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# Bottle Aging Affected Aromatic and Phenolic Wine Composition More than Yeast Starter Strains

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**Abstract:** Volatile and phenolic compounds play a key role in the sensory properties of wine, especially aroma and color. During fermentation, yeasts produce enzymes that affect the skin's phenolic compounds extraction and synthesize some of the most important wine volatile compounds. Generally, selected yeasts of the *Saccharomyces cerevisiae* (Sc) strains are inoculated, which are responsible for carrying out the wine fermentation, enhancing and highlighting its sensory characteristics and contributing to help achieve the wine typicity, according to the winemaker's criteria. After fermentation, all wines require aging in a bottle to modulate their composition and stability over time. Thus, four different Sc strains (Sc1–Sc4) were inoculated into tanks with Tempranillo grapes to carry out, in duplicate, their fermentation and subsequent aging in bottles (9 months), comparing the aromatic and phenolic composition between them. Results showed differences in the fermentation process (kinetic, ethanol yield), CI, TPI and content of alcohols, esters, anthocyanins, flavonols and flavanols in wines from the different Sc strains studied. Moreover, in the content in wines of most groups of aromas and phenols, except for total acetate esters and flavonols, aging in a bottle had more influence than the yeast strain used for fermentation.

**Keywords:** volatile compounds; phenolic compounds; yeasts strain; *Saccharomyces cerevisiae*; wine aging; alcoholic fermentation; malolactic fermentation; bottling; Tempranillo; color; aroma



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## 1. Introduction

In usual winemaking conditions, alcoholic fermentation starts when sugars and other nutrients required for yeast growth are released by crushing the grape. Moreover, with the purpose of ensuring the microbial stability and oxidative capacity of the wine, sulfur dioxide ($SO_2$) is commonly added during the fermentation process, reducing the population of must indigenous microorganisms. Thus, although grapes have their own indigenous yeasts able to carry out the must fermentation, many wines are still made by spontaneous fermentation. Therefore, these fermentations depend on both the wild yeasts present in must and the winery processing equipment, for the purpose of ensuring a correct, controlled and complete fermentation, as well as to enhance the wine sensory characteristics. Thus, modern winemaking is based on the use of selected yeast strains, mainly active dry yeasts of the *Saccharomyces cerevisiae* strain [1–3]. Wine fermentation is a very competitive scenario for microorganisms. Each strain of *S. cerevisiae* can have its own contribution to the wine sensory characteristics, and not all of them are equally adapted to all fermentation styles [4]. There are different factors involved, and among them, the widespread use of sulphiting agents, nutrient availability, osmotic pressure (ever increasing as a consequence of global climate warming), a relatively great total acidity and low pH, or the ethanol

release through fermentation, which determine the physiology and ecology of yeast during fermentation [4]. Thus, in enology, the use of selected yeast strains as active dry yeast is a widespread practice, preferably those yeast starters conditioned for fermentation grape varieties and conditions, wine style and characteristics that allow winemakers to develop and differentiate their products.

During fermentation, microorganisms and, particularly, *S. cerevisiae* catalyze the transformation of some grape primary aroma precursors and release various volatile compounds that constitute the secondary aroma [4]. Thus, yeast synthesizes the aromatic compounds that are quantitatively the most important for the aroma of wine, i.e., esters, higher alcohols, and acids, and qualitatively, in the case of esters [5–7]. In addition, although grapes are the main source of phenolic compounds, key compounds for the sensory properties of wines and linked to their positive properties for human health [8], the yeast strain that conducts the fermentation can also influence its composition, since the enzymes produced by the yeast affect the phenolic compounds extraction from the grape skins [9]. Further, some metabolites produced by yeasts, such as pyruvic acid and acetaldehyde, are involved in the polymerization reactions between phenolic compounds [10,11]. The importance of yeast strain in the wine composition and its sensory properties is, therefore, evident. Despite their relative genetic proximity, the wide metabolic diversity among wine *S. cerevisiae* strains makes their contribution to wine quality different and specific for each one, which explains the great number of strains on the market [4,12,13].

On the other hand, once the wine has been elaborated, it is bottled, and this stage is quite important in the wine life, since the wine quality and its properties, when it is consumed, depend on it. Thus, during storage, in general, phenolic compounds and, in particular, anthocyanins, flavonols, flavanols, and phenolic acids play a key role in the stabilization of the red wine color [14]. During the wine bottle aging, the volatile and phenolic compounds gradually suffer modifications, mainly due to reactions of oxidation, hydrolysis, esterification, polymerization, etc. [15,16]. These reactions, which modulate the composition of the wine, depend on a number of factors, including its initial composition at the time of bottling, the level of $SO_2$, storage conditions, storage temperature mainly, aging time, light exposure, and closure type [17–21].

Therefore, considering the importance of yeast strain and bottle aging for wine composition and quality, it is essential to study both factors together. Therefore, the purpose of this work was to study the influence of four *Saccharomyces cerevisiae* strains and bottle aging for 9 months on the aromatic and phenolic composition of Tempranillo wines.

## 2. Materials and Methods

### 2.1. Winemaking and Aging in Bottles

The trial was carried out with around 450 kg of Tempranillo grapes, in perfect sanitary conditions, harvested manually at their optimum maturation stage. At the cellar, the clusters were destemmed and crushed. The resulting paste and must were homogeneously distributed in eight tanks of 50 L, due to the trials with the four yeast strains being performed in duplicate, and 3 g/hL of total $SO_2$ was added on each tank. They were kept for 4 days in cold storage, in a chamber at 8 °C. Subsequently, they were tempered (20 °C) and inoculated, at 20 g/hL, with the 4 strains of *Saccharomyces cerevisiae* yeasts: two tanks were inoculated with Maurivin AWRI 796 (AB Mauri-ABBiotek, Toowoomba, Australia), called as Sc1; two tanks were inoculated with Safoeno SC22 (Fermentis, Marcq-en-Barœul, France), called as Sc2; two tanks were inoculated with Pinnacle Fruit Red (ABBiotek), called as Sc3, and two tanks were inoculated with Pinnacle Fructo Select (ABBiotek), called as Sc4.

The alcoholic fermentation (AF) was developed at 20 °C, with the density and temperature being measured daily. Furthermore, glucose/fructose was measured at the end of AF (the coefficients of variation in glucose/fructose ratio between the two tanks of each treatment (%) were: 0.00–2.67). During the fermentation process, the cap was punched down, one a day, with the purpose of improving the contact between the marc and the must, and to extract the compounds found in the skins. Once the AF was completed (when

glucose/fructose concentrations were not detected), wines were drawn off and pressed. Then, the wines were transferred to 25 L stainless steel tanks, each of the batches was treated separately, where lactic acid bacteria *Oeonococcus oeni* Pinnacle MaloSafe (ABBiotek) were inoculated, at 1 g/hL, to carry out the malolactic fermentation (MLF). These fermentations were carried out at 20 °C. MLF was monitored by malic and lactic acids determination (the coefficients of variation (%) between the two tanks of each treatment were: malic acid (0.22–6.09) and lactic acid (2.08–8.23)). Once finished (when malic acid was not detected), the wines were cold stabilized (10 °C, 1 month) and bottled (in 15 green glass bottles of 75 cL corked with cork stopper), remaining in the cellar bottle rack at controlled temperature and humidity (16 °C, 50–60%).

In the initial must, in the wines at the end of MLF and after 6 and 9 months of aging in bottles, the classical parameters were analyzed (Section 2.2). Moreover, two aliquots of each wine, from the two tanks and from two bottles of each repetition, at these three moments (end of MLF and after 6 and 9 months of aging in bottles) were taken and frozen at −20 °C for the determination of their volatile composition (Section 2.3) and their phenolic composition (Section 3.4).

### 2.2. Determination of Must and Wine Classical Parameters

The must enological parameters, Brix, probable alcohol, pH, and total acidity, were analyzed using the official methods established by the OIV [22]. Malic acid and yeast assimilable nitrogen (YAN) were determined using Miura One enzymatic equipment (TDI, Barcelona, Spain). Since the vinification for each of the Sc strains was carried out in two different tanks, as repetitions, these determinations were performed in duplicate, with the initial must composition of: °Brix, 23.4 ± 0.4; probable alcohol (% *v*/*v*), 13.7 ± 0.3; pH, 3.44 ± 0.05; total acidity (g/L as tartaric acid), 5.62 ± 0.30; malic acid (g/L), 2.66 ± 0.23, and YAN (mg N/L), 120 ± 12.

Wine enological parameters, alcohol degree, pH, total acidity, volatile acidity, optical density (OD) at 420, 520, and 620 nm, and color intensity (CI), were determined by the OIV methods [22]. Total polyphenol index (TPI) was measured using the method described by Ribéreau-Gayon et al. [23]. Malic and lactic acids, YAN and total polyphenols were analyzed using the Miura One.

As the vinification processes were carried out in duplicate for each of the Sc strains tested, the results of these parameters are shown as the average of two analyses (n = 2).

### 2.3. Analysis of Wine Volatile Compounds by GC-MS

The method used for the determination of wine volatile compounds was described by Garde-Cerdán et al. [7]. In a 10 mL tube, 8 mL of wine (centrifuged at 3220× *g*, at 4 °C, during 15 min), 10 µL of internal standard (2-octanol, Sigma–Aldrich, Madrid, Spain) and a magnetic stir bar were added. Wine volatile compounds extraction was conducted with 400 µL of dichloromethane (Merck, Darmstadt, Germany) by stirring the sample (15 min). After cooling at 0 °C over 10 min, the organic phase was separated by centrifugation (5031× *g*, 10 min, 4 °C), and the extract was put into a vial. The determination of analytes was performed by gas chromatographic using a Gas Chromatograph (GC) with a Mass Detector (MS) (Agilent, Palo Alto, CA, USA). The volume of injection was of 2 µL. A VF-Wax 52 CB (60 m × 0.25 mm i.d. × 0.25 µm) capillary column (Agilent) was used. The temperature of the injector was programmed: from 40 °C to 250 °C, at 180 °C/min. The oven temperature was 50 °C over 2 min, then set to rise at 3 °C/min, from 50 °C to 250 °C. The detector operated at electronic impact mode (70 eV), with an acquisition range (*m*/*z*) from 29 to 260. The volatile compounds identification was performed using the NIST library and by comparison with the mass spectrum of available standards (Sigma-Aldrich). A semi-quantification was carried out, relating the areas of each volatile compound with the area and the known concentration of the internal standard (2-octanol). Volatile compounds identified in wines at the end of MLF, and after 6 and 9 months of aging in bottles were alcohols, esters and acids. The results are shown by chemical families as the total sum of

the individual compounds: total alcohols (sum of isobutanol, isoamyl alcohols, n-hexanol, methionol, (E)-3-hexenol, and 2-phenylethanol); total acetate esters (sum of isoamyl acetate and 2-phenylethyl acetate), total C6, C8, C10 ethyl esters (sum of ethyl hexanoate, ethyl octanoate, and ethyl decanoate), total ethyl esters (sum of ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl lactate, diethyl succinate, and monoethyl succinate), total esters (sum of isoamyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl lactate, diethyl succinate, and monoethyl succinate), and total acids (sum of hexanoic acid, octanoic acid, and decanoic acid).

As the vinification processes were performed in duplicate, with two different tanks for each of the Sc strain tested, the results of these parameters are shown as the average of two analyses (n = 2).

### *2.4. Determination of Wine Phenolic Compounds by HPLC-DAD*

#### 2.4.1. Sample Preparation for the Analysis of Non-Anthocyanin Phenolic Compounds

According to Portu et al. [24], PCX SPE cartridges (500 mg, 6 mL; Bond Elut Plexa, Agilent, Palo Alto, CA, USA) were used. The SPE cartridges were put in the extraction system (Visiprep$^{TM}$ Vacuum Manifold, Sigma-Aldrich). First, wine samples (3 mL) were diluted with 3 mL of HCl (0.1 N). The cartridges were conditioned with 5 mL of methanol and 5 mL of water. Then, the samples were passed through the cartridges and washed with 5 mL of HCl (0.1 N) and 5 mL of water. The non-anthocyanin phenolic compounds were eluted with 3 × 5 mL of methanol, and then dried in a evaporator (miVac, Genevac Ltd., Suffolk, UK) at 35 °C and re-solved with 1.5 mL of methanol aqueous solution (20% *v*/*v*). The anthocyanin-free fraction was used in order to analyze non-anthocyanin phenolic compounds (flavonols, hydroxycinnamic and hydroxybenzoic acids, stilbenes, and flavanols).

#### 2.4.2. Analysis of Phenolic Compounds by HPLC-DAD

Phenolic compounds were analyzed according to Portu et al. [24] in an Agilent 1260 Infinity II chromatograph, equipped with a diode array detector (DAD). Samples were filtered and injected on a Licrospher® 100 RP-18 reversed-phase column (250 × 4.0 mm; 5 µm packing; Agilent) with pre-column Licrospher® 100 RP-18 (4 × 4 mm; 5 µm packing; Agilent), both at 40 °C. A flow rate of 0.630 mL/min was used. For the analysis of anthocyanins, 10 µL of each wine sample was injected. Eluents used were: (A) acetonitrile/water/formic acid (3:88.5:8.5, *v*/*v*/*v*), and (B) acetonitrile/water/formic acid (50:41.5:8.5, *v*/*v*/*v*). For the analysis of non-anthocyanin phenolic compounds, the injection volume was 20 µL. Eluents were: (A) acetonitrile/water/formic acid (3:88.5:8.5, *v*/*v*/*v*); (B) acetonitrile/water/formic acid (50:41.5:8.5, *v*/*v*/*v*), and (C) methanol/water/formic acid (90:1.5:8.5, *v*/*v*/*v*).

The identification of phenolic compounds was carried out according to the retention times of available pure compounds and the UV-Vis data obtained from authentic standards and/or published in previous studies [25]. For the quantification, DAD chromatograms were extracted at 520 nm (anthocyanins), 360 nm (flavonols), 320 nm (hydroxycinnamic acids and stilbenes), and 280 nm (gallic acid and flavanols) and the calibration graphs of the respective standards ($R^2$ > 0.99) were used. When a standard was not available, the quantification was made with the calibration graph of the most similar compound. Hence, malvidin-3-*O*-glucoside was used for anthocyanins, quercetin-3-*O*-glucoside for flavonols, *trans*-caftaric acid for free hydroxycinnamic acids and the corresponding tartaric esters, catechin for procyanidins B1 and B2, epicatechin for epigallocatechin, and *trans*-piceid and *trans*-resveratrol for their respective *cis* isomers. The results of the phenolic compounds found in the wines at the end of MLF, and after 6 and 9 months are shown by chemical families, with the exception of gallic acid, which was the only hydroxybenzoid acid found in the wines, as the sum total of the individual compounds: total anthocyanins (sum of delphinidin-3-glc, cyanidin-3-glc, petunidin-3-glc, peonidin-3-glc, malvidin-3-glc, delphinidin-3-acglc, cyanidin-3-acglc, petunidin-3-acglc,

peonidin-3-acglc, malvidin-3-acglc, delphinidin-3-cmglc, cyanidin-3-cmglc, petunidin-3-cmglc, peonidin-3-cmglc, malvidin-3-*cis*-cmglc, malvidin-3-*trans*-cmglc, malvidin-3-cfglc, vitisin A, and vitisin B; abbreviations: glc, glucoside; acglc, acetylglucoside; cmglc, *trans*-*p*-coumaroylglucoside; and cfglc, caffeoylglucoside); total flavonols (sum of myricetin-3-gal, myricetin-3-glcu, myricetin-3-glc, quercetin-3-glcu, quercetin-3-glc, laricitrin-3-glc, kaempferol-3-gal, kaempferol-3-glc, kaempferol-3-glcu, isorhamnetin-3-glc, syringetin-3-glc, free-myricetin, free-quercetin, free-laricitrin, free-kaempferol, free-isorhamnetin, and free-syringetin; abbreviations: gal, galactoside; glcU, glucuronide; and glc, glucoside); total flavanols (sum of epigallocatechin, catechin, epicatechin, procyanidin B1, and procyanidin B2); total hydroxycinnamic acids (sum of *trans*-caftaric acid, *trans*+*cis*-coutaric acids, caffeic acid, *trans*-fertaric acid, p-coumaric acid, and ferulic acid), and total stilbenes (sum of *trans*-piceid, *cis*-piceid, *trans*-resveratrol, and *cis*-resveratrol).

Since vinifications for each of the Sc strain tested were performed in duplicate, the results for phenolic compounds are the average of the analyses of two samples (n = 2).

### *2.5. Statistical Analysis*

The statistical elaboration of the data was performed using SPSS Version 21.0 (Chicago, IL, USA). Enological parameters, volatile and phenolic compounds data were processed using the variance analysis (ANOVA) and differences between samples were compared using Duncan's test ($p \leq 0.05$). Discriminant analyses were carried out with the volatile compounds and phenolic compounds found in the wine samples. Further, percentage of variance attributable to yeast strain, aging time and their interactions were analyzed (factorial analysis).

## 3. Results and Discussion

### *3.1. Kinetics of Fermentation and Wine Enological Parameters*

Figures 1 and 2 show the kinetics of alcoholic fermentation (AF) and malolactic fermentation (MLF), respectively, as a decrease in Brix (Figure 1) and malic acid content (Figure 2a) and an increase in lactic acid content (Figure 2b).

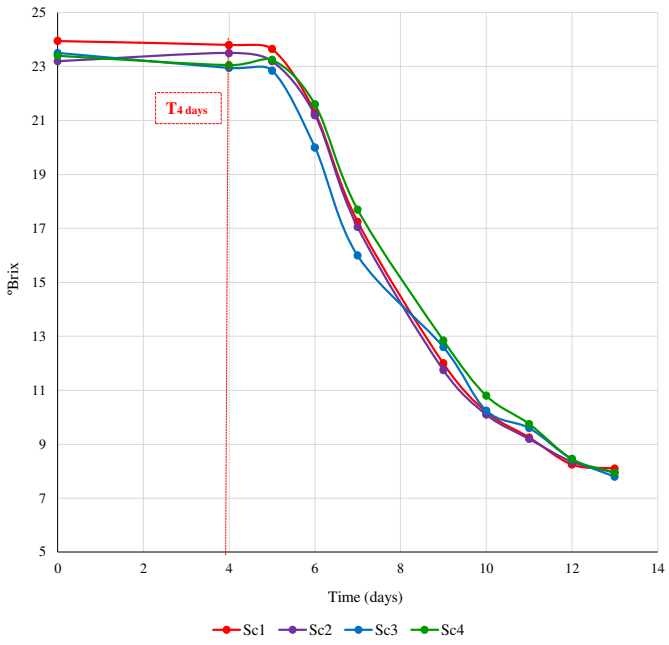


**Figure 1.** Alcoholic fermentation kinetics for the four vinifications carried out with the different yeasts (Sc1, Sc2, Sc3, and Sc4). °Brix given as average values (n = 2).

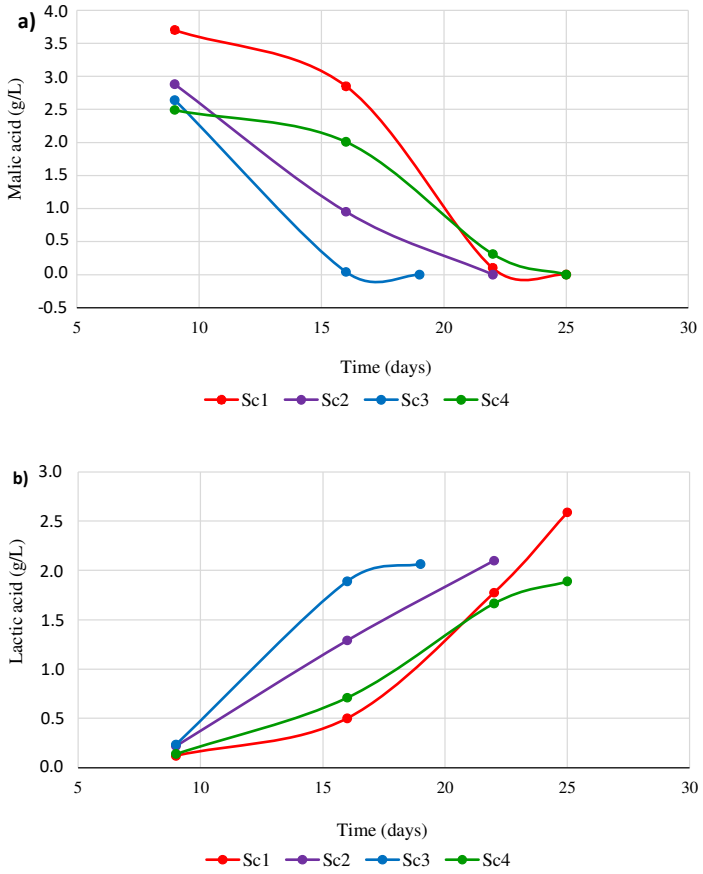


**Figure 2.** Evolution of (**a**) malic acid (g/L) and (**b**) lactic acid (g/L) during the malolactic fermentation for the four vinifications carried out with the different yeasts (Sc1, Sc2, Sc3, and Sc4). The contents of both acids are given as average values (n = 2).

The Sc3 yeast showed slightly faster initial kinetics than the other three yeasts (Figure 1), although all of them took the same time to complete alcoholic fermentation (13 days), reaching dryness in all cases.

Furthermore, it was in this trial (Sc3) that the MLF was completed most rapidly (19 days), with the Sc1 and Sc4 trials being the slowest to complete this second fermentation (25 days), with the Sc2 yeast showing an intermediate behavior (22 days). It should be noted that in all cases, the malic acid was completely consumed (Figure 2a and Table 1). When the fermentation was carried out with Sc1 yeast, the highest amount of lactic acid was formed (Figure 2b and Table 1), while the lowest concentration of lactic acid was found during vinifications with Sc4 yeast (Figure 2b and Table 1). The Sc2 and Sc3 yeasts showed similar behavior in terms of lactic acid formation (Figure 2b and Table 1).

Table 1 shows the results of the enological parameters of the wines made with the four yeasts at the end of MLF and at 6 and 9 months of bottle aging.

It should be noted that the ethanol yield of Sc3 yeast was lower than in the case of Sc1, with a reduction of 4.9%; the other two yeasts showed intermediate behavior in alcohol formation (Table 1).

Neither the pH nor the YAN of the wines was affected by the yeast used in vinification (Table 1).

Wines made with Sc1 had a higher total acidity than the rest of the wines, probably due to their higher lactic acid content, as described above (Figure 2b). As for the volatile acidity of the wines, both after MLF and after 6 and 9 months in a bottle, it was observed that it was particularly low when fermentations were carried out with Sc2 and Sc4 yeasts (Table 1). This parameter showed no difference throughout the aging of the wines in a bottle.

**Table 1.** Enological parameters of the wines at the end of malolactic fermentation (MLF) and after 6 and 9 months of aging in bottles, 6 MB and 9 MB, respectively.

| | Sc1 | | | Sc2 | | | Sc3 | | | Sc4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **End of MLF** | **6 MB** | **9 MB** | **End of MLF** | **6 MB** | **9 MB** | **End of MLF** | **6 MB** | **9 MB** | **End of MLF** | **6 MB** | **9 MB** |
| Alcohol degree (% *v*/*v*) | 13.8 ± 0.1 b | - | - | 13.4 ± 0.0 ab | - | - | 13.1 ± 0.3 a | - | - | 13.6 ± 0.2 ab | - | - |
| pH | 4.04 ± 0.06 a | - | - | 4.14 ± 0.02 a | - | - | 4.13 ± 0.08 a | - | - | 4.15 ± 0.00 a | - | - |
| Total acidity (g/L) * | 5.10 ± 0.11 b | - | - | 3.83 ± 0.21 a | - | - | 3.77 ± 0.24 a | - | - | 4.05 ± 0.11 a | - | - |
| Malic acid (g/L) | n.d. | - | - | n.d. | - | - | n.d. | - | - | n.d. | - | - |
| Lactic acid (g/L) | 2.62 ± 0.10 c | - | - | 2.26 ± 0.11 b | - | - | 2.15 ± 0.05 b | - | - | 1.90 ± 0.03 a | - | - |
| Volatile acidity (g/L) ** | 0.50 ± 0.02 A, b | 0.46 ± 0.00 A, b | 0.49 ± 0.04 A, b | 0.33 ± 0.04 A, a | 0.35 ± 0.01 A, a | 0.34 ± 0.01 A, a | 0.48 ± 0.07 A, b | 0.48 ± 0.07 A, b | 0.49 ± 0.10 A, b | 0.27 ± 0.01 A, a | 0.28 ± 0.01 A, a | 0.30 ± 0.01 A, a |
| YAN (mg N/L) | 8.50 ± 1.98 a | - | - | 21.5 ± 11.8 a | - | - | 9.50 ± 0.8 a | - | - | 22.5 ± 12.6 a | - | - |
| OD 420 nm | 0.24 ± 0.01 A, b | 0.28 ± 0.02 AB, a | 0.30 ± 0.02 B, a | 0.24 ± 0.01 A, ab | 0.29 ± 0.01 B, a | 0.30 ± 0.00 B, a | 0.22 ± 0.00 A, a | 0.27 ± 0.00 B, a | 0.29 ± 0.00 C, a | 0.23 ± 0.01 A, ab | 0.27 ± 0.01 B, a | 0.29 ± 0.01 B, a |
| OD 520 nm | 0.30 ± 0.02 A, b | 0.36 ± 0.04 A, a | 0.37 ± 0.05 A, a | 0.29 ± 0.02 A, ab | 0.35 ± 0.01 B, a | 0.35 ± 0.01 B, a | 0.25 ± 0.01 A, a | 0.34 ± 0.01 B, a | 0.35 ± 0.03 B, a | 0.29 ± 0.01 A, ab | 0.34 ± 0.00 B, a | 0.35 ± 0.01 B, a |
| OD 620 nm | 0.07 ± 0.00 A, b | 0.11 ± 0.01 B, a | 0.12 ± 0.01 B, a | 0.06 ± 0.00 A, ab | 0.11 ± 0.00 B, a | 0.12 ± 0.00 C, a | 0.06 ± 0.00 A, a | 0.11 ± 0.00 B, a | 0.12 ± 0.00 C, a | 0.06 ± 0.00 A, ab | 0.11 ± 0.00 B, a | 0.12 ± 0.00 C, a |
| Color intensity (CI) | 6.11 ± 0.27 A, b | 7.51 ± 0.74 A, a | 8.00 ± 0.74 A, a | 5.92 ± 0.35 A, ab | 7.47 ± 0.22 B, a | 7.69 ± 0.16 B, a | 5.25 ± 0.07 A, a | 7.19 ± 0.11 B, a | 7.51 ± 0.28 B, a | 5.76 ± 0.25 A, ab | 7.22 ± 0.13 B, a | 7.64 ± 0.29 B, a |
| TPI | 43.7 ± 0.7 A, a | 43.4 ± 0.4 A, a | 42.0 ± 0.5 A, a | 49.2 ± 1.4 A, b | 48.9 ± 1.1 A, b | 47.3 ± 1.0 A, b | 44.7 ± 0.1 A, a | 44.7 ± 1.1 A, a | 42.8 ± 0.6 A, a | 50.0 ± 1.2 A, b | 49.2 ± 1.7 A, b | 48.0 ± 2.5 A, b |
| Total polyphenols (mg/L) | 1719 ± 64 B, a | 1625 ± 15 AB, a | 1537 ± 11 A, a | 2086 ± 226 A, a | 1927 ± 59 A, b | 1767 ± 47 A, b | 1778 ± 54 B, a | 1692 ± 14 B, a | 1542 ± 28 A, a | 2046 ± 144 A, a | 1928 ± 66 A, b | 1769 ± 77 A, b |

All parameters are given as average values ± SD (n = 2). n.d.: not detected. -: not analyzed. YAN: yeast assimilable nitrogen. OD: optical density. TPI: total polyphenol index. * As g/L of tartaric acid. ** As g/L of acetic acid. For each yeast, different capital letters indicate significant differences, using the Duncan test ($p \leq 0.05$), between aging moments. For each moment, different lowercase letters indicate significant differences, using the Duncan test ($p \leq 0.05$), between wines elaborated with the 4 yeasts.

The color parameters, the color intensity and the corresponding optical densities at 420, 520 and 620 nm of the wines after MLF were higher when using the Sc1 yeast than when using the Sc3 yeast, but after bottle aging, these parameters were similar in the wines, showing no differences depending on the yeast used (Table 1). These four parameters increased throughout the process of bottle aging, especially when Sc2–Sc4 yeasts were used, and were generally higher at 9 months than at the end of the MLF. Finally, in general, both TPI and total phenolic compounds were higher in wines made with Sc2 and Sc4 yeasts at the three times studied (Table 1). Regarding the evolution of these parameters with aging time, it was observed that the TPI did not vary for any of the wines, regardless of the yeast used. The total phenolic compounds in the wines elaborated with Sc1 and Sc3 yeasts decreased, while their content did not change for the wines made using Sc2 and Sc4 yeasts (Table 1).

### 3.2. Wine Aromatic Composition

Figure 3 shows the results of the volatile composition of the wines made using the four yeasts at the end of MLF and after 6 and 9 months of aging in bottles. The content of total alcohols in the wines increased during the first 6 months in a bottle, remaining constant until the end of the studied period when Sc1 and Sc3 yeasts were used, while a marked decrease in their concentration was observed between 6 and 9 months in a bottle when the wines were made with Sc2 yeast. However, when Sc4 yeast was used, the content of total alcohols did not change (Figure 3a). Garde-Cerdán and Ancín-Azpilicueta [18] observed that total alcohols increased as the wines remained in bottles for 6 months. The concentration of total alcohols after MLF was higher in wines made with Sc2 and Sc4 yeasts than when Sc1 and Sc3 yeasts were inoculated. Among the factors that affect the formation of alcohols in the fermentation, yeast strain is one of the main parameters [1,26]. However, due to the different evolution of these compounds during aging, after 6 and 9 months in a bottle, it was the wines made with Sc2 and Sc3 yeast, respectively, that showed higher levels of total alcohols (Figure 3a). These compounds contribute to the aromatic complexity of the wines if the level is below 300 mg/L [27], a concentration not exceeded in any of the wines produced nor at any of the times studied (Figure 3a). Regarding esters, which are the most important fermentative compounds for the aromatic quality of wines, since they present low perception thresholds, contributing fruity and floral aromas to wines [6,7], their evolution is shown in Figure 3b–e. As discussed for alcohols, their formation during fermentation depends on the yeast strain [28,29]. The concentration of total acetate esters was not modified throughout the period studied when the wines were made with Sc1 and Sc4 yeasts, whereas their content decreased between 6 and 9 months of bottle aging for wines made with Sc2 and Sc3 yeasts (Figure 3b). Regardless of the aging time, the wines made with Sc4 yeast had the lowest concentration of total acetate esters, showing no significant differences between the other wines, Sc1–Sc3 (Figure 3b). The content of total C6, C8 and C10 ethyl esters remained unchanged during bottle aging for wines made with Sc3 yeast, but decreased when the other three yeasts, Sc1, Sc2 and Sc4, were used, with this decrease especially marked for wines made with Sc2 yeast (Figure 3c). Wines made with Sc4 yeast showed lower concentrations of these compounds, as described for total acetate esters, while the highest concentrations of total C6, C8 and C10 ethyl esters were found in Sc2 wines at the end of MLF, Sc1 after 6 months in bottles, and Sc1 and Sc3 after 9 months of aging (Figure 3c).

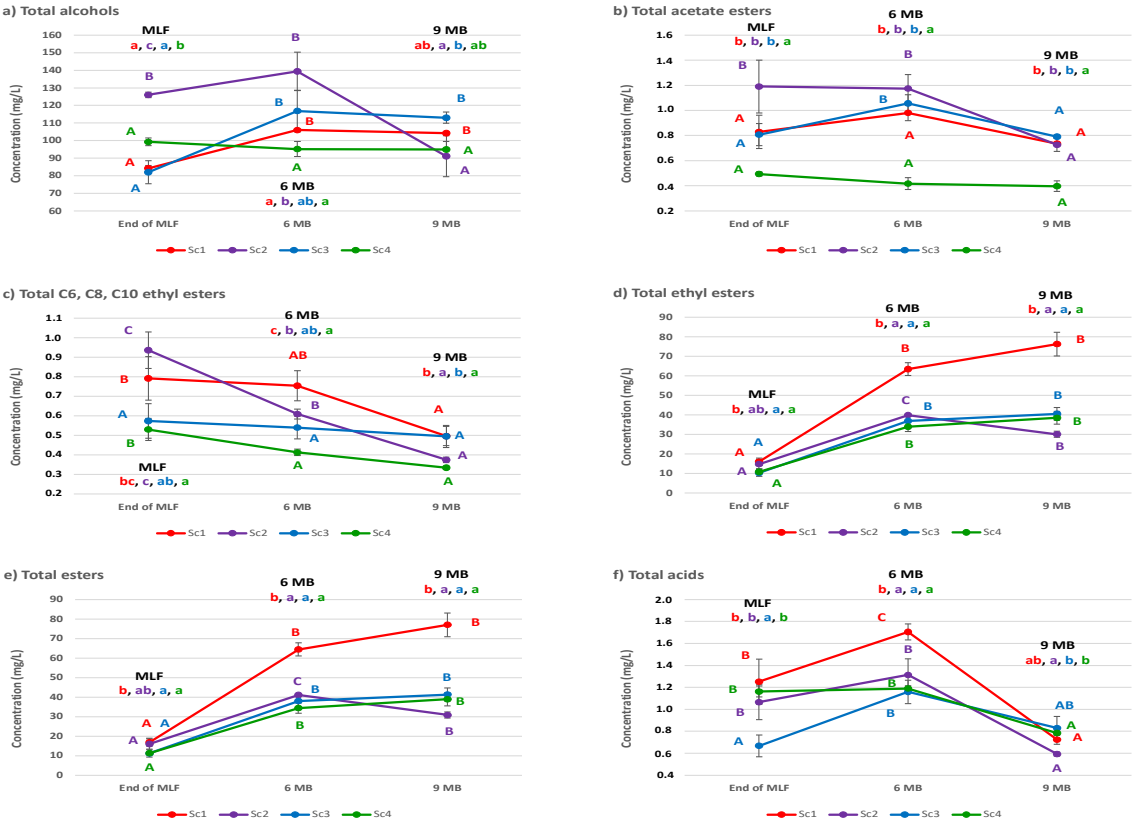


**Figure 3.** Volatile compounds concentration (mg/L) of the wines at the end of malolactic fermentation (MLF) and after 6 and 9 months of aging in bottles, 6 MB and 9 MB, respectively. All parameters are given as average values ± SD (n = 2). For each yeast, different capital letters indicate significant differences (Duncan test at $p \leq 0.05$) between aging moments. For each moment, different lowercase letters indicate significant differences (Duncan test at $p \leq 0.05$) between wines elaborated with the 4 yeasts.

Both the concentration and the evolution of total ethyl esters and total esters during the aging of the wines in a bottle were similar (Figure 3d,e), so they will be discussed together. It is worth noting the large increase in their content during the first 6 months in a bottle of the wines made with Sc1 yeast. The rest of the wines also showed this trend, so that their concentration increased in the first 6 months and then remained constant until the end of the studied period, except for the wines made with Sc2 yeast, for which the content of total ethyl esters and total esters decreased (Figure 3d,e). Logically, due to the large increase in the content of these esters in the wines made with Sc1, their concentration was higher in these wines than in the wines made with Sc2–Sc4 yeasts, which showed no significant differences between them (Figure 3d,e). The equilibrium concentrations of each ester and their hydrolysis products were different. The ethyl esters of fatty acids and the acetate esters of higher alcohols were hydrolyzed with the time of aging, whereas the ethyl esters of organic acids were formed during the storage of the wines in bottles [6,19]. Thus, in general, the content of total acetate esters and total C6, C8, C10 ethyl esters, i.e., ethyl esters of fatty acids, decreased during the aging of the wines in a bottle (Figure 3b,c), while the concentrations of total ethyl esters and total esters increased (Figure 3d,e). In the latter two cases, there was a significant increase in the content of ethyl lactate, diethyl succinate, and monoethyl succinate, i.e., ethyl esters of fatty acids, a result that coincides with that found by Pérez-Coello et al. [17]. Finally, total acids increased in concentration during the first 6 months of wine aging made with Sc1 and Sc3 yeasts, decreasing thereafter (Figure 3f), whereas when Sc2 and Sc4 yeasts were used, these compounds did not suffer initial concentration changes, decreasing in the final stage of aging, between 6 and 9 months of bottle aging (Figure 3f). Garde-Cerdán et al. [19] also observed an initial increase in the

concentration of these compounds and a subsequent decrease when aging wines in a bottle for 6 months. At the end of MLF, wines made with Sc3 yeast had the lowest total acid content; after 6 months in a bottle, wines made with Sc1 yeast had the highest concentration of these compounds, while at the end of the studied period, 9 months, their content was higher for wines made with Sc3 and Sc4 yeasts (Figure 3f). Acids are compounds that provide freshness to wines, thus, contributing to their sensory properties, as long as they are not found at concentrations higher than 20 mg/L [30]. The wines made with the four yeasts under study showed total acid contents well below this concentration throughout the aging in a bottle (Figure 3f).

### *3.3. Wine Phenolic Composition*

The results from the phenolic composition of the wines made with the four yeasts, Sc1–Sc4, after MLF and after 6 and 9 months in a bottle, are shown in Figure 4. The concentration of total anthocyanins decreased in the wines during the first 6 months in a bottle, remaining practically constant until the end of the studied period, regardless of the yeast used during vinifications (Figure 4a). In general, it is observed that, at the three times studied, the content of these compounds was higher in wines made with Sc2 and Sc4 yeasts than in wines made with Sc1 and Sc3 yeasts. Anthocyanins are principally responsible for the red color of red wines [31,32]. Independently of the wine and the cultivar, other authors, such as Hermosín-Gutiérrez et al. [33] and Monagas et al. [34], also observed that wine aging in bottles produced a considerable reduction in the total amount of anthocyanins.

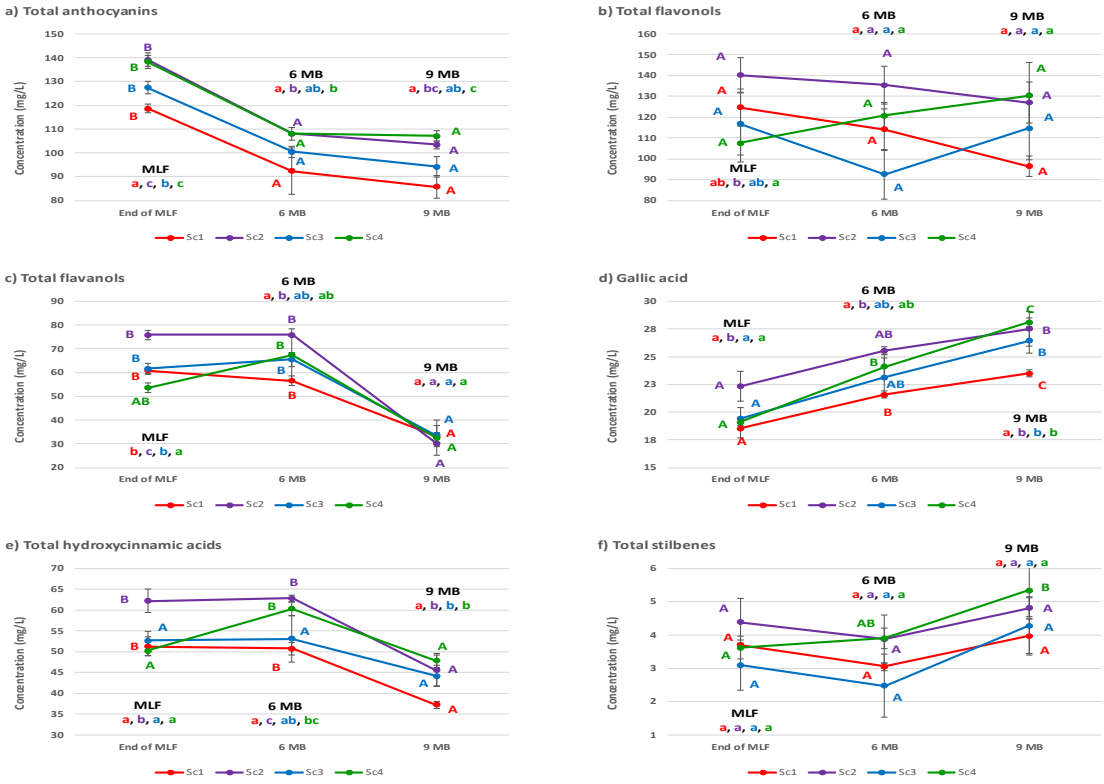


**Figure 4.** Phenolic compounds concentration (mg/L) of the wines at the end of malolactic fermentation (MLF) and after 6 and 9 months of aging in bottles, 6 MB and 9 MB, respectively. All parameters are given as average values ± SD (n = 2). For each yeast, different capital letters indicate significant differences (Duncan test at $p \leq 0.05$) between aging moments. For each moment, different lowercase letters indicate significant differences (Duncan test at $p \leq 0.05$) between wines elaborated with the 4 yeasts.

However, in none of the wines did the concentration of total flavonols change during bottle aging (Figure 4b), a result that coincides with that found by de Souza et al. [35] for

Syrah wines, aged in a bottle for 12 months. In addition, there were hardly any differences in their content depending on the yeast used, showing that at the end of MLF, wines made with Sc2 yeast had a greater concentration of these phenolic compounds than the wines made with Sc4 yeast (Figure 4b). Flavonols are the compounds that give a yellow color to wines, and are, therefore, of particular importance in white wines [36]. Besides, these compounds are essential for the stabilization of the wine color, since flavonols participate in the reactions of co-pigmentation with anthocyanins [31]. In the case of total flavanols, differences were observed according to both the time of aging and the yeast used to make the wines (Figure 4c). In contrast to anthocyanins, during the first 6 months of bottle aging, their concentration did not change, decreasing in the last 3 months of aging, regardless of the yeast used, with the exception of Sc4 (Figure 4c). At the end of MLF, wines made with Sc2 yeast had the highest concentration of total flavanols, while those made with Sc4 yeast had the lowest. However, during the time the wines remained in a bottle, the content of these compounds tended to equalize, so that at 9 months, no significant differences were observed, depending on the yeast (Figure 4c). Flavanols are mainly responsible for the wine astringency [37]. Gómez-Gallego et al. [31] also found a progressive diminution of these compounds over the aging period, more marked in the first year of Bobal, Cencibel, Tortosí and Moravia Agria cv. wines' storage. Boulton [38] suggested that this decrease in flavanols compounds through the aging period is due to reactions of oxidation and polymerization, many of which are related to the sensory properties of stabilization associated to red wine aging, principally color.

The behavior of gallic acid, the only hydroxybenzoid acid detected in the wines, was the inverse of that of the other phenolic compounds studied (Figure 4). Thus, its concentration increased throughout the aging of the wines in a bottle (Figure 4d), such that, regardless of the yeast inoculated during fermentation, its concentration was higher at 9 months than at the end of MLF. Uzkuç et al. [39] also found a rise in gallic acid content throughout the bottle aging of Cabernet Sauvignon wines, relating it to the hydrolysis reaction of gallo-tannins and esters of gallic acid with glucose. After MLF, gallic acid presented the highest concentration in wines made with Sc2 yeast, as well as after 6 months in a bottle, although without significant differences from wines made with Sc3 and Sc4 yeasts, while, at 9 months, this compound was in lower concentration in wines made with Sc1 than for the rest, Sc2–Sc4, without differences between them (Figure 4d).

Regarding total hydroxycinnamic acids, their concentration in the wines remained practically constant during the first 6 months in a bottle, with the exception of wines made with Sc4 yeast, for which an increase in their content was observed (Figure 4e). However, between 6 and 9 months of bottle aging, their concentration decreased, except for wines made with Sc3 yeast, for which no changes were observed (Figure 4e). As described for gallic acid, wines made with Sc2 yeast had the highest content of these acids, a behavior that was maintained during the first 6 months in a bottle, although without significant differences from wines made with Sc4 yeast, while at the end of the studied period, wines made with Sc1 yeast had the lowest concentration of hydroxycinnamic acids (Figure 4e). These compounds are easily oxidized and are associated with browning phenomena, mainly in white wines [16], and are also precursors of ethylphenols, negative compounds for wine quality, when found in high concentrations [40,41].

The decrease in concentration during the aging of wines in a bottle, in terms of anthocyanins (Figure 4a), flavanols (Figure 4c) and hydroxycinnamic acids (Figure 4e), is associated with copigmentation reactions, which allows for stabilization in the color of red wines [10,14]. Finally, as with total flavonols, the content of total stilbenes barely changed during wine storage in bottles, with the exception of the wine made with Sc4 yeast, for which it was observed that these compounds had a higher concentration after 9 months than at the end of the MLF (Figure 4f). Furthermore, the content of total stilbenes did not show significant differences at any of the three times studied, as a function of the yeast, i.e., these compounds were not affected either by the yeast that carried out the fermentation or by the bottle aging of the wines (Figure 4f). Among the phenolic compounds, stilbenes

have historically been related with moderate wine consumption health benefits [42,43]. However, in recent years, this health effect associated with these compounds, mainly resveratrol, is being questioned due to their low concentration in wines [44].

### *3.4. Discriminat and Factorial Analysis*

Figure 5 shows the discriminants carried out with the volatile composition (Figure 5a), phenolic composition (Figure 5b) and both (Figure 5c), taking the wine sample as the discrimination factor. As can be observed, the aromatic composition of the wines was different after MLF and bottle aging, without a clear separation according to aging time, with the exception of the wines made with the Sc1 yeast (Figure 5a). Function 1 had a weight in the discrimination of 74.6% and Function 2 of 15.3%, reaching, between them, 89.9%, meaning they were the variables with the highest weight in the total ethyl esters, total acids and total alcohols (Function 1) and total acetate esters, total acids and total alcohols (Function 2). In addition, the wines showed adequate separation, depending on the yeast, both after MLF and after 6 and 9 months in a bottle, with the exception of the Sc3 wines (Figure 5a). However, the wines were separated very clearly according to aging time when their phenolic composition was used to perform the discriminant analysis (Figure 5b), finding three groups perfectly separated, especially when the wines remained in a bottle for 9 months. Function 1 had a weight in the discrimination of 92.6% and Function 2 of 5.3%, being, between them, 97.9%; the variables with the greatest weight were gallic acid and total hydroxycinnamic acids and total anthocyanins and gallic acid, respectively. In this case, the separation according to the yeast used to carry out the fermentation was almost null, except for the Sc1 yeast, which appears somewhat more separated from the other three yeasts (Sc2–Sc4), which are quite close to each other.

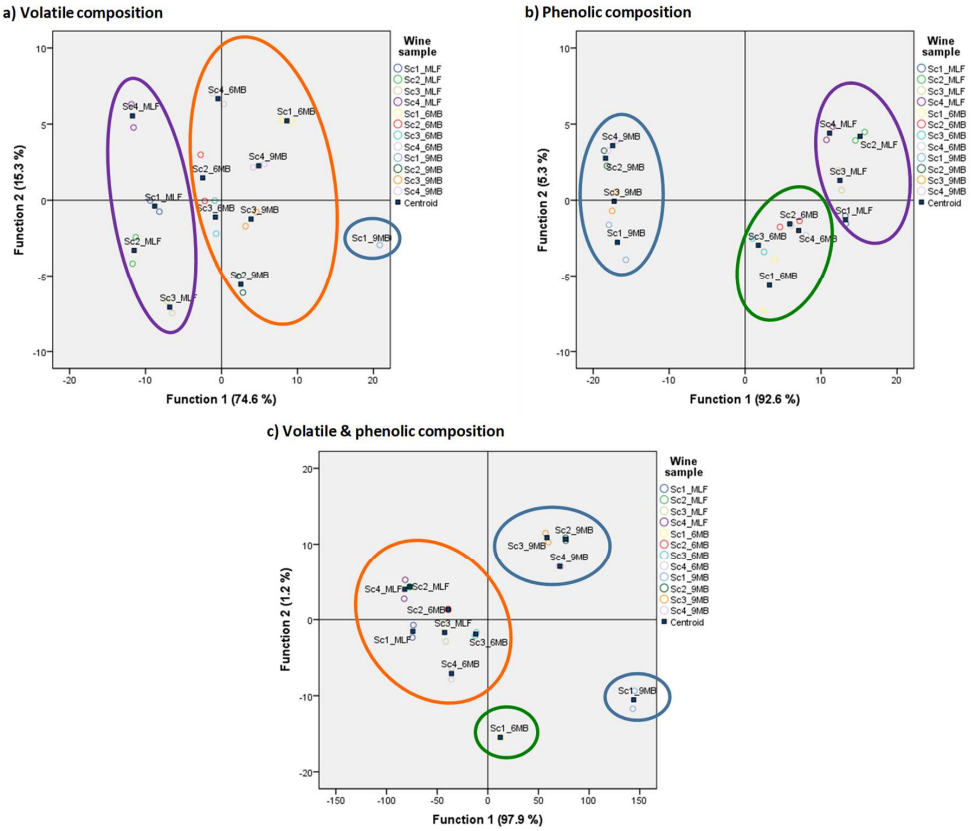


**Figure 5.** Discriminant analysis carried out with (**a**) volatile compounds concentration (mg/L), (**b**) phenolic compounds concentration (mg/L), and (**c**) volatile and phenolic compounds concentration (mg/L) of the wines elaborated with the four yeasts (Sc1, Sc2, Sc3, and Sc4) at the three winemaking moments (end of MLF and after 6 and 9 months of aging in bottles, 6 MB and 9 MB, respectively).

Finally, if we take into account all the compounds studied, both volatile and phenolic, it can be observed that the four groups clearly differentiated from one another (Figure 5c). The wines after MLF and 6 months in a bottle form one group, with the exception of the wines made with Sc1 yeast after 6 months in a bottle (Figure 5c); on the other hand, there are the wines that remained, for 9 months, in a bottle, which, in turn, form two groups: those made with Sc1 yeast and the other three wines, Sc2–Sc4 (Figure 5c). The weight of the discriminant functions was 97.9% (Function 1) and 1.2% (Function 2), with a total between them of 99.1%. The variables with the highest weight in Function 1 were: gallic acid, total hydroxycinnamic acids, total ethyl esters, and total alcohols; those with the highest weight in Function 2 were: total hydroxycinnamic acids, total alcohols, total flavonols, and total ethyl esters.

The percentages of aromatic and phenolic compounds variance attributable to yeast strain, aging time and their interaction (factorial analysis) are presented in Table 2.

**Table 2.** Percentage of wine aromatic and phenolic compounds variance attributable to yeast strain (YS), aging time (AT) and YS × AT interaction.

| | Yeast Strain (%) | Aging Time (%) | YS × AT (%) | Residual (%) |
|---|---|---|---|---|
| Total alcohols | 26.9 *** | 17.8 *** | **48.0** *** | 7.24 |
| Total acetate esters | **68.6** *** | 14.7 *** | 11.0 * | 5.71 |
| Total C6, C8, C10 ethyl esters | 31.3 *** | **42.8** *** | 19.4 ** | 6.48 |
| Total ethyl esters | 27.0 *** | **59.3** *** | 12.6 *** | 1.12 |
| Total esters | 27.1 *** | **59.0** *** | 12.7 *** | 1.16 |
| Total acids | 15.0 *** | **60.5** *** | 19.2 ** | 5.38 |
| Total anthocyanins | 20.8 *** | **76.1** *** | 0.46 ns | 2.66 |
| Total flavonols | **34.0** * | 2.72 ns | 28.6 ns | 34.7 |
| Total flavanols | 5.87 ** | **83.2** *** | 7.63 * | 3.33 |
| Gallic acid | 19.8 *** | **70.8** *** | 4.48 ns | 4.92 |
| Total hydroxycinnamic acids | 26.2 *** | **56.4** *** | 11.5 * | 5.88 |
| Total stilbenes | 28.1 * | **38.0** ** | 0.63 ns | 24.7 |

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns $p > 0.05$. The factor with the greatest weight is shown in bold.

As can be seen, the aging time factor had the greatest weight in practically all the aromatic and phenolic groups of compounds (except for the total flavonol content, which was not influenced), followed by the yeast strain factor, which had the greatest weight in the case of total acetate esters and total flavonols. Denat et al. [45] also observed, in their Tempranillo study, the highest influence of aging factor over yeast strain on the wine trace aroma composition. The interaction between both factors, yeast strain × aging time, was the most significant factor in the case of total alcohols, but was not significant in the case of total anthocyanins, total flavonols, gallic acid and total stilbenes. These results are of relevance, since, according to Denat et al. [46], there is scarce information on the incidence of yeast on the long-term storage of the aroma compounds, although Gammacurta et al. [47] studied the formation of fruity ethyl esters during aging by esterification. Ferreira and Lopez [48] also observed the importance of storage in the release or the decay of several aroma compounds. Denat et al. [45] suggested that storage time must be considered as a key factor to evaluate yeast's role on wine aroma.

The impact of the yeast strain on the wine phenolic profile and composition, as well as on the influence on the formation of stable pigments with aging time, has been extensively studied [49–51].

## 4. Conclusions

In the current study, Tempranillo vinifications, with the inoculation of four *Saccharomyces cerevisiae* strains, were compared in terms of the content of phenolic and volatile compounds after malolactic fermentation and 6 and 9 months of aging in bottles. The strong impact exerted by both the aging time as well as the selected strain of *Saccharomyces*

*cerevisiae* yeast on wine aromatic and phenolic compounds, potentially allow the use of these selected yeasts as a tool to enhance the complexity of Tempranillo wines. In addition, the importance of aging the wines in bottles, once the malolactic fermentation has finished, in order to increase the quality and stability of the wines must be highlighted. In the future, studies on aging conditions (time, temperature, bottle/barrels, level of $SO_2$ or alternatives, oxygenation, etc.), linked to the use of different yeasts and/or bacteria during vinification, could contribute to the knowledge of the real determining factors in the wines' aromatic and phenolic composition and, therefore, their sensory quality.

**Author Contributions:** Conceptualization, T.G.-C., M.I., P.R.-B. and E.P.P.-Á.; methodology, M.I.; formal analysis, I.S.d.U., R.M.-P. and S.M.-S.R.; investigation, T.G.-C., P.R.-B. and E.P.P.-Á.; resources, T.G.-C., M.I., P.R.-B. and E.P.P.-Á.; data curation, T.G.-C., P.R.-B. and E.P.P.-Á.; writing—original draft preparation, T.G.-C. and E.P.P.-Á.; writing—review and editing, T.G.-C., I.S.d.U., R.M.-P., M.I., S.M.-S.R., P.R.-B. and E.P.P.-Á.; funding acquisition, T.G.-C., M.I., P.R.-B. and E.P.P.-Á. All authors have read and agreed to the published version of the manuscript.

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