute to varietal aroma (Ribéreau-Gayon et al., 2006), with γ-nonalactone being the most abundant lactone in this study (128.93 – 1498.00 µL). The highest concentration was observed in *Lt+Sc*, in contrast to Jagatić Korenika et al. (2021), who reported higher γ-nonalactone concentrations in control treatments. Similarly, Ivić et al. (2024) found no significant differences in lactone concentrations, while this study showed that total lactone content was highest in *Lt+Sc*.

These results confirm that *Lt+Sc* significantly influenced the volatile aroma composition, decreasing the concentrations of higher alcohols and fatty acids, while desirable compounds such as β-Damascenone and ethyl lactate increased. This resulted in a more fruit-forward and balanced aromatic profile. Extended maceration influenced ester and terpene concentrations, leading to a specific reduction in key aroma compounds while also interacting with yeast metabolism to alter lactone composition. These results confirm the role of *Lt* in increasing the aromatic complexity and improving the balance of the wine, making it a promising strategy for the management of sensory characteristics.

Principal Component Analysis (PCA) was conducted to explore the relationships between aromatic compound groups and basic quality parameters of Trnjak red wine samples, subjected to different fermentation and maceration protocols. The PCA biplot (Figure 1) displays two principal components: PC1 (F1) and PC2 (F2) that together explain 67.44% of the overall variance. PC1 (F1) differentiates variables based on their overall intensity and character. On the positive side of F1, variables such as alcohol, reducing sugar, higher alcohols, dry extract, total acidity, and C13-norisoprenoids are prominent and positively associated with *Lt+Sc* samples.

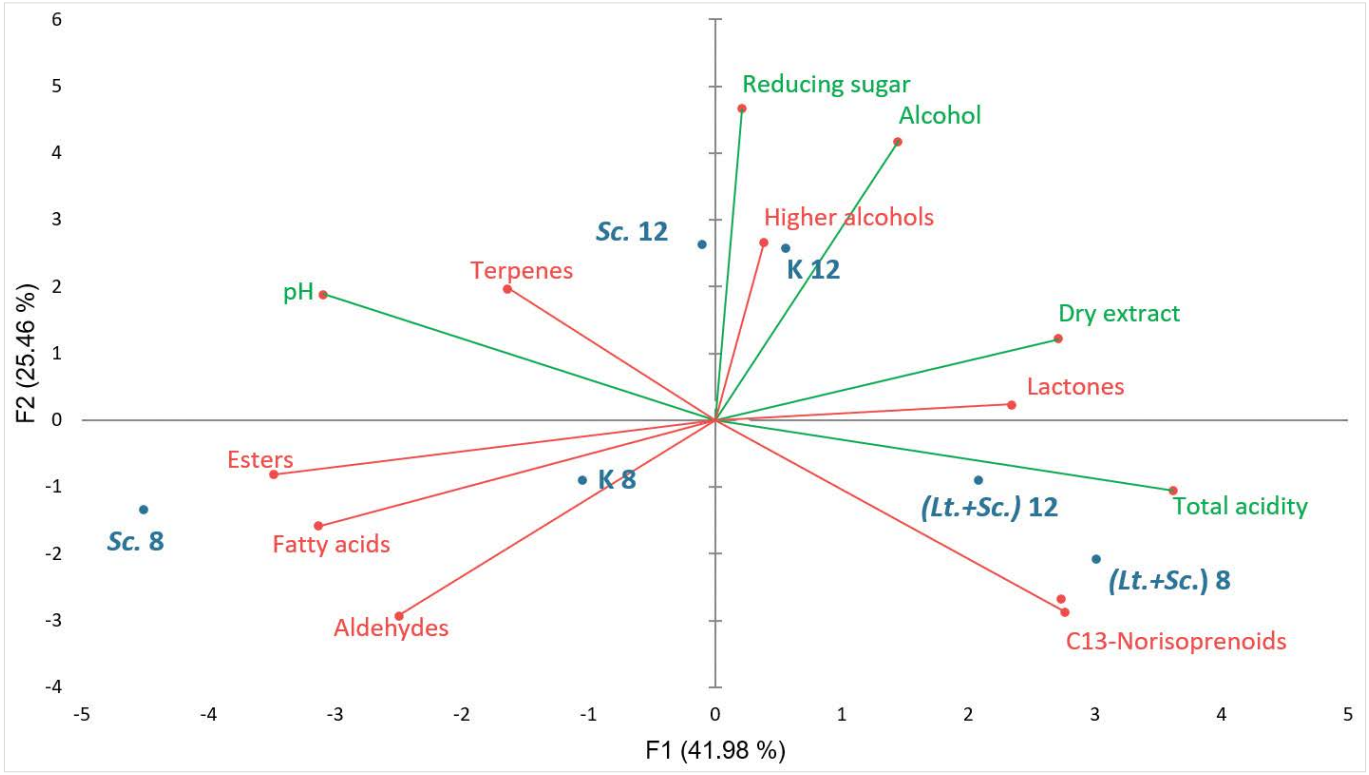

Figure 1. Principal component analysis (PCA) - aromatic compound groups and basic quality parameters of Trnjak red wine, vintage 2019

Conversely, the negative F1 axis is characterized by compounds like esters, aldehydes, fatty acids, and terpenes, which are more closely associated with the 8-day *Sc* and spontaneous fermentation samples. PC2 (F2) helps to distinguish the samples by subtler differences. It separates variables such as pH and lactones. The 12-day *Sc* and 12-day spontaneous treatments show strong positive scores on this axis. The PCA analysis illustrates how fermentation and maceration strategy significantly influenced the aromatic and compositional profile of Trnjak wine.

Sensory analysis

The PCA biplot (Figure 2), which explained 71.26% of the total variance, presents clear differentiation among the Trnjak 2019 red wines according to fermentation treatment and maceration time. The spontaneous fermentation treatment (K8) was distinctly separated along the negative F1 axis in the direction of dried fruit, consistent with its high concentration of diethyl succinate (oxidized and overripe fruit aromas).

The *Sc*.8 and (*Lt*+*Sc*.)8 variants were positioned in the upper quadrant, closer to the vegetal vector, reflecting their higher contents of C6 alcohols (*cis*- and *trans*-3-hexen-1-ol, 1-hexanol), which are responsible for grassy and leafy notes (Zhao et al., 2017). In the case of (*Lt*+*Sc*.)8, the elevated presence of 3-ethoxy-1-propanol (black pepper, black currant) may also explain the more pronounced spicy–herbal nuances (Mores-Arrocha et al., 2018). Similar findings were reported by Ivić et al. (2024), where this compound was also identified in the *Lt*+*Sc* sequential fermentation.

All wines produced with longer maceration (K12, *Lt*+*Sc*.12, *Sc*.12) have grouped on the positive F1 axis in association with body-fullness, taste quality, nutty and floral attributes, as well as bitterness and astringency. These sensory properties are supported by the chemical composition, as extended maceration increased the extraction of phenolic compounds (hydroxycinnamic and hydroxybenzoic acids, tannins) and anthocyanins (data not shown), resulting in fuller body, greater bitterness, and color stability, supporting the previous research

(Jagatić Korenika et al., 2023). Similarly, Herjavec et al. (2012) reported that extended maceration in production significantly improves red wine quality, resulting in a full-bodied and well-structured taste due to the enhanced presence of tannins. Bitterness perception in wine is influenced by several components, including ethanol and phenolic compounds (Gawel et al., 2013). Busse-Valverde et al. (2012) conclude that prolonged maceration reduces the perception of acidity, a trend reflected in this sensory analysis, where wines from longer maceration treatments had less pronounced acidity. Shorter maceration (8 days) resulted in fresher wines with higher acidity and lighter structure.

Higher concentrations of terpenes (linalool, α-terpineol, citronellol) and lactones (γ-nonalactone) in these treatments contributed to enhanced floral and nutty aromas (Ferreira and Lopez, 2019). Among all terpene compounds, citronellol was found at the highest concentrations, providing a floral scent like roses (Sáenz et al., 2010). Overall, extended maceration produced wines with the most balanced structure and complex aromatic expression, whereas shorter maceration, particularly in spontaneous fermentation, emphasized specific but less harmonious descriptors.

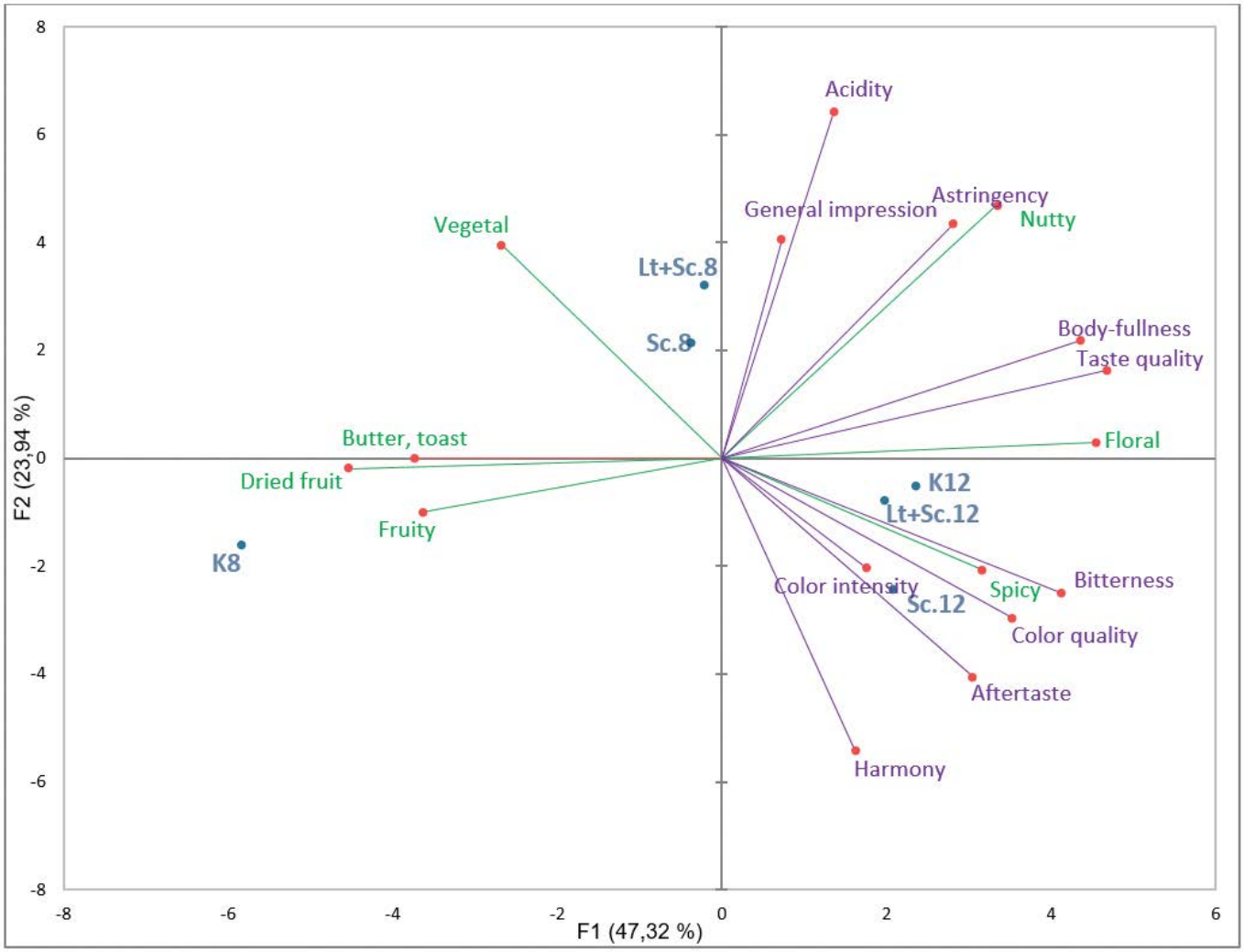

Figure 2. Principal component analysis (PCA) – aroma descriptors and sensory parameters of Trnjak red wine, vintage 2019

CONCLUSIONS

This study demonstrates the significant influence of *Lachancea thermotolerans* (*Lt*) in sequential fermentation with *Saccharomyces cerevisiae* (*Sc*) on the physicochemical, aromatic, and sensory properties of Trnjak wines, vintage 2019, produced from single vineyard. *Lt+Sc* fermentation reduced the alcohol content while increasing the total acidity and stability, which is particularly beneficial in warmer climates such as Herzegovina.

Sequential fermentation improved wine aroma complexity by reducing undesirable higher alcohols and fatty acids while increasing esters like ethyl lactate and beneficial aromatic compounds (e.g., linalool, β-Damascenone, and γ-nonalactone). Extended maceration further intensified floral and spicy aromas, while ester concentrations were slightly reduced. Sensory analysis confirmed that sequential fermentation and extended maceration enriched aromatic complexity, improved mouthfeel, and increased balance, resulting in a fuller, more structured wine.

Regarding the conclusions which indicate that sequential fermentation with *Lt+Sc* and extended maceration are valuable tools in modern winemaking that can improve the aroma, stability and texture of warm-climate red wine from the Trnjak variety, research will continue through more vintages and locations for additional validation.

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