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Effects of *Saccharomyces paradoxus* Fermentation on White Wine Composition: Insights from Integrated Standard and Metabolomics Approaches

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Featured Application: ***Saccharomyces paradoxus*** **yeast has shown good fermentation performance in previous studies. However, its potential for commercial winemaking remains largely untapped, accompanied by significant gaps in the literature on this topic. The results of this study provided a more detailed understanding of the contributions of** ***S. paradoxus*** **fermentation to wine composition and quality, thereby advancing knowledge on its potential applications in commercial winemaking. The observed characteristics suggest that** ***S. paradoxus*** **may be an interesting alternative in white winemaking, either for standalone fermentation, as a blending component, or as a fermentation starter.**

Abstract: Despite its promising potential, the capabilities of *Saccharomyces paradoxus* in commercial winemaking are still unutilized and require further investigation. In this study, the effects of fermentation by a *S. paradoxus* strain P01-161 on the composition of Malvazija istarska white wine in two harvest years were investigated. A range of complementary standard and metabolomics analysis approaches were applied, including OIV methods for basic parameters; HPLC-DAD-RI for organic acids, glycerol, and proteins; UPLC/MS/MS for phenolic compounds; and GC/FID, GC/MS, and GC × GC/TOF-MS for volatile compounds. The harvest year exhibited a significant impact, but many distinctive traits of *S. paradoxus* versus *S. cerevisiae* control wines were consistent across the seasons. These included reductions in malic acid and certain phenols and pathogenesis-related proteins. *Saccharomyces paradoxus* fermentation yielded higher levels of glycerol, volatile acidity, and specific thaumatin-like proteins. Among a total of 474 identified volatile compounds, *S. paradoxus* exhibited lower concentrations of several odoriferous alcohols, acids, and esters, as well as higher concentrations of β-damascenone, acetaldehyde, isobutyric acid, ethyl 2-methylbutyrate, ethyl acetate, isobutyl acetate, various esters of succinic and lactic acids, accompanied by numerous minor compounds, when compared to *S. cerevisiae*. These differences suggest the potential for distinct sensory profiles produced by the two yeasts, indicating that *S. paradoxus* could be a promising alternative for white wine production.

Keywords: yeast; *Saccharomyces paradoxus*; alcoholic fermentation; wine aroma; volatilome

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1. Introduction

The selection of yeast for alcoholic fermentation is one of the most critical factors influencing the final composition and quality of wine. Currently, numerous yeast species and strains are isolated, selected, and commercially available, with various *Saccharomyces cerevisiae* strains dominating due to their ability to conduct predictable and vigorous fermentations, tolerate relatively high levels of alcohol and sulfur dioxide, and function effectively across standard grape must pH ranges. In recent years, non-*Saccharomyces* species, such as *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia kluyveri*, *Metschnikowia pulcherrima*, and *Schizosaccharomyces pombe*, have gained attention as co-fermentation starters with *S. cerevisiae* due to their potential to enhance specific wine quality attributes [1,2]. Another yeast from the *Saccharomyces sensu stricto* complex, *Saccharomyces paradoxus*, has also demonstrated promising fermentation performance [3–5]. Unlike *S. cerevisiae* and other *S. sensu stricto* species, which are primarily associated with human fermentation environments, *S. paradoxus* is predominantly found in natural ecosystems, although it is rarely isolated from vineyards [5–8]. Several studies have reported that *S. paradoxus* strains can reduce volatile acidity and malic acid levels while increasing the glycerol concentration in comparison to *S. cerevisiae*-driven fermentations [3–5,9]. Furthermore, some *S. paradoxus* strains were found to exhibit higher concentrations of yeast cell wall chitin. This characteristic enables them to bind grape chitinases more efficiently than conventional *S. cerevisiae* strains, in this way enhancing wine protein haze stability [10,11]. The effect of *S. paradoxus* on volatile compounds—key contributors to the aroma and flavour profile of wine—is still unclear. A few existing studies showed contrasting results, with alterations in the concentrations of higher alcohols, fatty acids, and volatile esters, depending on the specific strain and compound. The obtained volatile profiles were generally comparable to those obtained by *S. cerevisiae* controls, highlighting the promising potential of *S. paradoxus* [4,5,12,13].

Despite these insights, the potential of *S. paradoxus* in commercial winemaking remains largely untapped, accompanied with significant gaps in the literature regarding this topic. Existing studies have primarily focused on standard physico-chemical parameters and, in some cases, a limited number of volatile compounds [4,5,12,13]. In this way, numerous potentially important effects remained unexplored. The effects of *S. paradoxus* fermentation on other key wine constituents, such as phenolic compounds and protein classes beyond chitinases, have not been studied to date. Additionally, most research on *S. paradoxus* and wine yeasts in general has been limited to single-season studies, overlooking potential year-to-year variability in fermentation outcomes due to variable grape composition.

The main hypothesis of this study is that *S. paradoxus* fermentation produces a distinct white wine chemical composition compared to standard *S. cerevisiae*, with a broader impact than currently recognized. Building on this foundation, the primary objective was to expand the investigation into the effects of *S. paradoxus* on a significantly larger array of wine components by employing integrated standard and metabolomics approaches. The main focus was set on volatile aroma and flavour compounds, which are crucial for wine quality and distinctiveness. To achieve a comprehensive volatilome characterization, an advanced state-of-the-art analytical technique, comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS), was employed. This technique involves the use of two gas chromatographic columns with differing polarities and lengths, connected in series. In this way, it provides significantly enhanced compound separation and interference-free mass spectra, improving sensitivity and enabling the identification of a much larger array of volatile compounds compared to conventional GC methods [14,15]. The characterization was further expanded by profiling phenolic compounds using targeted ultra-performance liquid chromatography coupled with triple-quadrupole mass spectrometry (UPLC/MS/MS). Key wine pathogenesis-related protein classes, such as chitinases and thaumatin-like proteins, were analysed via high-performance liquid chromatography (HPLC).

Additionally, this study hypothesizes that certain effects of *S. paradoxus* fermentation on white wine composition remain consistent across different harvest years. To test this, the investigation was expanded to include two harvest years. It was presumed that the

results of this study should contribute to a more detailed understanding of the biochemical contributions of *S. paradoxus* to white wine composition and quality, thereby advancing knowledge on its potential applications in commercial winemaking.

2. Materials and Methods

2.1. Yeast Inoculum Preparation

Saccharomyces cerevisiae yeast (Lalvin EC1118®) (SCE) was sourced from Lallemand Inc. (Montreal, QC, Canada), while *S. paradoxus* strain P01-161 (SPA) was sourced from UC Davis Phaff Yeast Culture collection (Davis, CA, USA). Yeast cultures were cultivated on YPD agar plates (containing 1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 28 °C. Individual colonies were then transferred into YPD broth (50 mL in 100 mL flasks) and incubated overnight at 24 °C with shaking at 120 rpm, achieving a concentration of approximately 10^8 cells/mL. Commercial pasteurized grape juice was diluted 50:50 (v/v) with deionized water to a total of 100 mL in 300 mL flasks. The juice was inoculated with an aliquot of fermenting YPD broth to a final cell concentration of 10^7 cells/mL and incubated overnight at 24 °C with stirring at 120 rpm. Inoculation of experimental Malvazija istarska grape juices was carried out directly from these liquid cultures at 2×10^6 cells/mL. The cell density was assessed by measuring the optical density at 600 nm (OD600) using a Cary 50 UV/Vis spectrophotometer (Varian Inc., Harbour City, CA, USA).

2.2. Vinification

Grapes from Malvazija istarska, the most widespread and important native white grape cultivar (*Vitis vinifera* L.) in Croatia, were hand-harvested from the experimental vineyard at the Institute of Agriculture and Tourism in Poreč, Istria, in two consecutive harvests, 2021 and 2022. All equipment used in the vinification process was thoroughly sanitized. A total of 3280 kg/3440 kg of grapes, respectively, were destemmed, crushed, and pressed immediately after harvest using a 500 L closed-type pneumatic press (Letina Inox d.o.o., Čakovec, Croatia) at pressures of 2×0.5 bar and 1×0.8 bar. The resulting juice was sulfited and cold-settled for 48 h at 10 °C with the addition of 2 g/hL of Endozym Rapid pectolytic enzyme (AEB s.p.a. Brescia, Italy). After settling, the must (2080 L/2300 L, respectively) had the following parameters: total acidity 4.7 g/L, pH 3.41, and sugars 22.1° Brix in 2021; and total acidity 4.3 g/L, pH 3.45, and sugars 20.8° Brix in 2022. Total acidity was adjusted to 6 g/L by adding tartaric acid, so the final pH values in 2021 and 2022 were 3.27 and 3.28, respectively. A part of the homogenized must was then transferred to 5 L demijohns equipped with airlocks for fermentation initiation, as described earlier. All fermentations were conducted in triplicate at 17 °C. Thirty-six hours upon inoculation of yeasts, diammonium phosphate (Corimpex Servise Srl, Romans d'Isonzo, Italy) was added to the must at a concentration of 30 g/hL. Daily monitoring of sugar content was performed using a portable density meter (DMA 35, Anton Paar, Graz, Austria). The control SCE fermentation was completed (reducing sugars < 4.0 g/L) in 23 days in both years, while SPA fermentation lasted 30 days in 2021 and 26 days in 2022. Upon completion of fermentation, the wines were racked, allowed to settle for three weeks, and following a second racking, samples were taken for analysis.

2.3. Standards, Chemicals, and Materials

Methanol, acetonitrile, and formic acid, all of LC/MS grade, were sourced from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic, sulfuric acid, and chemical standards of organic acids, glycerol, and thaumatin from *Thaumatococcus daniellii* were purchased from Sigma-Aldrich. Chemical standards of phenolic compounds were acquired from Sigma-Aldrich, Polyphenols Laboratories AS (Sandnes, Norway), Fluka (Buchs, Switzerland), Carlo Erba (Milan, Italy), Carl Roth (Karlsruhe, Germany), Extrasynthese (Genay, France), TransMIT PlantMetaChem (Giessen, Germany), and Leuven Bioproducts (Heverlee, Belgium). Chemical standards of volatile aroma compounds were sourced from Sigma-Aldrich, Merck (Darmstadt, Germany), Honeywell International Inc. (Morris Plains, NJ, USA), Fluka,

and AccuStandard Inc. (New Haven, CT, USA). Ultrapure water was utilized for chromatographic procedures and preparation of standard solutions. For GC/MS analysis of volatiles, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, StableFlex, 50/30 µm, 1 cm) SPME fibres were obtained from Supelco, Sigma-Aldrich (Bellefonte, PA, USA). For GC × GC/TOF-MS analysis, longer fibres (2 cm) of the same type were purchased from Supelco, Sigma-Aldrich.

2.4. Analysis

2.4.1. Basic Oenological Parameters

The basic physico-chemical parameters, including alcohol content by volume, total dry extract, total and volatile acidity, and pH, were measured following OIV protocols [16]. Organic acids and glycerol were analysed using high-performance liquid chromatography (HPLC) according to the method reported by Delač Salopek et al. [17]. An Agilent Infinity 1260 system was used, equipped with a G1311B quaternary pump, G1329B autosampler, G1316A column oven, G4212B DAD detector (for organic acids), and G7162A RID detector (for glycerol) (Agilent Technologies, Santa Clara, CA, USA). A sample volume of 0.5 mL was diluted with 1.0 mL of ultrapure water, filtered through a 0.45 µm PTFE filter, and then 10 µL was injected into an Agilent Hi-Plex H column (300 × 7.7 mm, 8 µm particle size) with a PL Hi-Plex H guard column (5 × 3 mm) (Agilent Technologies). The mobile phase was 4 mM sulfuric acid, running at 0.5 mL/min with the column temperature set at 70 °C. UV/Vis chromatograms were recorded at 210 nm, and the RID flow cell temperature was maintained at 50 °C throughout the analysis. Organic acids and glycerol were identified by comparing retention times and UV/Vis spectra to those of pure standards, while their concentrations were quantified using calibration curves (n = 5, r^2 > 0.995 for all the compounds).

2.4.2. Analysis of Pathogenesis-Related (PR) Proteins and Protein Stability

Analysis of pathogenesis-related (PR) proteins was carried out via reversed-phase high-performance liquid chromatography (RP-HPLC), following the protocols established by Marangon et al. [18] and Van Sluyter et al. [19]. The same Agilent Infinity 1260 system used for organic acids and glycerol was employed. Samples were first filtered through 0.45 µm PTFE filters, and 100 µL was injected into a C8 column (4.6 × 250 mm, 5 µm particle size, Vydac 208TP54) protected with a C8 guard (4.6 × 5 mm, 5 µm particle size, Vydac 208GK54). Detection wavelength was set at 210 nm. Two solvents were used: solvent A consisted of 0.1% trifluoroacetic acid (TFA) in 80% acetonitrile, and solvent B was 0.1% TFA in 8% acetonitrile. The gradient program applied was reported in a previous study [20]. The flow rate was set at 1 mL/min at room temperature. Thaumatin-like proteins were eluted between 9 and 12 min, while chitinases appeared between 18.5 and 24.5 min [18]. PR protein concentrations were calculated using a calibration curve generated with thaumatin from *Thaumatococcus daniellii* (n = 5, r^2 = 0.997), assuming a relative response factor of one.

Bentonite dosing to stabilize wines and remove PR proteins was determined to the nearest 10 g/hL after testing a range of doses (50–200 g/hL). Bentonite was added to wine aliquots in 100 mL glass cylinders, and the standard heat stability test was performed, as described previously [20,21]. The procedure included filtration of a sample aliquot (20 mL) through a PTFE 0.45 µm syringe filter, heating at 80 °C for 2 h in a drying oven, cooling at 2 °C for 2 h in a refrigerator, and stabilization at 24 °C (room temperature). The minimum bentonite dose required for protein stabilization was defined as the amount that produced a difference in haze between heated/cooled and unheated wine samples, measured in nephelometric turbidity units (NTUs), of less than 2 NTU. The haze measurements were conducted using a nephelometric turbidity meter (Hanna Instruments HI 83749, Padova, Italy).

2.4.3. UPLC/MS/MS Analysis of Phenolic Compounds

Phenolic compounds were analysed using ultra-performance liquid chromatography coupled with triple-quadrupole mass spectrometry (UPLC/MS/MS). The setup used included an Acquity UPLC system connected to a Xevo TQ MS equipped with an electrospray

ionization (ESI) source (Waters Corporation, Milford, MA, USA). The procedure reported by Vrhovsek et al. [22] was applied. Before analysis, samples were filtered through 0.2 µm PTFE filters, and 2 µL at 6 °C was injected by an autosampler onto a reverse-phase Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 µm) from Waters Corporation kept at 40 °C with a flow of 0.4 mL/min. Two mobile phases were employed, water and acetonitrile, both containing 0.1% formic acid (*v*/*v*). Specific solvent gradients and MS/MS detection settings, including multiple reaction monitoring (MRM) and quantification parameters, were carried out as described in prior studies [22,23]. Calibration curves were used for quantification (n = 5, r^2 > 0.995 for all the compounds). Data analysis was conducted using MassLynx 4.1 and Target Lynx 4.1 software (Waters Corporation).

2.4.4. GC/FID Analysis of Major Volatile Compounds

To analyse acetaldehyde, ethyl acetate, methanol, and major higher alcohols, gas chromatography with flame ionization detection (GC/FID) was employed after direct injection. A Nexis GC-2030 GC system equipped with an AOC-20 autosampler (Shimadzu, Kyoto, Japan) was fitted with an Rtx-WAX capillary column (60 m × 0.25 mm internal diameter × 0.25 µm film thickness) from Restek (Bellefonte, PA, USA). A split ratio of 1:20 was used, and prior to quantification via calibration curves (n = 3, r^2 > 0.995 for all the compounds), 1-pentanol was added as an internal standard for normalization.

2.4.5. GC/MS Analysis of Volatile Aroma Compounds

Prior to GC/MS analysis, volatile aroma compounds were extracted by HS-SPME following the protocol by Bubola et al. [24], with slight adjustments. Wine samples were diluted fourfold with deionized water and placed in 10 mL glass vials containing 1 g of ammonium sulfate. An internal standard solution (2-octanol, 1-nonanol, and heptanoic acid) was added in a volume of 50 µL. The DVB/CAR/PDMS fibre was conditioned above the sample for 15 min at 40 °C and then exposed to the headspace vapours for 40 min at 40 °C while stirring at 800 rpm. Desorption of volatiles took place in a GC-MS injector at 248 °C for 10 min (3 min in splitless mode). Identification and quantification of volatile compounds were performed on a Varian 3900 gas chromatograph (GC) coupled with a Varian Saturn 2100T ion trap mass spectrometer (Varian Inc., Harbour City, CA, USA). The GC was equipped with a 60 m × 0.25 mm i.d. × 0.25 µm d.f. capillary column (Rtx-WAX, Restek). The temperature program started at 40 °C; then, the temperature was increased by 2 °C/min to 240 °C and was held constant for additional 10 min. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. Mass spectra were acquired in EI mode (70 eV) within the 30–350 m/z range. Compound identification was carried out by comparing retention times and mass spectra with pure standards, as well as using the NIST05 library. A reverse match score above 750 was the criterion for identification. Spectra with lower scores were manually verified using characteristic ion ratios. Additional confirmation was achieved by comparing linear retention indices (calculated from C10 to C28 n-alkanes) with literature values obtained on equivalent columns. Calibration curves were constructed with internal standard normalization applied before quantification (n = 5, r^2 > 0.99 for all the compounds). Major volatile compounds were quantified using total ion current (TIC) peak areas, while minor compounds were quantified using quantifier ion peak areas. Method validation details can be found in Bubola et al. [24]. Relative standard deviations in repeatability conditions (n = 5) ranged from 4.02% to 13.04% for terpenoids, from 2.25% to 9.22% for alcohols, from 7.78% to 11.77% for fatty acids, from 1.68% to 7.33% for ethyl esters, and from 7.30% to 12.33% for acetate esters. For volatile compounds without commercially available standards, semi-quantitative analysis was performed by assuming equivalent detector response for similar compounds.

2.4.6. GC × GC/TOF-MS Analysis of Volatile Compounds

Prior to GC × GC/TOF-MS analysis, volatile compounds were extracted by HS-SPME following the method described in previous studies [14,15], with minor modifications. A

2.5 mL aliquot of wine was placed in a 20 mL vial containing 1.5 g of sodium chloride. An internal standard solution (2-octanol) was added (50 µL). A DVB-CAR-PDMS fibre was conditioned above the sample for 5 min at 35 °C and then exposed to the headspace for 20 min at the same temperature. Desorption of the volatiles occurred in a GC injector at 250 °C for 3 min in splitless mode. The fibre was reconditioned at 270 °C for 7 min between extractions. For identification and quantification, an Agilent 7890N GC (Agilent Technologies, Palo Alto, CA, USA) was coupled to a LECO Pegasus IV TOF-MS (Leco Corporation, St. Joseph, MI, USA) with a Gerstel MPS autosampler (GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany). The carrier gas was helium at a flow rate of 1.2 mL/min. The first-dimension GC column was a 30 m × 0.25 mm × 0.25 µm VF-WAXms (Agilent Technologies), and the second-dimension column was a 1.5 m × 0.15 mm × 0.15 µm Rxi 17Sil MS (Restek). The first oven was programmed to start at 40 °C for 4 min, followed by a ramp of 6 °C/min to 250 °C, held for 5 min. The second oven was kept at temperatures 5 °C higher than the first one throughout the analysis. Modulation occurred with a 7 s cycle time, including a 1.4 s hot pulse, with the modulator offset by +15 °C from the secondary oven, as detailed in a previous report [14]. Mass spectra were acquired in EI mode (70 eV) over the 40–350 m/z range. The ion source was set at 230 °C, with a detector voltage of 1317 V, an acquisition rate of 200 spectra/s, and an acquisition delay of 120 s. LECO ChromaTOF software (v4.32) was used for deconvolution, peak alignment, and baseline correction, with a signal-to-noise ratio of 100 and baseline offset at 0.8. Integration was performed manually. A standard mix of 122 volatile compounds was analysed under the same conditions. Identification was based on comparison of retention times and mass spectra with pure standards, and mass spectra libraries (NIST 2.0, Wiley 8, FFNSC 2) with a match factor of at least 750. Additional confirmation was achieved by comparing linear retention indices (calculated from C10 to C30 n-alkanes) with literature values for similar GC columns. Compound concentrations (µg/L) were calculated relative to the internal standard 2-octanol, assuming equal detector response.

During the HS-SPME GC×GC-TOF-MS analysis, a quality control (QC) sample was created by combining equal amounts of all wine samples and analysed periodically among other samples. Principal component analysis (PCA) showed that the QC samples were tightly clustered and distinctly separated from the wine samples, suggesting good method repeatability. The relative standard deviations of the internal standard concentration 2-octanol in the QC samples were 9.2% in 2021 and 7.0% in 2022, which are considered satisfactory for this analytical method.

2.5. Statistical Data Analysis

The data acquired in each harvest year were separately subjected to one-way analysis of variance (ANOVA) with a factor: yeast (Y). The data acquired across both harvest years were subjected to two-way ANOVA with factors: yeast (Y) and harvest year (H). Mean values were compared using the least significant difference (LSD) post hoc test at a significance level $p < 0.05$. Additionally, the data were processed by multivariate statistical techniques, including hierarchical cluster analysis (HCA) and partial least squares–discriminant analysis (PLS-DA). In order to better comprehend and visualize the effects of yeast which were consistent across two harvest years and to minimize the interferences caused by the effect of harvest year, prior to multivariate elaboration, the data were normalized across the datasets of each of the two harvest years separately. Normalization was performed for each variable using the following formula, $X_{norm} = (X_i - X_{min})/(X_{max} - X_{min})$, where X_{norm} represents normalized concentration, X_i represents original concentration, and X_{min} and X_{max} represent minimum and maximum concentrations of a variable in a given harvest year, respectively. ANOVA was performed using Statistica version 13.2 (StatSoft Inc., Tulsa, OK, USA), while HCA and PLS-DA were conducted using MetaboAnalyst version 6.0 (available at http://www.metaboanalyst.ca, accessed on 31 October 2024).

3. Results and Discussion

3.1. Basic Oenological Parameters

The basic oenological parameters of Malvazija istarska wine produced with *S. cerevisiae* (SCE) and *S. paradoxus* (SPA) yeasts are reported in Table 1. In 2021 wines, SPA-21 treatment led to lower ethanol levels compared to the SCE-21 control, while in 2022, the difference was not significant. Several studies reported a reduction in the alcohol content when fermenting with *S. paradoxus* compared to *S. cerevisiae*, although not always with statistical significance [4,5,9,25].

Table 1. Standard physico-chemical parameters (g/L, if not otherwise indicated) of Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years.

Physico-Chemical Parameter	Differences in Concentration						
	One-Way ANOVA				Two-Way ANOVA		
	2021		2022				
	SCE	SPA	SCE	SPA	Y	H	Y × H
Alcoholic strength (vol %)	**13.02** [a]	**12.56** [b]	11.45	11.57	n.s.	**21**	*
Total acidity (as tartaric acid)	6.00	5.70	6.20	6.20	**SCE**	**22**	*
Volatile acidity (as acetic acid)	**0.42** [b]	**0.48** [a]	0.35	0.38	**SPA**	**21**	n.s.
pH	**3.23** [b]	**3.28** [a]	3.09	3.11	**SPA**	**21**	n.s.
Citric acid	**0.37** [a]	**0.31** [b]	**0.31** [a]	**0.30** [b]	**SCE**	**21**	*
Tartaric acid	**2.69** [b]	**2.80** [a]	3.83	3.94	**SPA**	**22**	n.s.
Malic acid	**2.03** [a]	**1.78** [b]	**1.50** [a]	**1.23** [b]	**SCE**	**21**	n.s.
Lactic acid	0.08	0.08	0.44	0.33	n.s.	**22**	n.s.
Glycerol	**5.33** [b]	**6.98** [a]	**5.46** [b]	**5.85** [a]	**SPA**	**21**	*

Abbreviations: n.s.—not significant. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

Glycerol typically ranks second to ethanol in abundance as a yeast metabolite. It often serves as a carbon sink in wines with reduced ethanol content [26]. Evidence suggests that *S. paradoxus* may divert carbon flux toward glycerol production to a greater extent than *S. cerevisiae* [5], which has been supported by several studies showing increased glycerol levels in *S. paradoxus*-fermented wines [3–5]. In this study, SPA wines consistently showed higher glycerol levels than SCE wines across both harvest years (Table 1). Glycerol production is regulated by glycerol-3-phosphate dehydrogenases (*GPD*), which are upregulated under osmotic stress during anaerobic fermentation [27]. The observed difference may reflect unique redox balance and osmotic stress adaptations between the two yeasts, consistent with previous findings [4,28]. The obtained results and previous findings suggest that some of the genes involved in glycerol biosynthesis (*GPD1*, *GPD2*) and transport (*STL1*, *FPS1*) are consistently more expressed in *S. paradoxus* than in *S. cerevisiae* under winemaking conditions. The sensitivity of *S. paradoxus* to grape must fermentation conditions may be linked to its infrequent presence in vineyards, which has limited its exposure to natural selection and adaptation. Indeed, studies have shown that *S. paradoxus* exhibits lower mutation rates and greater genome stability compared to *S. cerevisiae* under laboratory evolution conditions [29,30]. Glycerol contributes positively to the sensory quality of wine, primarily by enhancing its mouthfeel and viscosity, giving it a smoother and fuller texture. Although the sensory effects of glycerol are generally considered subtle and nuanced, the ability of *S. paradoxus* to produce higher levels can be considered an advantage compared to *S. cerevisiae*.

Glycerol production by *S. cerevisiae* is associated with acetic acid formation, which aids in maintaining redox balance [27,31]. In 2021, SPA-21 wine exhibited slightly elevated volatile acidity compared to SCE-21 wine, while the difference in 2022 was not significant

(Table 1). Such a result did not entirely align with previous studies that reported lower acetic acid levels in wines fermented with *S. paradoxus* compared to *S. cerevisiae*, with an inverse correlation between glycerol and volatile acidity [3,4,13]. Álvarez et al. [13], building on the findings of Minebois et al. [32], stated that in *S. paradoxus*, acetic acid may be integrated into various metabolic reactions to a greater extent than in *S. cerevisiae*. One such reaction is the production of acetyl-CoA catalysed by acetyl-CoA synthetases. Acetyl-CoA is a precursor for fatty acids involved in cell membrane restructuring, which enhances ethanol resistance in yeast strains that are less adapted to alcoholic fermentation. It is also possible that *S. paradoxus* and *S. cerevisiae* possess distinct metabolic pathways for acetic acid production, transport, and metabolism. These differences may involve variations in the expression of the corresponding genes and the activity of enzymes, such as pyruvate decarboxylases, aldehyde dehydrogenases, and others.

Total acidity was not significantly affected by the yeast strains, though a tendency toward lower values in SPA-21 compared to SCE-21 was noted. This likely resulted from a reduction in malic acid content in SPA wines across both harvest years (Table 1), a trend often associated with *S. paradoxus* fermentations [3,5,9,25,33]. The ability of yeast to degrade extracellular malic acid depends on the efficient transport of the acid into the cell and the activity of the intracellular malic enzyme, which is coded by specific genes [34]. Redžepović et al. [33] reported that *S. paradoxus* reduced malic acid more effectively than *S. cerevisiae* and *S. bayanus* in the alcoholic fermentation of synthetic must and wine. *Saccharomyces paradoxus* continued to degrade malic acid even after glucose depletion, suggesting that malic acid may serve as a secondary carbon source for this yeast in glucose-depleted environments. This enhanced malic degradation was linked to an increased expression of malic enzymes, potentially indicating a more efficient transport system for this acid in *S. paradoxus*. Fermentation by *S. paradoxus* led to slightly elevated tartaric acid and decreased citric acid levels compared to control SCE wines (Table 1). The concentrations of malic, tartaric, and lactic acids were significantly influenced by harvest year. Higher malic acid levels were observed in 2021, while tartaric and lactic acid were more abundant in 2022.

3.2. Pathogenesis-Related (PR) Proteins

Pathogenesis-related (PR) proteins, especially thaumatin-like proteins (TLPs) and chitinases, occur naturally in grapes and mostly remain stable under fermentation conditions. These proteins are the primary contributors to protein instability and unwanted haze in white wines. To address this issue, white wines undergo protein stabilization through bentonite fining, which effectively removes excess proteins before bottling [18].

In 2021, no significant differences in the concentrations of PR proteins were observed between the wines studied (Table 2). In 2022, SPA-22 wine contained a higher concentration of TLP-2 and lower concentrations of chitinases compared to control SCE-22 wine. This trend was supported by the results of two-way ANOVA. Previous studies have demonstrated that chitin and its deacetylated derivative, chitosan—substrates for PR chitinases—can effectively remove class IV chitinases from wine and enhance wine protein stability [35,36]. Certain strains of *S. paradoxus* have been reported to possess elevated levels of yeast cell wall chitin. This feature allowed them to bind more grape chitinases than *S. cerevisiae* and to more effectively remove them from wine model solutions, as shown earlier [10]. This was further supported by Sommer and Tondini [11], who noted improved protein stability of wines from certain grape cultivars treated with *S. paradoxus* yeast hulls in laboratory tests, including the strains P01-161 and P01-167.

In this study, SPA fermentation with *S. paradoxus* did reduce the concentration of certain chitinases but did not improve protein stability compared to control SCE treatment. The wines produced by the two investigated yeasts required almost equal bentonite doses for complete protein stabilization (Table 2). The difference between this result and previous findings [35,36] might be due to yeast and PR proteins interacting differently during real fermentation compared to laboratory conditions. Additionally, Sommer and Tondini [11]

observed variability in the effectiveness of *S. paradoxus* yeast hull treatments across different wine cultivars and styles. This implies that the specific PR protein profile of Malvazija istarska grape juice and wine could have contributed to the reduced effect observed in this study. A significant impact of harvest year was observed, with wines from 2022 containing higher concentrations of PR proteins than those from 2021 (Table 2).

Table 2. Concentrations of pathogenesis-related (PR) proteins (mg/L) in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by high-performance liquid chromatography with diode array detection (RP-HPLC/DAD) and bentonite doses (g/hL) required to achieve protein stability of the wines.

PR Proteins and Bentonite Dose	Differences in Concentration						
	One-Way ANOVA				Two-Way ANOVA		
	2021		2022		Y	H	Y × H
	SCE	SPA	SCE	SPA			
Thaumatin-like proteins 1	13.16	13.62	35.01	32.89	n.s.	**22**	n.s.
Thaumatin-like proteins 2	12.72	13.05	**16.68** [b]	**18.15** [a]	**SPA**	**22**	n.s.
Thaumatin-like proteins 3	12.59	13.75	16.81	17.21	n.s.	**22**	n.s.
Thaumatin-like proteins 4	33.33	36.72	54.83	58.60	**SPA**	**22**	n.s.
Chitinases 1	30.32	28.16	**69.28** [a]	**60.53** [b]	**SCE**	**22**	n.s.
Chitinases 2	23.91	23.93	**57.23** [a]	**52.86** [b]	n.s.	**22**	n.s.
Total thaumatin-like proteins	71.80	77.15	123.33	126.84	n.s.	**22**	n.s.
Total chitinases	54.23	52.09	**126.5** [a]	**113.4** [b]	**SCE**	**22**	n.s.
Total PR proteins	126.03	129.24	249.9	240.2	n.s.	**22**	n.s.
Bentonite dose	90.00	93.33	96.67	103.3	n.s.	n.s.	n.s.

Abbreviations: n.s.—not significant. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. Significant differences are highlighted in bold.

3.3. Phenolic Compounds from Grapes

Phenolic compounds deriving from grapes contribute significantly to the character and quality of white wine. Hydroxycinnamic acids and flavonols are involved in the formation and stability of colour, flavan-3-ol monomers and oligomers impart subtle bitterness, while flavan-3-ol polymers (proanthocyanidins i.e., condensed tannins) contribute to astringency. Additionally, phenols act as antioxidants and protect white wines from oxidation, which can otherwise lead to unwanted browning and flavour degradation [37].

The concentrations of phenolic compounds found in the investigated wines are summarized in Table 3. Yeast had a significant effect on specific phenols, while harvest year significantly influenced the levels of nearly all identified phenols except syringic acid. In many cases, the effects of yeast and harvest year interacted. Among hydroxybenzoic acids, control SCE-21 wine contained higher concentrations of 4-aminobenzoic and 2,5-dihydroxybenzoic acids, whereas *p*-hydroxybenzoic acid was more abundant in SCE-22 wine. The concentrations of 4-aminobenzoic acid and vanillic acid were higher in 2022, while *p*-hydroxybenzoic acid and protocatechuic acid were more abundant in 2021 wines.

Hydroxycinnamic acids displayed significant interactions between yeast and harvest year, with some clear trends emerging. In 2021, SPA-21 wine had higher levels of hydroxycinnamoyltartrates, such as *trans*-caftaric and *trans*-fertaric acid, while control SCE-21 wine had higher concentrations of the identified free forms, including *p*-coumaric, caffeic, and ferulic acid. This pattern could suggest enhanced cinnamoyl esterase activity in *S. paradoxus*. However, this trend was not consistent in 2022. *Trans*-fertaric acid was more abundant in control SCE-22 than in SPA-22 wine, while other phenols from this group did not show significant differences.

Table 3. Concentrations (mg/L) of phenolic compounds from grapes in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by ultra-performance liquid chromatography/mass spectrometry (UPLC/MS/MS).

Phenolic Compound	Differences in Concentration						
	One-Way ANOVA				Two-Way ANOVA		
	2021		2022				
	SCE	SPA	SCE	SPA	Y	H	Y × H
4-Aminobenzoic acid	**0.07** [a]	**0.04** [b]	0.09	0.09	n.s.	**22**	n.s.
p-Hydroxybenzoic acid	0.44	0.36	**0.04** [a]	**0.00** [b]	n.s.	**21**	n.s.
Vanillic acid	0.11	0.11	0.13	0.15	n.s.	**22**	n.s.
2,5-Dihydroxybenzoic acid	**0.72** [a]	**0.32** [b]	0.31	0.38	**SCE**	**21**	*
Protocatechuic acid	0.57	0.61	0.30	0.24	n.s.	**21**	n.s.
Syringic acid	0.42	0.45	0.33	0.37	n.s.	n.s.	n.s.
p-Coumaric acid	**1.27** [a]	**0.26** [b]	0.12	0.08	**SCE**	**21**	*
Caffeic acid	**2.24** [a]	**1.28** [b]	0.58	0.49	**SCE**	**21**	*
Ferulic acid	**0.50** [a]	**0.30** [b]	0.11	0.15	**SCE**	**21**	*
trans-Caftaric acid	**0.18** [b]	**0.47** [a]	0.14	0.09	**SPA**	**21**	*
trans-Fertaric acid	2.45	2.97	**2.15** [a]	**1.75** [b]	n.s.	**21**	*
trans-Coutaric acid	**0.49** [b]	**0.74** [a]	0.55	0.47	n.s.	**21**	*
trans-Resveratrol	**0.12** [a]	**0.08** [b]	**0.08** [a]	**0.00** [b]	**SCE**	**21**	*
cis-Resveratrol	0.03	0.05	0.03	0.02	**SPA**	**21**	*
Catechol	**0.68** [a]	**0.35** [b]	0.29	0.36	n.s.	**21**	n.s.
Phlorizin	0.04	0.06	0.02	0.02	n.s.	**21**	n.s.
Quercetin	0.10	0.11	n.d.	n.d.	-	-	-
Catechin	**1.41** [a]	**1.04** [b]	0.12	0.09	**SCE**	**21**	*
Epicatechin	0.24	0.22	n.d.	n.d.	-	-	-
Epigallocatechin	**0.02** [a]	**0.00** [b]	n.d.	n.d.	-	-	-
Gallocatechin	0.19	0.16	n.d.	n.d.	-	-	-
Procyanidin B1	1.33	1.54	n.d.	n.d.	-	-	-
Procyanidin B2 + B4	0.16	0.21	n.d.	n.d.	-	-	-

Abbreviations: n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

Trans-resveratrol was more abundant in control SCE wines from both harvest years. A significant interaction indicated that the scale of the effect varied by year. It is possible that *S. cerevisiae* exhibited higher β-glucosidase activity, which is responsible for releasing *trans*-resveratrol from its precursor, *trans*-piceid [38]. For flavan-3-ols, significant differences were observed in 2021, with higher concentrations of catechin and epigallocatechin found in SCE-21 compared to SPA-21 wine. The concentrations of two other phenols, catechol and phlorizin, were affected by harvest year, with higher levels found in 2021 wines.

In addition to yeast-driven metabolic transformations of phenolic compounds, their differential adsorption onto yeast cell surfaces, a phenomenon observed in previous studies [39], may have also contributed to the observed variations.

3.4. Volatile Compounds

In this study, the integration of standard GC analysis techniques such as GC/FID and GC/MS with comprehensive GC×GC-TOF-MS metabolomics analysis yielded the most detailed volatile compound profile of wine produced by *S. paradoxus* to date. A total of 474 volatile compounds were identified, including 63 terpenoids, 16 norisoprenoids, 14 aldehydes, 18 ketones, 53 alcohols, 26 acids, 46 ethyl esters, 23 acetate esters, 54 other esters, 83 benzenoids, 32 furanoids and lactones, 17 sulfur-containing compounds, 12 volatile

phenols, and 17 miscellaneous compounds. Most compounds were identified in wines of both harvest years. However, a substantial number of compounds appeared exclusively in wines from one season. One-way and two-way ANOVA revealed significant differences according to yeast species used in fermentation for a number of compounds. Harvest year also influenced the levels of certain volatiles, while yeast × harvest year interactions were observed less frequently. Volatile compounds are reported, organized in separate tables based on their chemical class and listed by increasing retention time in the GC column. The complete data, including mean concentration values, standard deviations, and retention times of volatile compounds, are provided in Table S1.

3.4.1. Terpenoids

Grape-derived terpenoids are key contributors to the distinct and characteristic aromas typical for a cultivar. This effect is especially pronounced in wines with higher terpenoid levels, such as those made from muscats and other aromatic grape cultivars [40,41]. However, it also extends to cultivars which are less aromatic but have a notable monoterpenol potential, like Malvazija istarska [15]. Monoterpenols, like linalool, geraniol, citronellol, nerol, and ho-trienol, are the most impactful for wine aroma among terpenoids, adding appealing floral and fruity notes. In addition to enzymes originating from grapes, yeast β-glucosidases and other enzymes can also affect their content and composition during fermentation. This can occur through yeast-specific cleavage of glycosidic bonds to release volatile terpenoid aglycons or through various other transformations and interconversions [42–44].

In most cases in which significant differences were observed, SCE wines had higher concentrations of terpenoids in a single or in both harvest years (Table 4). According to one-way ANOVA, the concentrations of limonene, α-terpinolene, an unidentified monoterpene (LRI 1456), *trans*-furan linalool oxide, neryl ethyl ether, *p*-menth-1-en-9-al, and citronellyl acetate were higher in SCE-21 than in SPA-21 wine, while *trans*-β-ocimene was more abundant in SCE-22 than in SPA-22 wine. Epoxyterpinolene, *trans*-2-pinanol, and neryl acetate had higher concentrations in SCE wines from both years. In addition, two-way ANOVA showed significant differences in favour of SCE wines for α-ocimene, γ-terpinene, and α-curcumene. Isomenthone and *cis*,*trans*-farnesol, on the other hand, showed a tendency to higher concentration in SPA wines. The higher levels of specific terpenoids observed in SCE compared to SPA wines in this study suggest that *S. cerevisiae* exhibits greater β-glucosidase activity than *S. paradoxus*. However, the major monoterpenols remained unaffected, while most of the influenced terpenoids were specific derivatives, such as acetates, ethers, and oxides. This indicated that these differences might have stemmed from other terpenoid transformations and interconversions induced by yeast during fermentation, rather than solely from the cleavage of glycosides. As the contribution of the affected terpenoids to wine aroma remains mostly unknown, it is unclear whether the observed differences influence the expression of varietal aroma in the two wines studied. Several terpenoids showed significant variability based on harvest year, with higher concentrations in 2021 wines than in those from 2022.

Table 4. Concentrations (µg/L) of terpenoids found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022		Y	H	Y × H
					SCE	SPA	SCE	SPA			
TE1	β-Pinene	B	1141	-	1.40	0.25	2.96	3.42	n.s.	**22**	n.s.
TE2	β-Myrcene	A, B, C	1160	1159	10.50	6.97	8.72	10.25	n.s.	n.s.	n.s.
TE3	α-Phellandrene	B, C	1174	1172	0.30	0.14	0.41	0.36	n.s.	n.s.	n.s.
TE4	β-Phellandrene	B, C	1190	1186	n.d.	n.d.	4.94	4.60	-	-	-

Table 4. *Cont.*

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022		Y	H	Y × H
					SCE	SPA	SCE	SPA			
TE5	Limonene	A, B, C	1193	1195	**7.82** [a]	**5.13** [b]	3.46	3.90	**SCE**	**21**	*
TE6	α-Ocimene	B, C	1235	1245	10.10	5.22	4.22	3.88	**SCE**	**21**	n.s.
TE7	γ-Terpinene	B, C	1245	1239	2.69	1.38	1.30	1.26	**SCE**	**21**	n.s.
TE8	*trans*-β-Ocimene	A, B, C	1250	1250	11.34	8.48	**9.20** [a]	**6.20** [b]	**SCE**	**21**	n.s.
TE9	α-Terpinolene	B, C	1287	1284	**9.80** [a]	**6.25** [b]	8.23	6.49	**SCE**	n.s.	n.s.
TE10	Linalool ethyl ether	B, C	1324	1331	23.68	18.94	14.73	11.74	n.s.	21	n.s.
TE11	*cis*-Rose oxide	B, C	1358	1350	0.24	0.27	0.38	0.39	n.s.	22	n.s.
TE12	*trans*-Rose oxide	B, C	1373	1363	n.d.	n.d.	0.05	0.05	-	-	-
TE13	*cis*-Alloocimene	B, C	1382	1369	1.10	0.92	1.00	0.93	n.s.	n.s.	n.s.
TE14	*trans*-Alloocimene	B, C	1403	1400	1.15	0.87	0.95	0.97	n.s.	n.s.	n.s.
TE15	Dihydrolinalool	B, C	1435	1420	2.14	0.90	0.57	0.50	n.s.	n.s.	n.s.
TE16	*cis*-Furan linalool oxide	A, B, C	1445	1448	1.44	1.35	1.33	1.59	n.s.	n.s.	n.s.
TE17	Terpenoid n.i.	B	1456	-	**47.12** [a]	**32.76** [b]	28.21	23.09	**SCE**	**21**	n.s.
TE18	Dihydromyrcenol	B, C	1466	1455	1.89	1.08	0.79	0.75	n.s.	**21**	n.s.
TE19	*trans*-Furan linalool oxide	A, B, C	1471	1472	**0.56** [a]	**0.48** [b]	0.09	0.09	**SCE**	**21**	*
TE20	Isomenthone	B, C	1471	1470	**0.05** [b]	**0.24** [a]	0.17	0.20	n.s.	n.s.	n.s.
TE21	Nerol oxide	B, C	1477	1473	4.35	3.79	3.93	3.65	n.s.	n.s.	n.s.
TE22	Neryl ethyl ether	B, C	1482	1477	**1.31** [a]	**0.86** [b]	0.96	0.80	**SCE**	**21**	*
TE23	Epoxyterpinolene	B, C	1492	1486	**1.32** [a]	**0.51** [b]	**1.57** [a]	**0.58** [b]	**SCE**	**22**	n.s.
TE24	Geranyl vinyl ether	B, C	1510	1506	n.d.	n.d.	5.43	4.48	-	-	-
TE25	*trans*-2-Pinanol	B, C	1520	1522	**3.80** [a]	**1.37** [b]	**3.81** [a]	**1.41** [b]	**SCE**	n.s.	n.s.
TE26	(-)-Camphor	B, C	1531	1532	0.50	0.48	0.16	0.24	n.s.	**21**	n.s.
TE27	Dihydrolinalyl acetate	B	1531	-	0.10	0.19	n.d.	n.d.	-	-	-
TE28	Linalool	A, B, C	1548	1550	70.13	84.90	8.94	8.97	n.s.	**21**	n.s.
TE29	β-Pinone	B, C	1583	1594	n.d.	n.d.	0.13	0.19	-	-	-
TE30	β-Fenchol	B, C	1583	1588	n.d.	n.d.	0.07	0.09	-	-	-
TE31	4-Terpineol	A, B, C	1604	1604	0.91	0.60	0.11	0.31	n.s.	**21**	n.s.
TE32	Ho-trienol	B, C	1610	1612	11.41	12.29	10.74	12.86	n.s.	n.s.	n.s.
TE33	*p*-Menth-1-en-9-al	B, C	1622	1629	**1.13** [a]	**1.02** [b]	0.93	1.07	n.s.	n.s.	n.s.
TE34	Sabina ketone	B, C	1637	1647	n.d.	n.d.	0.47	0.58	-	-	-
TE35	Menthol	B, C	1641	1641	0.83	1.02	n.d.	n.d.	-	-	-
TE36	Citronellyl acetate	B, C	1666	1659	**0.79** [a]	**0.37** [b]	0.29	0.23	**SCE**	**21**	*
TE37	Farnesene isomer	B, C	1672	1685	2.00	1.99	0.64	0.66	n.s.	**21**	n.s.
TE38	*cis*-Ocimenol	B	1691	-	0.30	0.35	n.d.	n.d.	-	-	-
TE39	α-Terpineol	B, C	1704	1701	14.30	15.64	13.39	14.71	n.s.	n.s.	n.s.
TE40	Isoborneol	B, C	1710	1714	0.30	0.29	0.26	0.38	n.s.	n.s.	n.s.
TE41	Cyclomyral	B	1722	-	1.21	1.44	1.66	1.62	n.s.	**22**	n.s.
TE42	Neryl acetate	B, C	1731	1733	**0.41** [a]	**0.23** [b]	**0.18** [a]	**0.12** [b]	**SCE**	**21**	n.s.
TE43	α-Bisabolene	B, C	1736	1740	0.05	0.09	n.d.	n.d.	-	-	-
TE44	Geranial	B, C	1737	1743	n.d.	n.d.	0.11	0.22	-	-	-
TE45	Carvone	B, C	1741	1742	0.17	0.18	n.d.	n.d.	-	-	-
TE46	Farnesene isomer	B, C	1754	1757	0.24	0.22	0.21	0.24	n.s.	n.s.	n.s.
TE47	Geranyl acetate	B, C	1760	1759	1.28	0.41	n.d.	n.d.	-	-	-
TE48	Citronellol	A, B, C	1766	1760	1.15	1.16	1.26	1.16	n.s.	n.s.	n.s.
TE49	Terpenoid n.i.	B	1779	-	0.59	0.42	n.d.	n.d.	-	-	-
TE50	α-Curcumene	B, C	1785	1782	0.14	0.10	0.15	0.14	**SCE**	n.s.	n.s.
TE51	γ-Isogeraniol	B, C	1787	1800	n.d.	n.d.	0.41	0.41	-	-	-
TE52	Nerol	A, B, C	1804	1801	1.14	1.20	0.77	0.86	n.s.	**21**	n.s.
TE53	*cis*-Calamenene	B, C	1841	1840	0.27	0.20	0.14	0.12	n.s.	**21**	n.s.
TE54	Geraniol	A, B, C	1847	1847	0.98	1.15	0.86	0.84	n.s.	**21**	n.s.
TE55	Geranyl acetone	B, C	1860	1856	4.31	3.80	3.04	4.39	n.s.	n.s.	n.s.
TE56	10,11-Epoxycalamenene	B	1887	-	n.d.	n.d.	0.05	0.06	-	-	-
TE57	α-Calacorene	B, C	1926	1928	0.43	0.35	0.19	0.19	n.s.	**21**	n.s.
TE58	Nerolidol	B, C	2040	2039	0.50	0.79	0.58	0.55	n.s.	n.s.	n.s.
TE59	Thymol	B, C	2183	2187	0.10	0.12	0.26	0.18	n.s.	n.s.	n.s.
TE60	α-Bisabolol	B, C	2214	2209	n.d.	n.d.	0.04	0.03	-	-	-

Table 4. *Cont.*

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
TE61	Cadalene	B, C	2227	2226	0.19	0.12	0.12	0.12	n.s.	**21**	*
TE62	2,3-Dihydrofarnesol	B, C	2260	2265	n.d.	n.d.	0.13	0.11	-	-	-
TE63	*cis*,*trans*-Farnesol	B, C	2350	2351	0.11	0.49	0.10	0.12	**SPA**	**21**	n.s.

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

3.4.2. Norisoprenoids

Norisoprenoids, primarily C_{13}-norisoprenoids, are derived from the breakdown of grape carotenoids, such as lutein, β-carotene, violaxanthin, and neoxanthin. This process occurs through a complex series of mechanisms involving multiple intermediates. Their levels are largely influenced by soil, climate, and various pre-fermentation practices [45,46]. Yeast activity during fermentation can further influence the formation of free volatile norisoprenoids [47].

Among the 16 norisoprenoids identified in this study, only a few displayed significant variation among yeasts. A certain dichotomy was observed, since β-cyclocitral was more abundant in SCE wines, while both isomers of β-damascenone had higher concentrations in SPA wines from both harvest years (Table 5). The initial breakdown of carotenoids during vinification is catalysed by grape carotenoid cleavage dioxygenases (VvCCDs) encoded by specific genes [46]. However, during fermentation, other yeast enzymes supported by acidic environment can modify various intermediates, influencing the final levels of norisoprenoids in wine, as shown previously for *trans*-β-damascenone [47]. It is possible that *S. paradoxus* exhibited a stronger activity of enzymes involved in the reduction of its carbonyl precursors during fermentation, including alcohol dehydrogenases, aldose reductases, and NADPH reductases [47], which resulted in higher levels of β-damascenones in SPA wine. *Trans*-β-damascenone is the most prominent C_{13}-norisoprenoid in wine due to its low odour detection threshold and appealing odour. It contributes to wine aroma with stewed apple and honey notes. The significantly higher concentrations of *trans*-β-damascenone observed in SPA wines in this study suggest that these aromas are likely to be more pronounced in wines produced by *S. paradoxus* fermentation compared to those produced by *S. cerevisiae*. Wines from the 2022 harvest, aside from a vitispirane isomer, contained higher concentrations of norisoprenoids than 2021 harvest wines.

3.4.3. Aldehydes and Ketones

Acetaldehyde is the most prevalent aldehyde in wine, accounting for over 90% of the total aldehyde content. It is produced as an intermediate during fermentation by the conversion of sugars to ethanol via pyruvate decarboxylation by yeast pyruvate decarboxylases. Its portion is further converted into ethanol by alcohol dehydrogenases and into acetic acid by aldehyde dehydrogenases. These processes are regulated by specific yeast genes and influenced by fermentation conditions. Further, acetaldehyde is partly enzymatically transformed into acetoin, a ketone precursor to 2,3-butanediol and diacetyl. Acetaldehyde affects wine aroma differently depending on its concentration. At low levels, it boosts fruity notes, moderate concentrations evoke nutty aromas, and at high levels, it develops an odour similar to overripe apples [48]. Other aldehydes can be formed from amino acids and pyruvate via α-keto acids intermediates [49]. Unsaturated alkenals are a

product of the degradation of unsaturated long-chain fatty acids [48]. The formation and further conversions of both aldehydes and ketones are strongly affected by the activity of yeast.

In this study, SPA wines consistently showed significantly higher acetaldehyde concentrations across both harvest years (Table 6). Heptanal levels were elevated in SCE-21 wine, while 2-(acetoxy)-propanal concentrations were higher in SCE-22 wines compared to their SPA counterparts. SPA wines were characterized by increased levels of specific ketones compared to control SCE wines, including acetoin in 2021, 2-ethyl-3-methoxy-2-cyclopentenone and 1,2-dihydroxycyclobutene-3,4-dione in 2022, and 3-(acetoxy)-4-methyl-2-pentanone in both years (Table 7). In contrast, *S. cerevisiae* produced higher amounts of 2-heptanone, 2-nonanone, 2-decanone, and 2-dodecanone in both years, as well as 1-hydroxy-3-methyl-2-butanone, 2,4-nonanedione, 2-undecanone, and *p*-tert-butylcyclohexanone in certain years. Specific aldehydes and ketones were significantly influenced by harvest year. For 2-heptanone and 2-nonanone, significant yeast × harvest year interactions were observed, affecting the extent but not the direction of the effect of yeast.

Table 5. Concentrations (μg/L) of norisoprenoids found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
N01	Theaspirane isomer	B	1536	1540	1.38	0.93	5.04	4.67	n.s.	**22**	n.s.
N02	Vitispirane isomer	B, C	1537	1543	**3.09** [a]	**2.18** [b]	0.50	0.58	**SCE**	**21**	*
N03	α-Ionene	B, C	1559	1565	0.43	0.42	1.23	1.39	n.s.	**22**	n.s.
N04	β-Cyclocitral	A, B, C	1629	1630	**0.31** [a]	**0.18** [b]	**0.29** [a]	**0.21** [b]	**SCE**	n.s.	*
N05	Safranal	B, C	1654	1648	0.20	0.20	0.25	0.24	n.s.	**22**	n.s.
N06	Ionene derivative n.i.	B, C	1704	-	0.15	0.14	0.29	0.34	n.s.	**22**	n.s.
N07	Actinidol ethyl ether I	B, C	1705	1690	n.d.	n.d.	1.33	1.38	-	-	-
N08	Actinidol ethyl ether II	B, C	1737	1715	n.d.	n.d.	0.74	0.74	-	-	-
N09	1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	A, B, C	1722	1722	0.17	0.09	0.34	0.37	n.s.	**22**	n.s.
N10	1,5,8-Trimethyl-1,2-dihydronaphthalene	B, C	1754	1751	1.84	1.60	n.d.	n.d.	-	-	-
N11	*cis*-β-Damascenone	B, C	1771	1774	**1.95** [b]	**3.21** [a]	**1.47** [b]	**1.70** [a]	**SPA**	**21**	n.s.
N12	*trans*-β-Damascenone	B, C	1829	1829	**21.65** [b]	**40.39** [a]	**14.84** [b]	**16.86** [a]	**SPA**	**21**	*
N13	*trans*-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB)	B, C	1835	1832	0.48	0.26	0.95	1.04	n.s.	**22**	n.s.
N14	γ-Methylionone	B, C	1855	-	n.d.	n.d.	0.04	0.04	-	-	-
N15	β-Ionone	B, C	1942	1933	n.d.	n.d.	0.05	0.06	-	-	-
N16	Actinidol isomer	B, C	1947	1927	0.31	0.34	n.d.	n.d.	-	-	-

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

3.4.4. Alcohols

The formation of major higher alcohols during fermentation involves two biosynthetic pathways: the conversion of sugars via pyruvate and the catabolism of amino acids via the Ehrlich pathway. While the former process typically yields lower concentrations,

the Ehrlich pathway is a primary source. It includes the transamination of amino acids into α-keto acids, their decarboxylation into aldehydes, and reduction of aldehydes into higher alcohols [48]. Major higher alcohols produced in fermentation form a basis of wine aroma. In moderate amounts, they add depth and complexity to the wine's character. However, when their total concentration exceeds 350 mg/L, they can have a negative impact with their medicinal and solvent-like odours. Numerous previous studies have demonstrated a significant impact of yeast species on higher alcohol production in wine fermentations [1]. C_6-alcohols, on the other hand, arise from the breakdown of lipids, specifically long-chain fatty acids. This process occurs through a series of enzymatic reactions, primarily during grape harvesting and the initial processing steps. C_6-alcohols are carriers of herbaceous odours that generally have little impact on wine aroma due to their relatively high perception thresholds.

Table 6. Concentrations (µg/L, if not otherwise indicated) of aldehydes found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by targeted gas chromatography with flame-ionization detection (GC/FID) # and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
AD01	Acetaldehyde (mg/L) #	A	<1100	714	**18.1** [b]	**44.7** [a]	**35.3** [b]	**54.8** [a]	**SPA**	**22**	n.s.
AD02	Isobutanal	B, C	<1100	833	**0.00** [a]	**0.00** [b]	n.d.	n.d.	-	-	-
AD03	2-methyl-2-butenal	B, C	<1100	1095	n.d.	n.d.	0.48	0.54	-	-	-
AD04	Heptanal	B, C	1184	1187	**4.41** [a]	**1.30** [b]	n.d.	n.d.	-	-	-
AD05	Octanal	B, C	1294	1281	0.28	0.19	1.32	1.67	n.s.	**22**	n.s.
AD06	Nonanal	B, C	1399	1403	16.1	15.3	28.6	28.4	n.s.	n.s.	n.s.
AD07	2-Octenal	B, C	1436	1427	n.d.	n.d.	0.11	0.12	-	-	-
AD08	Decanal	A, B, C	1503	1504	5.46	4.77	4.72	8.02	n.s.	n.s.	n.s.
AD09	2-Nonenal	B, C	1543	1540	0.58	1.58	0.14	0.18	n.s.	**21**	n.s.
AD10	Undecanal	A, B, C	1608	1610	0.82	0.23	0.63	0.95	n.s.	n.s.	n.s.
AD11	2-Methyl-5-isopropenyl-1-cyclopenten-1-carboxaldehyde	B, C	1693	1691	n.d.	n.d.	0.10	0.09	-	-	-
AD12	Dodecanal	B, C	1716	1713	1.24	0.69	0.64	0.72	n.s.	n.s.	n.s.
AD13	2-(Acetoxy)-propanal	B	1829	-	1.41	0.18	**1.32** [a]	**0.64** [b]	**SCE**	n.s.	n.s.
AD14	2,6,6-Trimethyl-1-cyclohexene-1-acrolein	B	1933	-	0.17	0.18	0.66	0.75	n.s.	**21**	n.s.

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. Significant differences are highlighted in bold.

Several major fermentation alcohols, such as 1-propanol and isobutanol in 2021, 2-phenylethanol in 2022, and isoamyl alcohol in both harvest years, were found in higher concentrations in SCE wines (Table 8). This suggests that *S. cerevisiae* exhibited greater activity for some or all enzymes involved in the Ehrlich pathway, such as transaminases, decarboxylases, and alcohol dehydrogenases. It also implies that the mentioned higher alcohols may have a stronger influence on the aroma of *S. cerevisiae* compared to *S. paradoxus* fermented wines. However, because the impact of yeast varied for each higher alcohol

depending on the harvest year, it is likely that additional factors also had a significant effect. Similar differences between *S. cerevisiae* and *S. paradoxus* fermentations were observed by Majdak et al. [12] and Orlić et al. [3], except for 1-propanol, which generally showed higher concentrations in wines fermented by *S. paradoxus* [3]. In contrast, Costantini et al. [5] did not observe significant differences in isoamyl alcohol and 2-phenylethanol levels between *S. cerevisiae* and *S. paradoxus* fermented wines.

Table 7. Concentrations (μg/L) of ketones found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
KE01	2-Heptanone	B	1179	1181	**4.82** [a]	**1.30** [b]	**15.59** [a]	**2.54** [b]	**SCE**	**22**	*
KE02	Acetoin	A, B, C	1282	1285	**8.78** [b]	**41.39** [a]	4.17	3.66	**SPA**	**21**	*
KE03	6-Methyl-5-hepten-2-one	B, C	1345	1343	0.78	0.73	0.96	1.27	n.s.	n.s.	n.s.
KE04	Ethyl hexyl ketone	B, C	1362	1362	n.d.	n.d.	0.11	0.11	-	-	-
KE05	2-Nonanone	A, B, C	1392	1392	**220.1** [a]	**115.3** [b]	**39.70** [a]	**10.54** [b]	**SCE**	**21**	*
KE06	1-Hydroxy-3-methyl-2-butanone	B	1450	-	**1.12** [a]	**0.85** [b]	n.d.	n.d.	-	-	-
KE07	3-(Acetoxy)-4-methyl-2-pentanone	B	1466	-	**0.33** [b]	**0.67** [a]	**0.23** [b]	**0.62** [a]	**SPA**	n.s.	n.s.
KE08	2-Decanone	B, C	1498	1503	**1.69** [a]	**1.34** [b]	**2.12** [a]	**1.57** [b]	**SCE**	**22**	n.s.
KE09	2-Ethyl-3-methoxy-2-cyclopentenone	B	1553	-	1.00	1.17	**0.39** [b]	**0.52** [a]	**SPA**	**21**	n.s.
KE10	3-Undecanone	B, C	1570	1586	0.33	0.27	0.24	0.32	n.s.	n.s.	n.s.
KE11	6-Methyl-3,5-heptadiene-2-one	B, C	1577	1596	n.d.	n.d.	0.08	0.10	-	-	-
KE12	2,4-Nonanedione	B	1583	-	n.d.	n.d.	**0.44** [a]	**0.14** [b]	-	-	-
KE13	2-Undecanone	B, C	1598	1598	**9.90** [a]	**3.94** [b]	2.37	1.73	**SCE**	**21**	*
KE14	2,2,4,5-Tetramethylhex-5-en-3-one	B	1599	-	n.d.	n.d.	0.37	0.37	-	-	-
KE15	*p*-tert-Butylcyclohexanone	B, C	1641	1645	**0.47** [a]	**0.38** [b]	1.50	1.59	n.s.	**22**	n.s.
KE16	1,2-Dihydroxycyclobutene-3,4-dione	B	1672	-	0.51	0.47	**0.71** [b]	**1.17** [a]	**SPA**	**22**	*
KE17	2-Dodecanone	B, C	1710	1709	**0.73** [a]	**0.51** [b]	**0.84** [a]	**0.62** [b]	**SCE**	**22**	n.s.
KE18	2,6-Di-tert-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one	B, C	2099	2094	n.d.	n.d.	0.12	0.15	-	-	-

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

Most C_6-alcohols were unaffected, with the exception of *cis*-2-hexen-1-ol, which was present at higher concentrations in SCE wines in both years. Among minor alcohols, SCE-21 wines had higher levels of 1-pentanol, 2-decanol, and 2-undecanol than SPA-21 wines, reflecting similar trends observed for the related aldehydes (Table 6). SCE-22 wines exhibited increased concentrations of 2,7-dimethyl-2,6-octadien-1-ol, 2-methyl-5-nonanol, 1-undecanol, and 1-dodecanol. 3-Ethoxy-1-propanol and 2-nonanol were significantly more abundant in SCE wines across both harvest years (Table 8). SPA wines, on the other hand, showed higher levels of 6-methyl-5-hepten-2-ol, 1-octanol, and *cis*-6-nonen-1-ol in both years, as well as higher levels of 3-octanol, 1-heptanol, 3-nonanol, 3-ethyl-4-methylpentan-1-ol, two 2,3-butanediol isomers, *trans*,*cis*-3,6-nonadien-1-ol, and 1-decanol in specific years. In 2021 wines, the higher levels of 2,3-butanediol corresponded to the increased levels of acetoin. It is possible that in 2021, the conditions were more favourable for this specific metabolic pathway, which involves converting acetaldehyde to acetoin through the action of pyruvate decarboxylase via the acetaldehyde-TPP complex, followed by

acetoin transformation into 2,3-butanediol. In the majority of significant cases, 2021 wines contained higher concentrations of higher alcohols than 2022 wines.

Table 8. Concentrations (μg/L, if not otherwise indicated) of alcohols found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by targeted gas chromatography with flame-ionization detection (GC/FID) #, targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
AL01	Methanol (mg/L) #	A	<1100	911	60.20	59.99	39.49	41.52	n.s.	**21**	n.s.
AL02	1-Propanol (mg/L) #	A	n.d.	1035	**23.53** [a]	**18.43** [b]	20.57	18.25	**SCE**	n.s.	n.s.
AL03	Isobutanol (mg/L) #	A, B, C	1090	1098	**14.49** [a]	**12.75** [b]	16.93	14.63	**SCE**	**22**	n.s.
AL04	Isoamyl alcohol (mg/L) #	A, B, C	1229	1229	**164.9** [a]	**131.8** [b]	**160.1** [a]	**128.0** [b]	**SCE**	n.s.	n.s.
AL05	1-Pentanol	B, C	1245	1244	**12.58** [a]	**9.29** [b]	n.d.	n.d.	-	-	-
AL06	3-Methyl-3-buten-1-ol	B, C	1245	1244	0.73	0.55	0.64	0.70	n.s.	n.s.	n.s.
AL07	Isohexanol	B, C	1314	1309	**54.87** [a]	**25.60** [b]	**25.61** [a]	**20.10** [b]	**SCE**	**21**	*
AL08	2-Heptanol	A, B, C	1319	1312	**9.17** [a]	**2.96** [b]	**6.59** [a]	**1.93** [b]	**SCE**	**21**	n.s.
AL09	2-Methyl-2-buten-1-ol	B, C	1319	1320	0.29	0.30	n.d.	n.d.	-	-	-
AL10	3-Methylpentanol	A, B, C	1329	1322	144.65	124.08	46.57	52.39	n.s.	**21**	n.s.
AL11	1-Hexanol (mg/L) ‡	A, B, C	1356	1357	1.52	1.71	1.17	1.33	n.s.	**21**	n.s.
AL12	*trans*-3-Hexen-1-ol ‡	A, B, C	1366	1361	75.36	78.05	45.12	46.19	n.s.	**21**	n.s.
AL13	3-Ethoxy-1-propanol	B, C	1377	1379	**23.99** [a]	**0.17** [b]	**44.26** [a]	**0.47** [b]	**SCE**	**22**	*
AL14	*cis*-3-Hexen-1-ol ‡	A, B, C	1389	1389	42.37	40.13	56.17	54.67	n.s.	**22**	n.s.
AL15	3-Octanol	A, B, C	1392	1393	1.20	1.29	**0.51** [b]	**0.63** [a]	**SPA**	**21**	*
AL16	*cis*-2-Hexen-1-ol ‡	A, B, C	1416	1413	**17.85** [a]	**13.54** [b]	**16.28** [a]	**13.09** [b]	**SCE**	n.s.	n.s.
AL17	1-Octen-3-ol	B, C	1450	1452	21.70	20.09	15.60	17.34	n.s.	**21**	n.s.
AL18	1-Heptanol	B, C	1456	1457	16.58	19.27	**5.91** [b]	**8.61** [a]	**SPA**	**21**	n.s.
AL19	2,7-Dimethyl-2,6-octadien-1-ol	B	1457	-	n.d.	n.d.	**0.16** [a]	**0.05** [b]	-	-	-
AL20	6-Methyl-5-hepten-2-ol	A, B, C	1461	1460	**0.16** [b]	**0.24** [a]	**0.33** [b]	**0.45** [a]	**SPA**	**22**	n.s.
AL21	1-(2-Methoxy-1-methylethoxy)-2-propanol	B, C	1478	1478	n.d.	n.d.	0.18	0.30	-	-	-
AL22	2-Methyl-5-nonanol	B	1478	-	n.d.	n.d.	**0.29** [a]	**0.12** [b]	-	-	-
AL23	*cis*,*cis*-1,5-Octadien-3-ol	B, C	1483	1488	n.d.	n.d.	0.18	0.21	-	-	-
AL24	2-Ethyl-1-hexanol	B, C	1487	1490	12.08	12.68	37.43	47.97	**SPA**	**22**	n.s.
AL25	3-Nonanol	B, C	1492	1493	0.37	0.41	**0.29** [b]	**0.36** [a]	**SPA**	**21**	n.s.
AL26	*cis*-4-Hepten-1-ol	B, C	1499	1502	n.d.	n.d.	0.65	0.88	-	-	-
AL27	3-Ethyl-4-methylpentan-1-ol	B, C	1509	1506	1.61	1.77	**1.83** [b]	**2.23** [a]	**SPA**	**22**	n.s.
AL28	2-Nonanol	A, B, C	1520	1518	**69.54** [a]	**46.95** [b]	**38.36** [a]	**13.37** [b]	**SCE**	**21**	n.s.
AL29	1-(2-Methoxypropoxy)-2-propanol	B, C	1521	1532	n.d.	n.d.	0.75	0.97	-	-	-
AL30	2,3-Butanediol I	A, B, C	1537	1541	**63.92** [b]	**169.8** [a]	**1.00**	1.18	**SPA**	**21**	*
AL31	1-Octanol	B, C	1553	1558	**34.09** [b]	**60.30** [a]	**27.19** [b]	**42.50** [a]	**SPA**	**21**	*
AL32	2,3-Butanediol II	A, B, C	1573	1576	**383.4** [b]	**676.5** [a]	0.69	1.50	**SPA**	**21**	*
AL33	2-Methyl-5-nonanol	B	1575	-	0.44	0.47	21.06	25.56	n.s.	**22**	n.s.
AL34	*cis*-3-Octen-1-ol	B	1575	-	0.12	0.13	0.10	0.11	n.s.	n.s.	n.s.
AL35	*trans*-2-Octen-1-ol	A, B, C	1615	1618	1.65	1.73	0.34	0.38	n.s.	**21**	n.s.
AL36	2-Decanol	B, C	1618	1622	**0.46** [a]	**0.27** [b]	0.50	0.09	**SCE**	n.s.	n.s.
AL37	1-Nonanol	A, B, C	1660	1661	3.72	4.95	2.62	3.00	n.s.	**21**	n.s.
AL38	*cis*-6-Nonen-1-ol	B, C	1716	1714	**0.89** [b]	**1.08** [a]	**0.95** [b]	**1.37** [a]	**SPA**	**22**	*
AL39	2-Undecanol	B, C	1722	1723	**5.26** [a]	**2.86** [b]	n.d.	n.d.	-	-	-
AL40	*trans*,*cis*-3,6-Nonadien-1-ol	B, C	1730	1762	n.d.	n.d.	**0.04** [b]	**0.07** [a]	-	-	-
AL41	3,5-Dimethyl-4-heptanol	B	1742	-	0.32	0.43	n.d.	n.d.	-	-	-
AL42	*trans*,*cis*-2,6-Nonadien-1-ol	B, C	1749	1750	n.d.	n.d.	0.04	0.05	-	-	-
AL43	4-tert-Butylcyclohexanol	B	1749	-	n.d.	n.d.	0.65	0.82	-	-	-
AL44	1-Decanol	A, B, C	1766	1767	5.83	6.73	**3.63** [b]	**4.94** [a]	**SPA**	**21**	n.s.
AL45	1,3-Propanediol	B, C	1785	1789	0.48	0.45	n.d.	n.d.	-	-	-
AL46	*cis*-4-Decen-1-ol	B, C	1797	1797	0.16	0.28	0.20	0.23	**SPA**	n.s.	n.s.
AL47	1-Undecanol	B, C	1865	1871	0.41	0.28	**0.36** [a]	**0.25** [b]	n.s.	n.s.	n.s.

Table 8. *Cont.*

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
AL48	2-Phenylethanol (mg/L) ‡	A, B, C	1891	1893	34.56	30.45	**19.07** [a]	**14.64** [b]	**SCE**	**21**	n.s.
AL49	1,4-Butanediol	B, C	1918	1911	1.11	1.37	0.45	0.52	n.s.	**21**	n.s.
AL50	2-Ethyl-2-(hydroxymethyl)-1,3-propanediol	B	1926	-	0.20	0.23	n.d.	n.d.	-	-	-
AL51	1-Dodecanol	B, C	1968	1973	1.90	1.41	**1.97** [a]	**1.54** [b]	**SCE**	n.s.	n.s.
AL52	2-Ethyl-1-dodecanol	B, C	2092	2090	n.d.	n.d.	0.13	0.09	-	-	-
AL53	2-Phenoxyethanol	B, C	2147	2144	0.93	0.54	3.36	1.29	n.s.	n.s.	n.s.

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

3.4.5. Acids

Major wine volatile fatty acids in wine, including saturated linear short- to medium-chain butyric, hexanoic, octanoic, and decanoic acids, are produced through a series of enzymatically catalysed reactions. These reactions involve the elongation of acetyl-CoA units, primarily driven by acetyl-CoA carboxylase in the fatty acid synthase (FAS) complex. On the other hand, saturated branched short-chain fatty acids are generated through the catabolism of amino acids through the Ehrlich pathway, similar to their higher alcohol analogues [48]. The biosynthesis of fatty acids is strongly influenced by yeast species used in fermentation [1].

Higher levels of octanoic acid were found in SCE wines across both years, while the same wines contained higher levels of hexanoic and decanoic acid compared to SPA wines in specific years (Table 9). Previous studies have reported inconsistent findings regarding the differences in medium-chain fatty acid levels between fermentations conducted with *S. cerevisiae* and *S. paradoxus* [5,12]. Orlić et al. [4] observed varying results among *S. paradoxus* strains. Some strains produced higher levels of hexanoic acid than *S. cerevisiae*, while others showed similar production levels. Octanoic acid levels varied significantly, and decanoic acid production was consistently lower in *S. paradoxus* than in *S. cerevisiae* fermentations. Conversely, Álvarez et al. [13] found higher levels of medium-chain fatty acids in *S. paradoxus* wines. The authors hypothesized that this increase may be due to a greater carbon flux toward acetyl-CoA formation in *S. paradoxus*. This process contributes to fatty acid synthesis and cell membrane remodelling, as a strategy for improved ethanol tolerance of *S. paradoxus* which is less adapted to alcoholic fermentation than *S. cerevisiae*. Other acids found to be more abundant in SCE wines were propanoic and isohexanoic acid in 2021 and nonanoic acid in 2022 (Table 9). Higher levels of isobutyric and 2-methylbutyric acid were found in SPA-21 compared to SCE-21 wines. The differences in isovaleric acid were significantly affected by harvest year, with higher values found in SCE-21 and SPA-22 wines, respectively. The noted divergence in fatty acid production with respect to their origin (FAS complex vs. Ehrlich pathway) suggests differing regulatory mechanisms between the two yeasts studied. Several acids also showed variation due to harvest year.

3.4.6. Ethyl Esters

Esters are key contributors to wine aroma, providing pleasant fruity and floral odours. Ethyl esters are formed through the condensation reaction between ethanol and activated fatty acids catalysed by alcohol acetyl transferases. Major linear medium-chain ethyl esters

are formed in the FAS complex simultaneously to their fatty acid analogues, while short branched-chain ethyl esters are the product of the esterification of fatty acids formed in the Ehrlich pathway. The synthesis of ethyl esters is regulated by a series of enzymes coded by the corresponding genes expressed in a yeast-species-dependent manner. However, their formation depends more on the concentrations of substrates than on the activity of enzymes involved [1,48,50]. The balance between ester synthesis and hydrolysis by yeast enzymes is crucial. While yeasts produce esters, they also produce esterases that are responsible for their hydrolysis.

Table 9. Concentrations (μg/L, if not otherwise indicated) of acids found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
AC01	Propanoic acid	A, B, C	1537	1540	**5.02** [a]	**3.36** [b]	7.42	6.62	n.s.	**22**	n.s.
AC02	Isobutyric acid	A, B, C	1570	1570	**1.95** [b]	**3.11** [a]	11.81	13.68	n.s.	**22**	n.s.
AC03	Pivalic acid	B, C	1581	1579	1.73	1.39	2.17	2.35	n.s.	n.s.	n.s.
AC04	4-Methyl-3-pentenoic acid	B	1595	-	1.50	2.14	n.d.	n.d.	-	-	-
AC05	Butyric acid (mg/L) ‡	A, B, C	1617	1612	1.59	1.45	1.11	1.04	n.s.	**22**	n.s.
AC06	2-Propenoic acid	B	1641	-	0.74	0.75	1.18	1.21	n.s.	**22**	n.s.
AC07	Isovaleric acid	A, B, C	1672	1675	**181.2** [a]	**128.5** [b]	**30.03** [b]	**56.14** [a]	n.s.	**21**	*
AC08	2-Methylbutyric acid	B, C	1675	1675	**61.10** [b]	**100.8** [a]	n.d.	n.d.	-	-	-
AC09	2-Methylpropenoic acid	B	1697	-	0.15	0.10	n.d.	n.d.	-	-	-
AC10	Pentanoic acid	A, B, C	1741	1751	3.24	2.65	3.64	4.89	n.s.	n.s.	n.s.
AC11	2-Butenoic acid	B, C	1780	1773	n.d.	n.d.	0.39	0.38	-	-	-
AC12	Isohexanoic acid	B, C	1810	1809	**0.39** [a]	**0.27** [b]	n.d.	n.d.	-	-	-
AC13	Hexanoic acid (mg/L) ‡	A, B, C	1617	1828	7.28	6.30	**7.44** [a]	**5.20** [b]	**SCE**	n.s.	n.s.
AC14	*trans*-3-Hexenoic acid	B, C	1942	1948	n.d.	n.d.	0.04	0.05	-	-	-
AC15	2-Ethylhexanoic acid	B, C	1953	1960	3.71	3.12	3.44	3.36	n.s.	n.s.	n.s.
AC16	Heptanoic acid	A, B, C	1954	1955	4.62	4.51	5.57	5.98	n.s.	n.s.	n.s.
AC17	*trans*-2-Hexenoic acid	B, C	1968	1967	0.53	0.46	0.73	0.96	n.s.	**22**	*
AC18	Octanoic acid (mg/L) ‡	A, B, C	2043	2042	**7.66** [a]	**5.49** [b]	**9.07** [a]	**5.58** [b]	**SCE**	n.s.	n.s.
AC19	3-Hydroxypivalic acid	B	2099	-	n.d.	n.d.	0.22	0.39	-	-	-
AC20	3-Octenoic acid	B	2120	-	1.67	0.82	0.65	0.69	n.s.	n.s.	n.s.
AC21	Nonanoic acid	A, B, C	2168	2168	21.43	39.04	**37.86** [a]	**24.07** [b]	n.s.	n.s.	n.s.
AC22	Decanoic acid (mg/L) ‡	A, B, C	2257	2258	2.79	2.62	**1.59** [a]	**0.92** [b]	**SCE**	**21**	n.s.
AC23	9-Decenoic acid	B, C	2330	2335	13.41	16.99	1.95	2.74	n.s.	**21**	n.s.
AC24	Undecanoic acid	B, C	2346	2359	0.04	0.01	0.33	0.32	n.s.	**22**	n.s.
AC25	Tridecanoic acid	B, C	2590	2651	n.d.	n.d.	0.21	0.46	-	-	-
AC26	Tetradecanoic acid	B, C	2696	2693	0.63	0.52	0.12	0.10	n.s.	**21**	n.s.

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

Higher concentrations of major linear medium-chain ethyl esters, such as ethyl butyrate, hexanoate, and octanoate, were found in control SCE compared to SPA wines. This effect was observed in a specific harvest year or in both harvest years, depending on the ester, as confirmed by one-way or two-way ANOVA (Table 10). The higher levels of these esters in SCE wines imply their greater contribution to the fruity and floral components of the aroma of *S. cerevisiae* compared to *S. paradoxus* fermented wines. An exception

was ethyl decanoate, for which significant differences were not observed, although a tendency towards higher levels in SCE wines was also observed. In previous studies, certain *S. paradoxus* strains showed the ability to produce amounts of major linear medium-chain ethyl esters comparable to those obtained by *S. cerevisiae* control fermentations [3]. Other authors observed superior concentrations produced by *S. paradoxus* [12,13]. These inconsistencies may have stemmed from differences in yeast strains, grape juice composition, and fermentation parameters between the studies.

Table 10. Concentrations (μg/L, if not otherwise indicated) of ethyl esters found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
EE01	Ethyl propanoate ‡	B, C	<1100	949	**26.51** [a]	**16.11** [b]	**40.90** [a]	**28.84** [b]	**SCE**	**22**	n.s.
EE02	Ethyl isobutyrate ‡	A, B, C	<1100	962	19.55	23.53	12.35	13.01	n.s.	**21**	n.s.
EE03	Ethyl butyrate (mg/L) ‡	A, B, C	1030	1030	**0.60** [a]	**0.48** [b]	0.51	0.47	**SCE**	n.s.	n.s.
EE04	Ethyl 2-methylbutyrate ‡	A, B, C	1049	1049	**3.94** [b]	**6.77** [a]	**3.88** [b]	**5.80** [a]	**SPA**	n.s.	n.s.
EE05	Ethyl 3-methylbutyrate ‡	A, B, C	1065	1065	**12.02** [a]	**8.74** [b]	10.61	9.60	**SCE**	n.s.	n.s.
EE06	Ethyl pentanoate	B, C	1133	1140	n.d.	n.d.	**0.56** [a]	**0.30** [b]	-	-	-
EE07	Ethyl *trans*-2-butenoate	B, C	1160	1158	**19.35** [a]	**6.59** [b]	**44.04** [a]	**23.70** [b]	**SCE**	**22**	n.s.
EE08	Ethyl hexanoate (mg/L) ‡	A, B, C	1242	1236	**1.39** [a]	**0.93** [b]	0.93	0.83	**SCE**	**21**	n.s.
EE09	Ethyl pyruvate	B, C	1272	1267	8.06	9.18	**8.20** [b]	**16.93** [a]	**SPA**	**21**	*
EE10	Ethyl *trans*-3-hexenoate	B, C	1307	1292	4.11	3.22	4.82	5.54	n.s.	**22**	n.s.
EE11	Ethyl 3-ethoxypropionate	B, C	1336	1320	n.d.	n.d.	**0.37** [a]	**0.00** [b]	-	-	-
EE12	Ethyl heptanoate	B, C	1340	1342	**8.84** [a]	**6.09** [b]	9.59	7.54	**SCE**	n.s.	n.s.
EE13	Ethyl 2-hexenoate I	B, C	1350	1357	14.68	12.80	21.45	22.60	n.s.	**22**	n.s.
EE14	Ethyl 2-hexenoate II	B, C	1361	1357	0.17	0.18	n.d.	n.d.	-	-	-
EE15	Ethyl *trans*-4-heptenoate	B, C	1378	1377	n.d.	n.d.	0.18	0.18	-	-	-
EE16	Ethyl 2-hydroxyisovalerate	B, C	1426	1426	n.d.	n.d.	0.01	0.09	-	-	-
EE17	Ethyl octanoate (mg/L) ‡	A, B, C	1435	1435	1.65	0.98	0.86	0.67	**SCE**	n.s.	n.s.
EE18	Ethyl acetylacetate	B, C	1462	1466	**0.41** [a]	**0.04** [b]	**0.40** [a]	**0.05** [b]	**SCE**	n.s.	n.s.
EE19	Ethyl 7-octenoate	B, C	1482	1486	**2.14** [a]	**0.78** [b]	n.d.	n.d.	-	-	-
EE20	Ethyl *trans*,*trans*-2,4-hexadienoate	B, C	1510	1505	n.d.	n.d.	0.11	0.12	-	-	-
EE21	Ethyl *trans*-4-octenoate	B	1516	-	n.d.	n.d.	0.09	0.08	-	-	-
EE22	Ethyl 3-hydroxybutyrate	B, C	1520	1524	**2.48** [a]	**0.91** [b]	4.61	5.15	n.s.	**22**	*
EE23	Ethyl nonanoate	B, C	1537	1535	7.98	10.42	12.21	11.08	n.s.	n.s.	n.s.
EE24	Ethyl 2-hydroxy-4-methylvalerate	B, C	1542	1547	**13.95** [a]	**9.89** [b]	7.34	10.81	n.s.	**21**	*
EE25	Ethyl 2-octanoate	B, C	1559	1557	0.39	0.35	0.22	0.23	n.s.	**21**	n.s.
EE26	Ethyl *trans*-6-nonenoate	B, C	1583	1592	n.d.	n.d.	0.04	0.04	-	-	-
EE27	Ethyl levulate	B, C	1612	1614	n.d.	n.d.	0.03	0.05	-	-	-
EE28	Ethyl decanoate (mg/L) ‡	A, B, C	1637	1638	2.42	2.07	1.77	1.21	n.s.	**21**	n.s.
EE29	Ethyl *trans*-4-decenoate	B, C	1672	1680	**0.31** [b]	**0.43** [a]	n.d.	n.d.	-	-	-
EE30	Ethyl 3-hydroxyhexanoate	B, C	1685	1677	**0.24** [b]	**0.31** [a]	n.d.	n.d.	-	-	-
EE31	Ethyl 9-decenoate I	B, C	1697	1697	**43.45** [b]	**104.3** [a]	3.31	11.61	**SPA**	**21**	n.s.
EE32	Ethyl 3-acetoxyhexanoate	B, C	1729	1712	**2.04** [a]	**0.89** [b]	n.d.	n.d.	-	-	-
EE33	Ethyl 9-decenoate II	B, C	1729	1712	**0.49** [b]	**1.07** [a]	0.05	0.04	**SPA**	**21**	n.s.
EE34	Ethyl 5-oxohexanoate	B	1737	-	n.d.	n.d.	**0.09** [a]	**0.04** [b]	-	-	-
EE35	Ethyl undecanoate	B, C	1747	1739	0.55	0.60	0.63	0.81	n.s.	n.s.	n.s.
EE36	Ethyl 2-decenoate	B, C	1766	1750	0.15	0.15	n.d.	n.d.	-	-	-
EE37	Ethyl 4-hydroxybutyrate	B, C	1804	1796	**9.21** [a]	**3.55** [b]	**3.36** [a]	**2.36** [b]	**SCE**	**21**	*
EE38	Ethyl dodecanoate	A, B, C	1847	1850	191.9	214.9	n.d.	n.d.	-	-	-
EE39	Ethyl 3-acetoxyoctanoate	B, C	1897	1898	2.13	2.32	n.d.	n.d.	-	-	-
EE40	Ethyl tridecanoate	B, C	1949	1950	n.d.	n.d.	0.36	0.43	-	-	-
EE41	Ethyl tetradecanoate	B, C	2054	2054	8.30	6.67	5.95	5.23	n.s.	n.s.	n.s.
EE42	Ethyl 3-hydroxydecanoate	B, C	2104	2102	3.24	3.06	**3.17** [a]	**1.64** [b]	**SCE**	**21**	*
EE43	Ethyl pentadecanoate	B, C	2149	2151	n.d.	n.d.	0.34	0.37	-	-	-
EE44	Ethyl hexadecanoate	B, C	2251	2241	21.30	13.04	7.38	7.42	n.s.	**21**	n.s.

Table 10. *Cont.*

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022		Y	H	Y × H
					SCE	SPA	SCE	SPA			
EE45	Ethyl *cis*-11-hexadecenoate	B, C	2281	2236	0.80	0.91	n.d.	n.d.	-	-	-
EE46	Ethyl octadecanoate	B, C	2463	2464	0.32	0.16	n.d.	n.d.	-	-	-

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

Table 11. Concentrations (μg/L, if not otherwise indicated) of acetate esters found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by targeted gas chromatography with flame-ionization detection (GC/FID) #, targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022		Y	H	Y × H
					SCE	SPA	SCE	SPA			
AE01	Methyl acetate ‡	B, C	<1100	813	22.63	19.95	**11.01** ^{b}	**15.93** ^{a}	n.s.	**21**	*
AE02	Ethyl acetate (mg/L) #	A, B	<1100	890	**26.33** ^{b}	**49.95** ^{a}	36.14	44.80	**SPA**	n.s.	*
AE03	Propyl acetate	B, C	<1100	982	43.93	20.79	n.d.	n.d.	-	-	-
AE04	Isobutyl acetate ‡	A, B, C	<1100	1009	111.5	126.1	**79.97** ^{b}	**91.04** ^{a}	**SPA**	**21**	n.s.
AE05	Butyl acetate ‡	B, C	<1100	1064	**16.96** ^{b}	**41.00** ^{a}	23.32	26.85	**SPA**	n.s.	*
AE06	Isoamyl acetate (mg/L) ‡	A, B, C	1133	1133	**6.63** ^{a}	**5.34** ^{b}	**4.86** ^{a}	**4.41** ^{b}	**SCE**	**21**	*
AE07	Pentyl acetate	B, C	1169	1185	8.29	7.57	n.d.	n.d.	-	-	-
AE08	3-Methyl-3-buten-1-yl acetate	B, C	1190	1190	n.d.	n.d.	**0.33** ^{a}	**0.24** ^{b}	-	-	-
AE09	*trans*-2-penten-1-yl acetate	B	1220	1228	n.d.	n.d.	**0.32** ^{a}	**0.17** ^{b}	-	-	-
AE10	Hexyl acetate (mg/L) ‡	A, B, C	1272	1272	0.50	0.46	0.30	0.27	n.s.	**21**	n.s.
AE11	*cis*-3-Hexenyl acetate ‡	B, C	1314	1308	185.6	198.5	**57.03** ^{a}	**43.88** ^{b}	n.s.	**21**	n.s.
AE12	*trans*-3-Hexenyl acetate ‡	B, C	1320	1315	132.9	141.2	**54.76** ^{a}	**42.86** ^{b}	n.s.	**21**	n.s.
AE13	3-Ethoxypropyl acetate	B	1361	-	**11.88** ^{a}	**0.15** ^{b}	**11.04** ^{a}	**0.36** ^{b}	**SCE**	n.s.	n.s.
AE14	Heptyl acetate	B, C	1378	1374	n.d.	n.d.	1.47	1.25	-	-	-
AE15	3-Methylheptyl acetate	B, C	1385	1395	0.85	0.75	3.55	3.48	n.s.	**22**	n.s.
AE16	2-Ethyl-1-hexyl acetate	B	1480	-	14.84	15.41	1.50	1.32	n.s.	**21**	n.s.
AE17	*cis*-2-tert-Butylcyclohexyl acetate	B	1570	-	5.61	0.96	0.41	0.35	n.s.	n.s.	n.s.
AE18	*trans*,*trans*-2,4-Octadienyl acetate	B	1570	-	0.26	0.22	n.d.	n.d.	-	-	-
AE19	*cis*-6-Nonen-1-yl acetate	B, C	1634	1634	0.85	0.58	n.d.	n.d.	-	-	-
AE20	*cis*-4-tert-butylcyclohexyl acetate	B, C	1688	1675	0.44	0.58	0.14	0.15	n.s.	**21**	n.s.
AE21	Diol acetate n.i.	B	1741	-	**44.51** ^{a}	**17.30** ^{b}	**31.00** ^{a}	**13.12** ^{b}	**SCE**	**21**	n.s.
AE22	1,3-Butanediol diacetate	B, C	1785	1768	3.71	1.67	n.d.	n.d.	-	-	-
AE23	2-Phenethyl acetate (mg/L) ‡	A, B, C	1803	1801	0.45	0.49	**0.26** ^{a}	**0.20** ^{b}	n.s.	**21**	n.s.

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

Table 12. Concentrations (μg/L, if not otherwise indicated) of other esters found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022		Y	H	Y × H
					SCE	SPA	SCE	SPA			
OE01	Propyl formate	B, C	<1100	916	0.63	0.95	n.d.	n.d.	-	-	-
OE02	Methyl hexanoate	A, B, C	1179	1188	15.59	9.56	**37.59** [a]	**22.52** [b]	**SCE**	**21**	n.s.
OE03	Isoamyl isobutyrate	B, C	1188	1183	0.40	0.37	**3.32** [a]	**1.27** [b]	**SCE**	**22**	*
OE04	Isoamyl acrylate	B	1215	-	n.d.	n.d.	**0.39** [a]	**0.03** [b]	-	-	-
OE05	Isoamyl butyrate	B, C	1266	1266	**11.84** [a]	**5.34** [b]	**7.83** [a]	**5.56** [b]	**SCE**	**21**	*
OE06	Isoamyl isovalerate	B, C	1298	1294	**0.41** [a]	**0.23** [b]	0.44	0.43	**SCE**	**22**	*
OE07	Propyl hexanoate	B, C	1324	1319	**3.04** [a]	**1.27** [b]	**4.84** [a]	**2.03** [b]	**SCE**	**22**	n.s.
OE08	Ethyl lactate (mg/L) ‡	A, B, C	1341	1341	11.99	13.02	26.58	32.69	n.s.	**22**	n.s.
OE09	Hexyl propanoate	B, C	1345	1342	**0.40** [a]	**0.20** [b]	n.d.	n.d.	-	-	-
OE10	Isobutyl hexanoate	B, C	1356	1357	**2.29** [a]	**1.15** [b]	1.55	1.70	**SCE**	n.s.	*
OE11	Methyl octanoate	B, C	1397	1399	**79.69** [a]	**45.06** [b]	84.16	68.84	**SCE**	**22**	n.s.
OE12	Butyl hexanoate	B, C	1419	1416	**0.08** [b]	**0.14** [a]	n.d.	n.d.	-	-	-
OE13	2-Ethyl-1-hexyl propanoate	B	1452	-	**1.40** [a]	**1.01** [b]	3.38	3.22	n.s.	**22**	n.s.
OE14	Isoamyl hexanoate	A, B, C	1461	1458	**27.12** [a]	**12.86** [b]	8.18	8.48	**SCE**	**21**	*
OE15	Methyl 2-hydroxy-4-methylpentanoate	B, C	1477	1470	0.86	0.83	n.d.	n.d.	-	-	-
OE16	Isoamyl pyruvate	B	1478	-	n.d.	n.d.	**0.72** [b]	**1.17** [a]	-	-	-
OE17	Propyl octanoate	B, C	1520	1530	**1.64** [a]	**0.80** [b]	**1.27** [a]	**0.69** [b]	**SCE**	**21**	n.s.
OE18	2-Ethylhexyl butyrate	B	1521	-	n.d.	n.d.	0.29	0.30	-	-	-
OE19	Hexyl propyl oxalate	B	1525	-	**1.01** [b]	**2.08** [a]	n.d.	n.d.	-	-	-
OE20	Isobutyl octanoate	B, C	1553	1551	**0.53** [a]	**0.33** [b]	0.96	1.11	n.s.	**22**	n.s.
OE21	Isoamyl lactate	B, C	1570	1572	2.36	1.86	**10.45** [b]	**16.88** [a]	**SPA**	**22**	*
OE22	Diethyl malonate	B, C	1581	1582	**0.68** [a]	**0.58** [b]	0.86	0.87	n.s.	**22**	n.s.
OE23	Methyl decanoate	B, C	1598	1599	6.70	6.39	12.40	10.29	n.s.	**22**	n.s.
OE24	*trans*-3-Hexenyl tiglate	B	1605	-	n.d.	n.d.	0.23	0.21	-	-	-
OE25	Ethyl methyl succinate	B, C	1635	1642	**0.61** [b]	**0.86** [a]	1.35	1.06	n.s.	**22**	*
OE26	Diethyl methyl succinate	B	1643	-	n.d.	n.d.	0.09	0.13	-	-	-
OE27	Diethyl fumarate	B, C	1654	1647	0.18	0.20	0.20	0.22	n.s.	n.s.	n.s.
OE28	Isoamyl octanoate	B, C	1660	1657	**33.31** [a]	**20.06** [b]	43.82	34.95	**SCE**	**22**	n.s.
OE29	Diethyl succinate	A, B, C	1679	1672	**231.1** [b]	**321.8** [a]	330.7	369.1	**SPA**	**22**	n.s.
OE30	Propyl decanoate	B, C	1729	1743	0.41	0.35	**1.05** [a]	**0.56** [b]	**SCE**	**22**	*
OE31	Vinyl decanoate	B	1730	-	n.d.	n.d.	**2.49** [a]	**0.88** [b]	-	-	-
OE32	Isobutyl decanoate	B, C	1755	1756	n.d.	n.d.	0.25	0.25	-	-	-
OE33	Ethyl propyl succinate	B, C	1762	1767	n.d.	n.d.	**0.67** [a]	**0.48** [b]	-	-	-
OE34	Diisobutyl succinate	B	1766	-	**0.20** [b]	**0.25** [a]	n.d.	n.d.	-	-	-
OE35	Diethyl glutarate	B, C	1785	1780	0.21	0.25	0.45	0.56	n.s.	**22**	n.s.
OE36	2-Phenethyl formate	B, C	1797	1806	1.53	1.62	0.68	0.67	n.s.	**21**	n.s.
OE37	Ethyl butyl succinate	B, C	1797	1820	**0.23** [b]	**0.33** [a]	**0.39** [b]	**0.61** [a]	**SPA**	**22**	n.s.
OE38	Methyl dodecanoate	B, C	1810	1806	0.21	0.23	n.d.	n.d.	-	-	-
OE39	Hexyl octanoate	B, C	1816	1803	**0.11** [a]	**0.09** [b]	0.12	0.10	**SCE**	n.s.	n.s.
OE40	Isoamyl decanoate	B, C	1866	1864	21.07	17.78	10.58	8.36	n.s.	**21**	n.s.
OE41	Phenylethyl isobutyrate	B, C	1888	1896	**1.04** [a]	**0.55** [b]	n.d.	n.d.	-	-	-
OE42	Ethyl isoamyl succinate	B, C	1899	1897	3.80	3.99	6.54	7.03	n.s.	**22**	n.s.
OE43	Phenethyl isovalerate	B, C	1968	1983	2.32	1.99	**1.58** [a]	**1.03** [b]	**SCE**	**21**	n.s.
OE44	Isopropyl tetradecanoate	B, C	2042	2045	n.d.	n.d.	0.19	0.15	-	-	-
OE45	Diethyl malate	B, C	2047	2048	1.60	1.27	1.52	1.30	**SCE**	n.s.	n.s.

Table 12. *Cont.*

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
OE46	Isoamyl dodecanoate	B, C	2069	2071	1.44	1.30	0.36	0.34	n.s.	**21**	n.s.
OE47	Diethyl 2-hydroxyglutarate	B, C	2161	2171	0.29	0.25	1.08	0.76	n.s.	**22**	n.s.
OE48	1-Phenylethyl isobutyrate	B	2176	-	**0.92** [a]	**0.67** [b]	**0.39** [a]	**0.32** [b]	**SCE**	**21**	*
OE49	Isopropyl palmitate	B, C	2237	2237	n.d.	n.d.	0.35	0.32	-	-	-
OE50	Ethyl phenyllactate	B, C	2281	2273	0.73	0.97	**0.58** [b]	**0.84** [a]	**SPA**	n.s.	n.s.
OE51	Diethyl tartrate	B, C	2324	2358	n.d.	n.d.	0.13	0.09	-	-	-
OE52	Ethyl hydrogen succinate	B, C	2380	2367	76.88	69.83	88.81	67.96	n.s.	n.s.	n.s.
OE53	2-Phenethyl octanoate	B, C	2388	2373	1.88	1.53	n.d.	n.d.	-	-	-
OE54	Triethyl citrate	B, C	2463	2461	0.09	0.08	0.03	0.05	n.s.	n.s.	n.s.

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

Significant differences were noted for a range of other esters, with most showing higher concentrations in control SCE wines. These included isoamyl isovalerate, hexyl propanoate, isobutyl hexanoate, methyl octanoate, 2-ethyl-1-hexyl propanoate, isoamyl hexanoate, isobutyl octanoate, diethyl malonate, isoamyl octanoate, hexyl octanoate, and phenylethyl isobutyrate in 2021, and methyl hexanoate, isoamyl isobutyrate, isoamyl acrylate, propyl decanoate, vinyl decanoate, ethyl propyl succinate, and phenethyl isovalerate in 2022. The concentrations of isoamyl butyrate, propyl hexanoate, propyl octanoate, and 1-phenylethyl isobutyrate were consistently higher in SCE wines in both years. The concentration differences in many of these esters aligned with variations in their alcohol and acid precursors in the wines studied (Tables 8 and 9). In addition to the mentioned lactates and succinates, SPA wines were characterized by higher concentrations of a few other esters, such as butyl hexanoate and hexyl propyl oxalate in 2021 and isoamyl pyruvate in 2022 (Table 12). Some esters from this group were affected by the harvest year. Several esters had a higher concentration in wines from 2021 and even more so in wines from 2022.

3.4.7. Benzenoids

Certain benzenoids are derived from the phenylpropanoid synthesis pathway in grapes, while others are produced during fermentation from aromatic amino acids like phenylalanine and tyrosine. They can be further transformed by yeast, forming benzenoid aldehydes, alcohols, acids, esters, and other derivatives. Some benzenoid compounds can enter grapes and wines as a result of environmental pollution. Despite a high number of identified benzenoid compounds, only a few were affected by yeast species (Table 13). Control SCE treatment wines had higher concentrations of toluene, 1,2,3-trimethylbenzene, 4-ethyl-*m*-xylene, ethyl phenethyl ether, acetophenone, ethyl *o*-methylbenzoate, 3-methylacetophenone, 2-methylnaphthalene, 1-methylnaphthalene, and phenylacetic acid in 2021, and ethyl hydrocinnamate and 3-hydroxy-4-phenyl-2-butanone in 2022. The concentrations of benzyl acetate and ethyl 2-phenylacetate were higher in SCE wines of both harvest years. The higher levels of several derivates from the phenylalanine yeast metabolism, such as ethyl phenethyl ether, phenylacetic acid, and 2-phenylacetate, coincided with the higher levels of major phenylalanine products in SCE wines, such as 2-phenylethanol (Table 6) and 2-phenethyl acetate (Table 11). SPA wines contained

higher concentrations of several benzenoids, such as 3,4-dimethylacetophenone in 2021, 4-ethylbenzoic acid and *p*-tert-butylbenzoic acid in 2022, as well as 2′,5′-dimethylcrotonophenone in both years. The effect of harvest year was significant for particular compounds from this group.

Table 13. Concentrations (μg/L) of benzenoids found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
BE1	Toluene	B, C	<1100	1048	**4.15** [a]	**2.99** [b]	5.07	4.32	n.s.	n.s.	n.s.
BE2	*p*-Xylene	B, C	1137	1136	2.63	8.17	9.74	6.53	n.s.	n.s.	n.s.
BE3	*m*-Xylene	B, C	1176	1160	n.d.	n.d.	3.66	2.52	-	-	-
BE4	*o*-Xylene	B, C	1179	1189	2.04	2.40	0.70	0.38	n.s.	**21**	n.s.
BE5	*p*-Ethyltoluene	B, C	1220	1231	n.d.	n.d.	0.30	0.28	-	-	-
BE6	Styrene	B, C	1258	1262	9.75	8.48	n.d.	n.d.	-	-	-
BE7	Cardene	B, C	1259	1269	7.94	7.44	0.98	1.59	n.s.	**21**	n.s.
BE8	*p*-Cymene	B, C	1276	1273	5.79	5.93	5.76	5.40	n.s.	n.s.	n.s.
BE9	*o*-Propyltoluene	B, C	1305	1305	n.d.	n.d.	1.95	1.81	-	-	-
BE10	1,2,3-Trimethylbenzene	B, C	1345	1344	**0.56** [a]	**0.34** [b]	0.91	0.89	n.s.	**22**	n.s.
BE11	2-Ethyl-*o*-xylene	B, C	1366	1362	0.97	0.72	n.d.	n.d.	-	-	-
BE12	4-Ethyl-*m*-xylene	B, C	1377	1373	**1.34** [a]	**0.61** [b]	n.d.	n.d.	-	-	-
BE13	3,5-Diethyltoluene	B, C	1405	1409	n.d.	n.d.	0.89	0.93	-	-	-
BE14	2-(4′-Methylphenyl)-propanal	B	1408	-	0.53	0.49	n.d.	n.d.	-	-	-
BE15	*p*-Cymenene	B, C	1419	1414	0.37	0.29	0.64	0.72	n.s.	**22**	n.s.
BE16	*n*-Amylbenzene	B, C	1420	1421	n.d.	n.d.	0.45	0.41	-	-	-
BE17	*m*-di-tert-Butylbenzene	B, C	1435	1436	0.36	0.08	n.d.	n.d.	-	-	-
BE18	Durene	B, C	1445	1435	**5.30** [a]	**3.78** [b]	**7.75** [a]	**6.48** [b]	**SCE**	**22**	n.s.
BE19	p-Ethylstyrene	B, C	1459	1462	0.16	0.17	0.81	0.89	n.s.	**22**	n.s.
BE20	3,3-Dimethoxy-1-phenylpropane-1,2-dione	B	1471	-	**4.36** [a]	**2.40** [b]	**1.49** [b]	**1.89** [a]	**SCE**	**21**	*
BE21	α-Phenyldiethyl ether	B	1482	-	1.01	0.76	n.d.	n.d.	-	-	-
BE22	β-Methylindan	B	1482	-	0.57	0.50	0.90	0.91	n.s.	**22**	n.s.
BE23	1H-Indene	B, C	1489	1479	n.d.	n.d.	0.13	0.12	-	-	-
BE24	1,2,3,4-Tetramethylbenzene	B, C	1503	1505	0.64	0.51	n.d.	n.d.	-	-	-
BE25	1-Phenylhexane	B, C	1525	1524	1.05	1.14	n.d.	n.d.	-	-	-
BE26	Ethyl phenethyl ether	B	1526	-	**0.88** [a]	**0.74** [b]	6.99	7.69	n.s.	**22**	n.s.
BE27	Benzaldehyde	A, B, C	1531	1538	4.60	4.78	0.37	0.34	n.s.	**21**	n.s.
BE28	1,2,3,4-Tetrahydronaphthalene	B, C	1533	1525	n.d.	n.d.	0.16	0.19	-	-	-
BE29	2,3-Dihydro-2,2-dimethylindene	B	1533	-	n.d.	n.d.	0.24	0.24	-	-	-
BE30	2,4,6-Trimethylstyrene	B	1542	-	0.58	0.72	0.14	0.14	n.s.	**21**	n.s.
BE31	*m*-Divinylbenzene	B, C	1570	1541	0.58	0.65	0.41	0.42	n.s.	**21**	n.s.
BE32	Benzonitrile	B, C	1610	1614	1.11	1.19	n.d.	n.d.	-	-	-
BE33	2-Methylbenzaldehyde	B, C	1629	1622	0.84	0.85	1.62	1.69	n.s.	**22**	n.s.
BE34	Methyl benzoate	B, C	1629	1624	0.13	0.13	n.d.	n.d.	-	-	-
BE35	2-Phenylethanal	A, B, C	1654	1656	50.80	39.55	35.94	35.16	n.s.	**21**	n.s.
BE36	4-Methylbenzaldehyde	B, C	1655	1655	0.51	0.47	0.82	0.85	n.s.	**22**	n.s.
BE37	Acetophenone	A, B, C	1660	1660	**3.24** [a]	**2.26** [b]	4.01	3.96	n.s.	**22**	n.s.
BE38	Ethyl benzoate	B, C	1672	1680	6.90	6.68	5.90	5.69	n.s.	**21**	n.s.
BE39	Estragole	B, C	1679	1676	0.14	0.12	0.21	0.21	n.s.	**22**	n.s.
BE40	4-Ethylbenzaldehyde	B, C	1716	1714	1.29	1.41	1.71	1.74	n.s.	**22**	n.s.
BE41	Benzyl acetate	B, C	1735	1739	**0.31** [a]	**0.24** [b]	**0.24** [a]	**0.17** [b]	**SCE**	**21**	n.s.
BE42	Ethyl phenyl ketone	B, C	1735	1744	0.17	0.17	0.27	0.30	n.s.	**22**	n.s.
BE43	Octyl benzene	B, C	1741	1741	1.47	1.66	1.77	1.62	n.s.	n.s.	n.s.
BE44	Ethyl *o*-methylbenzoate	B, C	1747	1751	**0.17** [a]	**0.12** [b]	0.22	0.21	**SCE**	**21**	*
BE45	*p*-Methoxyanisole	B, C	1747	1752	0.80	0.84	1.05	1.05	n.s.	**22**	n.s.
BE46	1-Methylene-1H-indene	B, C	1749	1763	n.d.	n.d.	2.44	2.23	-	-	-
BE47	2-Phenylisopropanol	B, C	1766	1770	0.11	0.10	0.40	0.42	n.s.	**22**	n.s.
BE48	4-Methylacetophenone	B, C	1766	1763	0.18	0.20	0.33	0.35	n.s.	**22**	n.s.
BE49	Methyl salicylate	B, C	1785	1789	1.83	1.61	0.37	0.36	n.s.	**21**	n.s.
BE50	3-Methylacetophenone	B, C	1785	1786	**0.28** [a]	**0.22** [b]	4.45	4.72	n.s.	**22**	n.s.

Table 13. *Cont.*

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
BE51	Ethyl 2-phenylacetate	B, C	1791	1788	**13.75** [a]	**8.47** [b]	**8.21** [a]	**6.59** [b]	**SCE**	**21**	*
BE52	Benzyl propanoate	B, C	1799	1788	n.d.	n.d.	0.03	0.04	-	-	-
BE53	1-Phenylethanol	B, C	1812	1812	n.d.	n.d.	0.07	0.08	-	-	-
BE54	3,4-Dimethylacetophenone	B, C	1841	1823	**0.30** [b]	**0.38** [a]	0.44	0.44	n.s.	**22**	n.s.
BE55	2′,4′,6′-Trimethylacetophenone	B	1849	-	n.d.	n.d.	0.14	0.11	-	-	-
BE56	2-Methylnaphthalene	B, C	1860	1856	**0.24** [a]	**0.20** [b]	0.30	0.28	n.s.	**22**	n.s.
BE57	4-Ethylacetophenone	B, C	1872	1867	0.22	0.26	0.27	0.28	n.s.	**22**	n.s.
BE58	Benzyl alcohol	A, B, C	1879	1877	2.64	2.68	2.58	2.79	n.s.	n.s.	n.s.
BE59	Ethyl hydrocinnamate	B, C	1887	1880	n.d.	n.d.	**0.54** [a]	**0.30** [b]	-	-	-
BE60	1-Methylnaphthalene	B, C	1897	1893	**0.15** [a]	**0.11** [b]	n.d.	n.d.	-	-	-
BE61	Isoamyl benzoate	B, C	1913	1916	n.d.	n.d.	0.10	0.11	-	-	-
BE62	*trans*-4-Phenylbut-3-en-2-one	B	1961	-	0.57	0.63	n.d.	n.d.	-	-	-
BE63	Biphenyl	B, C	1992	2012	n.d.	n.d.	0.53	0.57	-	-	-
BE64	1-Ethyl-2,3-dihydro-1H-indene	B	1992	-	n.d.	n.d.	0.09	0.09	-	-	-
BE65	4-Phenylbutenone	B, C	1997	2032	0.31	0.35	n.d.	n.d.	-	-	-
BE66	2′,5′-Dimethylcrotonophenone	B	1997	-	**0.17** [b]	**0.28** [a]	**0.68** [b]	**0.83** [a]	**SPA**	**22**	n.s.
BE67	2,6-Diisopropylnaphthalene	B, C	2230	2242	n.d.	n.d.	0.07	0.05	-	-	-
BE68	4-Acetylbenzaldehyde	B	2235	-	0.85	0.89	n.d.	n.d.	-	-	-
BE69	3-Hydroxy-4-phenyl-2-butanone	B, C	2260	2259	n.d.	n.d.	**0.06** [a]	**0.04** [b]	-	-	-
BE70	1-Hydroxy-2,4-di-tert-butylbenzene	B, C	2299	2294	n.d.	n.d.	43.78	44.38	-	-	-
BE71	1,3-Diacetylbenzene	B, C	2332	2333	n.d.	n.d.	0.19	0.17	-	-	-
BE72	1-Phthalanone	B, C	2357	2356	n.d.	n.d.	0.11	0.09	-	-	-
BE73	Benzoic acid	B, C	2438	2432	5.11	7.20	4.66	5.24	n.s.	n.s.	n.s.
BE74	1H-Indole	B, C	2455	2454	0.80	0.82	0.27	0.33	n.s.	**21**	n.s.
BE75	*p*-Isopropenylphenol	B	2455	-	0.07	0.08	n.d.	n.d.	-	-	-
BE76	Benzophenone	B, C	2482	2470	n.d.	n.d.	0.09	0.08	-	-	-
BE77	3-Methylbenzoic acid	B	2532	-	0.18	0.14	0.31	0.27	n.s.	**22**	n.s.
BE78	Phenylacetic acid	B, C	2560	2560	**0.62** [a]	**0.46** [b]	n.d.	n.d.	-	-	-
BE79	3-Phenylbutyric acid	B	2628	-	0.04	0.26	n.d.	n.d.	-	-	-
BE80	4-Ethylbenzoic acid	B	2635	-	n.d.	n.d.	**0.16** [b]	**0.29** [a]	-	-	-
BE81	3-(1-Methylethyl)benzoic acid	B	2642	-	0.03	0.11	0.04	0.03	n.s.	n.s.	n.s.
BE82	2,4,6-Trimethylbenzoic acid	B	2714	-	0.06	0.08	n.d.	n.d.	-	-	-
BE83	*p*-tert-Butylbenzoic acid	B	2749	-	n.d.	n.d.	**0.21** [b]	**0.51** [a]	-	-	-

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

3.4.8. Furanoids and Lactones

Furanoids typically arise from the degradation of sugars, whereas lactones are cyclic esters created through the intramolecular condensation of carboxylic and alcohol functional groups of hydroxycarboxylic acids. The most significant lactones in wine are those that form the most stable structures, particularly five-membered ring γ-lactones, followed by six-membered ring δ-lactones. The factors influencing the synthesis and transformation of these two compound classes during winemaking are not well understood. It is known that γ-butyrolactone can be produced by yeast from γ-aminobutyric acid and α-aminoglutaric acid, while it is presumed that some lactones may be synthesized de novo during fermentation [48,51].

The differences between wines produced by the two investigated yeasts were generally more expressed in harvest 2021 (Table 14). Control SCE-21 wine was characterized by higher concentrations of γ-butyrolactone, γ-ethoxybutyrolactone, δ-hexalactone, γ-octalactone,

δ-octalactone, γ-decalactone, δ-decalactone, δ-dodecalactone, and mevalonic acid δ-lactone compared to SPA-21 wine. SCE treatment produced more 2-butyltetrahydrofuran in both harvest years. SPA wines contained more furfural, ethyl 2-furoate, and solerone in 2021, 5-(1-hydroxyethyl)-2(3H)-furanone in 2022, and β-methyl-γ-butyrolactone and 4-(1-hydroxyethyl)-γ-butyrolactone in both harvest years. Particular furanoids and lactones were significantly affected by the harvest year, with some having higher levels in 2021 and others in 2022 wines.

Table 14. Concentrations (μg/L) of furanoids and lactones found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
FL01	2-Pentylfuran	B, C	1229	1231	0.86	0.71	n.d.	n.d.	-	-	-
FL02	2-Butyltetrahydrofuran	B	1267	-	**35.47** [a]	**25.44** [b]	**21.66** [a]	**14.86** [b]	**SCE**	**21**	n.s.
FL03	*cis*-2-(1-Pentenyl)furan	B	1399	-	n.d.	n.d.	0.07	0.07	-	-	-
FL04	Furfural	A, B, C	1472	1460	**2.24** [b]	**3.31** [a]	7.69	9.36	**SPA**	**22**	n.s.
FL05	*cis*-2-(2-Pentenyl)furan	B	1505	-	n.d.	n.d.	0.14	0.13	-	-	-
FL06	3-Acetyl-2,5-dimethylfuran	B	1577	-	n.d.	n.d.	0.28	0.13	-	-	-
FL07	γ-Valerolactone	B, C	1616	1617	0.23	0.25	0.41	0.86	n.s.	n.s.	n.s.
FL08	Ethyl 2-furoate	B, C	1629	1628	**26.15** [b]	**34.89** [a]	33.59	41.47	**SPA**	**22**	n.s.
FL09	γ-Butyrolactone	B, C	1635	1634	**38.59** [a]	**16.18** [b]	n.d.	n.d.	-	-	-
FL10	γ-Hexalactone	B, C	1710	1710	2.99	2.52	3.02	3.41	n.s.	n.s.	n.s.
FL11	α-Methyl-γ-crotonolactone	B, C	1729	1726	0.19	0.19	0.18	0.21	n.s.	n.s.	n.s.
FL12	γ-Ethoxybutyrolactone	B, C	1735	1728	**0.21** [a]	**0.17** [b]	0.15	0.18	n.s.	**22**	*
FL13	γ-Crotonolactone	B, C	1766	1758	0.47	0.50	0.43	0.50	n.s.	n.s.	n.s.
FL14	5-Ethyl-2(5H)-furanone	B, C	1768	1754	n.d.	n.d.	0.04	0.06	-	-	-
FL15	δ-Hexalactone	B, C	1804	1798	**0.66** [a]	**0.52** [b]	1.52	1.53	n.s.	**22**	n.s.
FL16	γ-Heptalactone	B, C	1815	1811	0.33	0.34	0.45	0.42	n.s.	n.s.	n.s.
FL17	β-Methyl-γ-butyrolactone	B	1816	-	**1.85** [b]	**2.29** [a]	**0.18** [b]	**0.28** [a]	**SPA**	**21**	*
FL18	γ-Octalactone	B, C	1926	1924	**5.05** [a]	**3.89** [b]	8.02	7.59	n.s.	**22**	n.s.
FL19	δ-Octalactone	A, B, C	1976	1976	**0.71** [a]	**0.53** [b]	0.97	0.89	n.s.	**22**	n.s.
FL20	Pantolactone	B, C	2035	2034	n.d.	n.d.	0.39	0.39	-	-	-
FL21	γ-Nonalactone	A, B, C	2040	2046	4.63	4.12	7.68	6.66	n.s.	**22**	n.s.
FL22	Solerone	B, C	2076	2096	**1.28** [b]	**1.68** [a]	0.08	0.07	**SPA**	**21**	*
FL23	2-Hydroxy-γ-butyrolactone	B	2076	-	0.11	0.27	n.d.	n.d.	-	-	-
FL24	γ-Decalactone	B, C	2154	2152	**2.45** [a]	**1.69** [b]	2.70	2.57	**SCE**	**22**	n.s.
FL25	δ-Decalactone	B, C	2197	2193	**0.71** [a]	**0.38** [b]	0.72	0.53	**SCE**	n.s.	n.s.
FL26	γ-Undecalactone	B, C	2235	2235	4.66	4.22	10.98	8.48	n.s.	**22**	n.s.
FL27	5-(1-Hydroxyethyl)-2(3H)-furanone, solerol isomer	B, C	2324	2343	n.d.	n.d.	**0.31** [b]	**0.56** [a]	-	-	-
FL28	γ-Dodecalactone	B, C	2380	2384	0.24	0.20	0.18	0.17	n.s.	**21**	n.s.
FL29	4-(1-Hydroxyethyl)-γ-butyrolactone	B, C	2386	2431	**1.33** [b]	**6.51** [a]	**1.42** [b]	**5.75** [a]	**SPA**	n.s.	n.s.
FL30	δ-Dodecalactone	B, C	2430	2423	**0.36** [a]	**0.16** [b]	0.24	0.17	**SCE**	**21**	*
FL31	γ-Tridecalactone	B, C	2488	2488	0.70	0.29	0.03	0.03	**SCE**	**21**	*
FL32	Mevalonic acid δ-lactone	B	2551	-	**0.21** [a]	**0.14** [b]	n.d.	n.d.	-	-	-

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

3.4.9. Sulfur-Containing Compounds

In wines, sulfur-containing compounds arise from multiple sources, particularly through yeast metabolism. This includes both the catabolic and anabolic processes involving sulfur-containing amino acids, such as methionine, cysteine, and their derivative homocysteine. The mentioned amino acids are formed by yeast using inorganic sulfur as a source [49,52]. The production of sulfur compounds in wine was previously shown to be significantly affected by the yeast strain used in fermentation [53].

Control SCE-21 wine contained higher concentrations of ethyl 3-methylthiopropanoate, isothiocyanatocyclohexane, methionol, benzothiazole, and sulfurol compared to SPA-21 wine. SCE treatment was consistent in producing higher levels of 2-thiophenecarboxaldehyde across two harvest years (Table 15). SPA treatment wines excelled in the production of propyl ethynyl sulfoxide in 2021 and 3-ethylthio-1-propanol in 2022. They had higher concentrations of both compounds tentatively identified as 3-[(2-hydroxyethyl)thio]-1-propanol in both harvest years. Sulfur-containing compounds mostly had higher levels in 2021 compared to 2022 harvest wines.

Table 15. Concentrations (μg/L) of sulfur containing compounds found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
SU01	Allyl isothiocyanate	B, C	1368	1353	n.d.	n.d.	0.13	0.19	-	-	-
SU02	S-Ethyl octanethioate	B	1525	-	12.88	22.74	11.03	10.74	**SPA**	**21**	*
SU03	2-Methylthioethanol	B, C	1527	1531	n.d.	n.d.	0.36	0.42	-	-	-
SU04	Dihydro-2-methyl-3(2H)-thiophenone	B, C	1531	1538	0.91	1.09	5.11	8.54	n.s.	**22**	n.s.
SU05	Propyl ethynyl sulfoxide	B	1559	-	**1.07** ^{b}	**1.80** ^{a}	n.d.	n.d.	-	-	-
SU06	Ethyl 3-methylthiopropanoate	B, C	1570	1571	**2.72** ^{a}	**1.73** ^{b}	1.22	1.06	**SCE**	**21**	*
SU07	Methionol acetate	B, C	1635	1627	2.67	2.54	1.01	0.92	n.s.	**21**	n.s.
SU08	Isothiocyanatocyclohexane	B, C	1679	1670	**0.79** ^{a}	**0.64** ^{b}	0.58	0.59	n.s.	**21**	n.s.
SU09	Ethyl methanesulfonate	B	1691	-	2.53	1.30	n.d.	n.d.	-	-	-
SU10	2-Thiophenecarboxaldehyde	B, C	1704	1701	**0.27** ^{a}	**0.16** ^{b}	**0.45** ^{a}	**0.32** ^{b}	**SCE**	**22**	n.s.
SU11	Methionol	A, B, C	1722	1717	**14.56** ^{a}	**10.08** ^{b}	4.76	4.15	**SCE**	**21**	*
SU12	3-[(2-Hydroxyethyl)thio]-1-propanol I	B	1779	-	**0.22** ^{b}	**2.95** ^{a}	**0.03** ^{b}	**0.25** ^{a}	**SPA**	**21**	*
SU13	3-Ethylthio-1-propanol	B, C	1780	1802	n.d.	n.d.	**0.03** ^{b}	**0.14** ^{a}	-	-	-
SU14	3-[(2-Hydroxyethyl)thio]-1-propanol II	B	1822	-	**0.08** ^{b}	**0.67** ^{a}	**0.03** ^{b}	**0.28** ^{a}	**SPA**	**21**	*
SU15	4-(Methylthio)-1-butanol	B, C	1841	1812	0.45	0.35	0.03	0.03	n.s.	**21**	n.s.
SU16	Benzothiazole	B, C	1962	1962	**0.71** ^{a}	**0.63** ^{b}	1.23	1.33	n.s.	**22**	n.s.
SU17	Sulfurol	B, C	2305	2302	**0.45** ^{a}	**0.24** ^{b}	0.18	0.21	**SCE**	**21**	*

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. An asterisk indicates a significant interaction between the two factors (Y × H). Significant differences are highlighted in bold.

3.4.10. Volatile Phenols

The most well-studied volatile phenols in wine, such as vinyl and ethyl phenols, derive from microbial activity. They are formed via the decarboxylation of hydroxycinnamic acids, such as ferulic and *p*-coumaric acid from grapes. *Saccharomyces cerevisiae* yeasts showed significant activity of cinnamate decarboxylases, which catalyse the conversion of hydroxycinammic acids into vinylphenols [54]. However, their conversion to ethylphenols by the action of vinylphenol reductases is most often associated with the spoilage yeast *Brettanomyces*

bruxellensis. Ethyl phenols are considered off-flavours, and their odours are often described as animal-like, medicinal, or sweaty. Various yeast species were previously shown to exhibit different degrees of decarboxylase activity [55], which was also noted for *S. paradoxus* [56].

One-way ANOVA revealed a statistically significant difference only for 4-tert-amylphenol, with a higher level found in SCE-21 than in SPA-21 wine (Table 16). Two-way ANOVA, on the other hand, showed a significantly higher concentration of 4-vinylguaiacol in SPA wines. Two-way ANOVA revealed a higher concentration of ferulic acid in control SCE wines. Based on that, it was assumed that the investigated *S. paradoxus* strain could have exhibited higher decarboxylation activity than *S. cerevisiae* control. In most cases, the concentrations of volatile phenols were higher in 2022 harvest wines.

3.4.11. Miscellaneous Compounds

Significant differences between the concentrations of miscellaneous compounds in the investigated wines were found only in 2021. Tridecane, pentadecane, and azulene levels were higher in SCE-21 wines, while those of 1-octen-3-ol methyl ether, *cis*-5-hydroxy-2-methyl-1,3-dioxane, and glutaconic anhydride were higher in SPA-21 wines (Table 17).

Table 16. Concentrations (μg/L) of volatile phenols found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022		Y	H	Y × H
					SCE	SPA	SCE	SPA			
VP01	Guaiacol	B, C	1866	1869	0.08	0.08	0.09	0.11	n.s.	**22**	n.s.
VP02	2,3,6-Trimethylphenol	B, C	2004	2028	0.07	0.08	0.09	0.07	n.s.	n.s.	n.s.
VP03	2-Methylphenol	B, C	2011	2011	0.09	0.08	0.14	0.16	n.s.	**22**	n.s.
VP04	Phenol	A, B, C	2011	2012	4.08	4.16	3.86	4.08	n.s.	n.s.	n.s.
VP05	4-Ethylguaiacol	B, C	2035	2033	n.d.	n.d.	0.05	0.32	-	-	-
VP06	2-Ethylphenol	B, C	2076	2071	0.10	0.10	0.17	0.14	n.s.	n.s.	n.s.
VP07	4-Ethylphenol	B, C	2177	2181	0.32	0.41	0.51	0.44	n.s.	n.s.	n.s.
VP08	4-Vinylguaiacol	A, B, C	2197	2196	0.71	2.68	4.76	7.06	**SPA**	**22**	n.s.
VP09	4-tert-Butylphenol	B	2284	-	n.d.	n.d.	0.17	0.22	-	-	-
VP10	4-Vinylphenol	B, C	2393	2406	0.61	1.05	n.d.	n.d.	-	-	-
VP11	4-tert-Amylphenol	B	2413	-	**0.19** [a]	**0.09** [b]	0.10	0.20	n.s.	n.s.	n.s.
VP12	Acetoguaiacone	B, C	2644	2664	n.d.	n.d.	0.08	0.07	-	-	-

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. Significant differences are highlighted in bold.

3.5. Multivariate Statistical Analysis

To better understand and visualize the key differences in the physico-chemical profiles of Malvazija istarska wines fermented with *S. cerevisiae* (SCE) and *S. paradoxus* (SPA) yeasts, which were consistent across two harvest years, multivariate statistical analyses were conducted using HCA and PLS-DA. To reduce the impact of variations due to harvest year, the data were normalized within each year. Two separate reduced datasets were analysed. The first dataset included variables, such as basic physico-chemical parameters (Table 1), PR proteins (Table 2), and grape-derived phenolic compounds (Table 3), which

showed statistically significant differences based on one-way ANOVA of the normalized data. The second dataset included 40 volatile compounds (Tables 4–17) selected for their strong statistical difference (the highest *F*-ratio values) determined by one-way ANOVA applied on the normalized data. Additional volatile compounds frequently cited as key wine odorants were also included in the second dataset.

Table 17. Concentrations (μg/L) of miscellaneous components found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS).

Co.	Volatile Compounds	ID	LRI_e	LRI_l	Differences in Concentration						
					One-Way ANOVA				Two-Way ANOVA		
					2021		2022				
					SCE	SPA	SCE	SPA	Y	H	Y × H
OC1	*trans*-2-Dodecene	B	1272	-	2.75	2.83	0.61	0.55	n.s.	**21**	n.s.
OC2	Tridecane	A, B, C	1303	1300	**12.36** [a]	**8.86** [b]	14.17	13.32	n.s.	**22**	n.s.
OC3	*trans*,*cis*-2,4-Dodecadiene	B, C	1405	1402	n.d.	n.d.	0.79	0.60	-	-	-
OC4	1-Octen-3-ol methyl ether	B	1411	-	**0.00** [b]	**0.43** [a]	n.d.	n.d.	-	-	-
OC5	2,6-Dimethyl-1,3,5,7-octatetraene I	B, C	1445	1446	2.14	1.66	4.42	4.25	n.s.	**22**	n.s.
OC6	2,6-Dimethyl-1,3,5,7-octatetraene II	B, C	1456	1460	3.70	3.46	n.d.	n.d.	-	-	-
OC7	1-Tetradecene	B, C	1477	1474	2.69	2.58	2.78	2.60	n.s.	n.s.	n.s.
OC8	*cis*-5-Hydroxy-2-methyl-1,3-dioxane	B, C	1498	1494	**0.24** [b]	**1.14** [a]	n.d.	n.d.	-	-	-
OC9	Pentadecane	A, B, C	1503	1500	**1.04** [a]	**0.79** [b]	n.d.	n.d.	-	-	-
OC10	Hexadecane	A, B, C	1599	1600	n.d.	n.d.	0.70	0.58	-	-	-
OC11	Edulan I	B, C	1612	1602	n.d.	n.d.	0.16	0.17	-	-	-
OC12	Dimethylmaleic anhydride	B, C	1741	1755	0.12	0.13	n.d.	n.d.	-	-	-
OC13	Azulene	B, C	1754	1746	**2.25** [a]	**1.77** [b]	n.d.	n.d.	-	-	-
OC14	*cis*-2-Methyl-7-octadecene	B	1866	-	0.14	0.12	n.d.	n.d.	-	-	-
OC15	(3-Methylphenyl) methanol, 2-methylpropyl ether	B	1968	-	0.50	0.50	n.d.	n.d.	-	-	-
OC16	Glutaconic anhydride	B	1997	-	**1.91** [b]	**2.45** [a]	1.79	1.92	**SPA**	**21**	n.s.
OC17	γ-Aminobutyrolactam	B, C	2035	2037	n.d.	n.d.	0.59	0.72	-	-	-

Abbreviations: Co.—compound's code; ID—identification of compounds: A—retention time accordant with that of a pure standard; B—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra electronic libraries or literature; C—linear retention index (LRI) accordant with an index from literature (compounds with only B in the ID column were considered tentatively identified); LRI_e—experimental linear retention index; LRI_l—linear retention index from literature; n.s.—not significant; n.d.—not detected. One-way ANOVA section: different superscript lowercase letters represent statistically significant differences between the two investigated wines determined by one-way ANOVA at $p < 0.05$ for each harvest year separately. Two-way ANOVA section: designations are reported representing yeast (SCE, SPA) and harvest year (21, 22) with higher concentration determined by two-way ANOVA at $p < 0.05$ with yeast (Y) and harvest year (H) as factors. Significant differences are highlighted in bold.

Using the first dataset, clear clustering of wines according to yeast species was achieved, as illustrated in the heatmap in Figure 1. The control SCE wines from both harvest years showed higher levels of organic acids, including malic acid, as well as several phenols, particularly *p*-coumaric acid and *trans*-resveratrol. These wines also had elevated levels of specific PR proteins, notably a group of chitinases. SPA wines were consistent in having higher levels of pH, glycerol, tartaric acid, volatile acidity, and particular thaumatin-like PR proteins, compared to control SCE wines.

PLS-DA analysis of the first dataset successfully differentiated wines from both harvest years based on yeast species (Figure 2a). The top fifteen volatile compounds with the highest VIP scores are presented in Figure 2b. Compounds with the highest VIP scores (>1) included *trans*-resveratrol, malic acid, citric acid, and *p*-coumaric acid, which were more characteristic of SCE wines. In contrast, SPA wines were distinguished by elevated levels of glycerol, pH, and volatile acidity.

Applying HCA to the second dataset, including volatile compounds, clearly separated and clustered the wines by yeast species (Figure 3). In general, control SCE wines from both harvest years produced higher levels of several important and well-known wine volatile aroma compounds. These included isobutanol, isoamyl alcohol, 2-phenylethanol, ethyl butyrate, ethyl 3-methylbutyrate, ethyl hexanoate, ethyl octanoate, isoamyl acetate, hexanoic acid, and decanoic acid, as well as various minor compounds from different chemical classes. In contrast, SPA wines produced by *S. paradoxus* were distinguished by higher levels of both β-damascenone isomers, acetaldehyde, isobutyric acid, ethyl 2-methylbutyrate, ethyl acetate, isobutyl acetate, esters of succinic and lactic acids, and 4-vinylguaiacol, along with several minor alcohols and sulfur compounds.

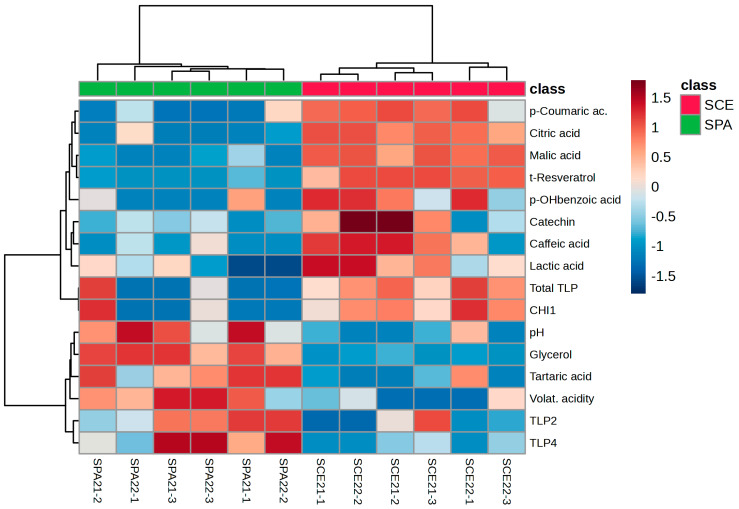
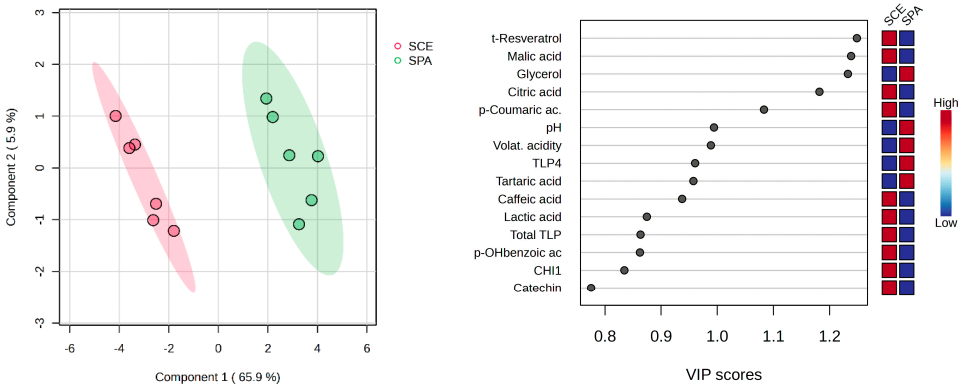


Figure 1. Hierarchical clustering analysis of Malvazija istarska wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years (2021, 2022) based on basic physico-chemical parameters, PR proteins, and phenolic compounds from grapes. Each row of the heatmap represents a specific parameter or a compound, while columns denote individual samples. Cell colours indicate compound abundance (normalized data): dark blue signifies low abundance, pale colours reflect medium abundance, and dark red denotes high abundance.



(**a**) (**b**)

Figure 2. (**a**) Differentiation of Malvazija istarska wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years according to yeast species in two-dimensional space by partial least squares−discriminant analysis (PLS−DA); (**b**) variable importance in projection (VIP) scores highlighting the most influential variables (physico-chemical parameters, PR proteins, grape-derived phenolic compounds; normalized data) contributing to the differentiation.

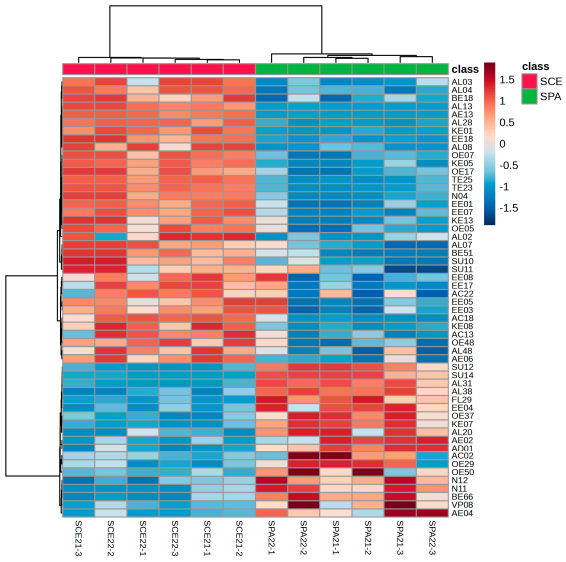
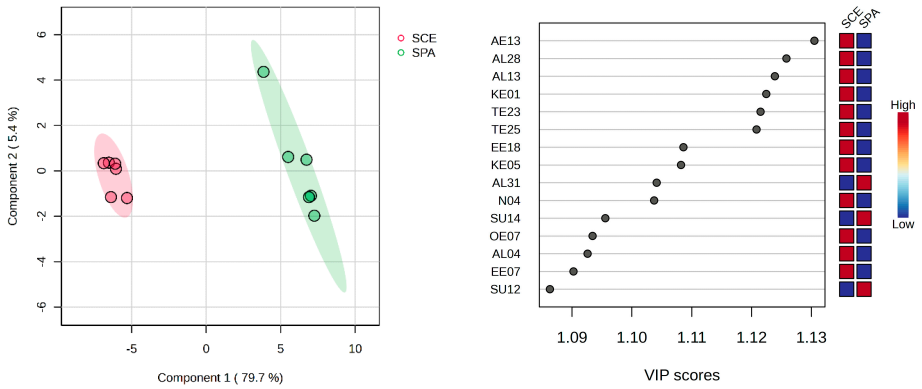


Figure 3. Hierarchical clustering analysis of Malvazija istarska wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years (2021, 2022) based on the composition of selected volatile compounds determined by GC/FID, GC/MS, and GC × GC/TOF-MS analysis. Each row of the heatmap represents a specific parameter or a compound, while columns denote individual samples. Cell colours indicate compound abundance (normalized data): dark blue signifies low abundance, pale colours reflect medium abundance, and dark red denotes high abundance. Compounds' codes correspond to those in Tables 4–17.

PLS-DA analysis of the second dataset achieved a clear separation of wines by yeast species across both harvest years (Figure 4a). The compounds contributing most to this distinction were primarily minor ones with unknown sensory significance. Compounds, such as 3-ethoxypropyl acetate, 2-nonanol, 3-ethoxy-1-propanol, 2-heptanone, epoxyterpinolene, *trans*-2-pinanol, ethyl acetylacetate, and 2-nonanone, were more characteristic of control SCE wines, while higher levels of 1-octanol and two compounds tentatively identified as 3-[(2-hydroxyethyl)thio]-1-propanol distinguished SPA wines.



(**a**) (**b**)

Figure 4. (**a**) Differentiation of Malvazija istarska wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years according to yeast species in two-dimensional space by partial least squares−discriminant analysis (PLS−DA); (**b**) variable importance in projection (VIP) scores highlighting the most influential variables (volatile compounds; normalized data) contributing to the differentiation. Compounds' codes correspond to those in Tables 4–17.

4. Conclusions

This study demonstrated that fermentation with the investigated *S. paradoxus* yeast strain significantly influenced the physico-chemical composition of Malvazija istarska wine compared to the standard *S. cerevisiae*. While the harvest year had a marked qualitative and quantitative impact, several distinctive effects of *S. paradoxus* fermentation were consistent across both seasons, suggesting the existence of metabolic features characteristic for this yeast. For instance, the decrease in malic acid and the increase in glycerol levels confirmed previous findings on *S. paradoxus* fermentation performance. This yeast was also associated with reduced levels of other organic acids and specific phenolic compounds like *p*-coumaric acid and *trans*-resveratrol. It caused a less pronounced reduction in pathogenesis-related chitinases than reported in earlier studies. On the other hand, *S. paradoxus* fermentation resulted in increased levels of volatile acidity, pH, and particular thaumatin-like proteins compared to control *S. cerevisiae* wines. With 474 volatile compounds identified, the in-depth GC × GC/MS-TOF analysis, combined with standard GC techniques, provided the most comprehensive insight into the changes to the wine volatile profile caused by *S. paradoxus* fermentation reported to date. The investigated *S. paradoxus* strain was found to produce slightly lower levels of several key odoriferous alcohols, fatty acids, and esters compared to *S. cerevisiae*. This suggests a potentially weaker expression of the corresponding aromas in these wines. Nevertheless, *S. paradoxus* produced higher levels of other important volatiles, including β-damascenone, acetaldehyde, isobutyric acid, ethyl 2-methylbutyrate, ethyl acetate, isobutyl acetate, various esters of succinic and lactic acids, and 4-vinylguaiacol, as well as numerous other compounds that may possibly contribute to wine sensory quality. These characteristics suggest that *S. paradoxus* produces a distinct aroma sensory profile and may be an interesting alternative in white wine production. It could be used either for standalone fermentation, the production of blending components, or as a fermentation starter. Variations observed between this study and prior research, alongside the observed significant effects of harvest year, highlight the need for further research to optimize the selection of *S. paradoxus* strains for improved oenological performance. Future studies should also focus on the response of *S. paradoxus* to different fermentation conditions and evaluate its potential for red winemaking.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app142311362/s1, Table S1: Concentration of parameters and compounds (means and standard deviations) found in Malvazija istarska white wines produced by fermentation with *Saccharomyces cerevisiae* (SCE) and *Saccharomyces paradoxus* (SPA) yeasts in two harvest years (2021, 2022).

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