Use of a flor yeast strain for the second fermentation of sparkling wines: effect of endogenous CO_2 over-pressure on the volatilome

Running Title: Use of a flor yeast strain for the diversification of sparkling wines

by

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ABSTRACT

Saccharomyces cerevisiae flor yeast is used for the first time in sparkling wine-making. Twenty-six oenological variables and fifty-three volatile metabolites are quantified in the middle (P = 3 bar) and at the end (P = 6 bar) of the second fermentation, carried out in open and closed bottles. A heat-map of volatiles and the fingerprints obtained for ten chemical families and ten odorant series visualize the changes for each condition. Terpenes, fatty acids and volatile phenols increased their contents by pressure effect at the end of the study by 25.0, 7.8 and 2.2 %, respectively. The remaining families decrease between 17.4 % and 30.1 % for furanic compounds and esters in the same stage. A Principal Component Analysis established that nine volatiles are mainly affected by pressure and five by fermentation stage. The use of ethanol-tolerant flor yeasts constitutes an innovative procedure for the enhancement of the sparkling wines diversification.

Keywords: sparkling wine, flor yeast, volatilome, second fermentation, chemometry.

Chemical compounds studied in this article:

Acetaldehyde (PubChem CID: 177); Acetoin (PubChem CID: 173); Benzaldehyde (PubChem CID: 240); 2,3-Butanediol (PubChem CID: 262); Decanal (PubChem CID: 8175); Decanoic acid (PubChem CID: 2969); Dodecanoic acid (PubChem CID: 3893); Dihydrofuran-2(3H)-one (PubChem: 7302); γ-Decalactone (PubChem CID: 12813); 1,3-Dimethoxy-2-hydroxybenzene (PubChem CID: 7041); Ethyl acetate (PubChem CID: 8857); Ethyl lactate (PubChem CID: 7344); 2-Ethylhexan-1-ol (PubChem CID: 7720); Ethyl propanoate (PubChem CID: 7749); Ethyl isobutanoate (PubChem CID: 7342); Ethyl butanoate (PubChem CID: 7762); Ethyl hexanoate (PubChem CID: 31265); Ethyl octanoate (PubChem: CID: 7799); Ethyl decanoate (PubChem CID: 8048); Ethyl dodecanoate (PubChem CID: 7800); Ethyl tetradecanoate (PubChem CID: 31283); Ethyl hexadecanoate (PubChem CID: 12366); 4-Ethenyl-2-methoxyphenol (PubChem CID: 332); 5-H-furan-2-one (PubChem CID: 10341); Guaiacol (PubChem CID: 460); Hexan-1-ol (PubChem CID: 8103); Hexanoic acid (PubChem CID: 8892); Hexyl acetate (PubChem CID: 8908); Methanol (PubChem CID: 137654); Methyl acetate (PubChem CID: 6584); 2-Methyl-propan-1-ol (PubChem CID: 6560); 2-Methyl-1-Butanol (PubChem CID: 8723); 3-Methyl-1-Butanol (PubChem CID: 31260); Nerolidol (PubChem CID: 5284507); Nonanal (PubChem CID: 31289); Octanoic acid (PubChem CID: 379); Propan-1-ol (PubChem CID: 1031); 2-Phenylethan-1-ol (PubChem CID: 7409); 2-Phenylethyl acetate (PubChem CID: 7654); Phenylethyl phenylacetate (PubChem CID: 7601); Tetradecanoic acid (PubChem CID: 11005).

1. Introduction

The use of commercial yeast strains, available as Active Dry Yeasts, is considered the most effective advance adopted by the wine industry in recent decades. Nevertheless, as a counterpart the obtained wines show a lack of typicity which was traditionally linked to a specific "terroir". This effect is especially important in the sparkling wine production, because the number of commercially available yeast strains are very scarce. To face the great uniformity on the sensorial properties of sparkling wines, the use of indigenous yeasts, isolated from quality winegrowing areas, is recommended (Vigentini, Barrera Cardenas, Valdetara, Faccincani, Panont, Picozzi & Foschino, 2017).

According to the last wine-market studies from the Organization International of Wine (OIV, 2018), sparkling wines have shown the greatest increase in sales volume and in global value over the last few years. This trend is known by the winemakers, who seek to satisfy the consumer demand with different strategies to obtain a specific and high quality product by using the grape varieties or selected ethanol-tolerant yeast strains from its specific production area (Munoz-Redondo, Cuevas, Leon, Ramirez, Moreno-Rojas, & Ruiz-Moreno, 2017; Ruiz-Moreno, Munoz-Redondo, Cuevas, Marrufo-Curtido, Leon, Ramirez, et al., 2017; Giovenzana, Beghi, Vagnoli, Iacono, Guidetti, & Nardi, 2016; Martinez-Rodriguez, Carrascosa, Barcenilla, Pozo-Bayon, & Polo, 2001).

The high quality sparkling wines have, as the main characteristic, endogenous CO_2 gas formed by yeast during the second fermentation in closed vessels (Ubeda, Callejon, Troncoso, Pena-Neira, & Morales, 2016). The traditional elaboration procedure (so-called *Champenoise* method) provides one of the most recognized high quality sparkling wines around the world and essentially consists of a second alcoholic fermentation of a base-wine in closed bottles, followed by a long ageing time on the yeast lees while the cell autolysis occurs (Torresi, Frangipane, & Anelli, 2011; Welke, Zanus, Lazzarotto, Pulgati, & Zini, 2014a). For this special elaboration, the yeast selection is difficult as a consequence of the preparation of the base wine stage and the addition of the expedition liquor stage. Both phases remain a secret only known by the technicians of the wine-cellar. In addition, the yeast

contribution to the wine aroma is scarce as the winemakers recognise, being considered only useful for the CO_2 production (Vigentini et al., 2017). Likewise, other factors should be mentioned such as the long testing time required, the difficulty to clarify the yeast metabolism contribution during fermentation and those related to autolysis phenomena during aging on lees. For these reasons, researches in the last years were focused on the yeast selection, using different yeasts immobilization systems, or killer yeast strains in co-culture with sensitive strains to accelerate the autolysis processes and shorten the ageing time (Lombardi, De Leonardis, Lustrato, Testa, & Iorizzo, 2015; Lopez de Lerma, Peinado, Puig-Pujol, Mauricio, Moreno, & Garcia-Martinez, 2018; Puig-Pujol, Bertran, Garcia-Martinez, Capdevila, Minguez, & Carlos Mauricio, 2013). In contrast, there are few studies about the improvement of the second fermentation process (Giovenzana et al., 2016; Kemp, Alexandre, Robillard, & Marchal, 2015). As a result, the yeasts were selected by their capacity for autolysis, resistance to high ethanol content, low pH, CO_2 overpressure and quality of the final product. Another important factor is the ability to form aggregates and to flocculate, that facilitate the removal of sediment and the disgorging phase in the sparkling wine production (Torresi et al., 2011). Studies in this field made by Canonico, Comitini, & Ciani, (2018); Di Gianvito, Perpetuini, Tittarelli, Schirone, Arfelli, Piva, et al., (2018), evaluate the yeast behaviour during the whole process, while few works aim to study the influence of stress conditions on their metabolism during the second fermentation (Giovenzana et al. 2016; Martinez-Garcia, Garcia-Martinez, Puig-Pujol, Mauricio, & Moreno, 2017).

The aroma is considered as an important attribute for the sparkling wine quality, having a great impact on the consumer preferences (Kemp et al., 2015; Munoz-Redondo et al., 2017). However, the literature about the aroma of these wines is very limited, compared to other wine types and the existing studies are focused on the differences between aged sparkling wines and their respective base wines (Lopez de Lerma et al., 2018; Pozo-Bayon, Martin-Alvarez, Moreno-Arribas, Andujar-Ortiz, & Pueyo, 2010; Riu-Aumatell, Bosch-Fuste, Lopez-Tamames, & Buxaderas, 2006; Torrens, Urpi, Riu-Aurnatell, Vichi, Lopez-Tamames, & Buxaderas, 2008; Welke, et al., 2014a). Although

the contribution of yeast to wine volatilome is well established, there are few studies on the effects of the second fermentation, and the existing works only show a few chemical families (Munoz-Redondo et al., 2017).,

According to Vigentini et al., (2017), "in a perspective of precision enology, where the wine is designed on specific vine cultivars and microorganisms, exploring the yeast biodiversity is a strategic activity to improve the production". In this respect, some wine-producing areas around the world use some *Saccharomyces cerevisiae* strains, so-called "flor yeasts", to elaborate Sherry type wines by using the biological aging method (Moreno-Garcia, Mauricio, Moreno, & Garcia-Martinez, 2016). Flor yeast has a high capacity to form aggregates and consequently to flocculate (Pretorius & Bauer, 2002) and can result a good candidate for the innovation and diversification in the sparkling wine-making.

This work aims to study the effect of the CO_2 overpressure, released by a flor yeast during the second alcoholic fermentation in closed bottles, on the volatilome of sparkling wines. This yeast is used for the first time in the sparkling wine production, since it is traditionally used for the biological aging of sherry-type wines.

2. Material and methods

2.1. Chemical standards

Aroma compounds were identified and quantified using standard solutions prepared from commercially available pure compounds of analytical grade, purchased from Sigma-Aldrich, Merck and Fluka. Pure water was obtained from a Milli-Q purification system (Millipore).

2.2. Yeast strain and experimental design

Saccharomyces cerevisiae G1 strain (ATCC: MYA-2451), a high ethanol- tolerant flor yeast from the Department of Microbiology (University of Cordoba, Spain) collection, was used for the second fermentation of sparkling wine. This yeast was isolated from wines with 14.5 % V/V ethanol content subjected to biological aging in the Montilla-Moriles area (Southern Spain).

2.2.1. Starter culture and yeast acclimation

A volume of 45 mL of pasteurized must was inoculated with a pure culture of G1 strain from agar YEPD broth and was incubated at 21ºC for 48h. The yeasts acclimation to the base wine conditions was achieved following the usual INCAVI protocol (Institut Català de la Vinya i el Vi), which consists in making up the above-mentioned culture to 1L with the same must and fermenting at 21ºC for 5 days in constant agitation (100 rpm). Reached this time, the ethanol content was 10 % v/v and yeasts cells were counted in a Thoma chamber to calculate the inoculum volume that provides a yeast population of 1.5 10^6 cells/mL in the final wine.

The characteristics of the pasteurized grape must (60% Macabeo, 40% Chardonnay) used for these conditions were: 174.9 g/L of reducing sugars, pH 3.4 and total acidity 3.6 g/L.

2.2.2. Base wine and fermentation conditions

The base wine was obtained from a blend of 60 % Macabeo and 40 % Chardonnay grapes, from the Penedes grape-growing area (North-eastern, Spain). This wine was distributed in 750 mL bottles, then added with 22 g of sucrose per bottle and a starter culture to provide 1.5 10^6 cells/mL yeast population. Fifteen bottles were arranged in a conditioned chamber at 14 ºC, to perform the second fermentation process, as was described by (Martínez-García et al., 2017). In this way, three batch of samples were established to obtain three independent biological triplicates. Three bottles containing the base wine (BW) without sugar and yeast addition, were used as initial control samples. Six of the remaining bottles were capped with a perforated plastic lid for the experiments in open bottles (OB), under non-pressure condition and the other six bottles were sealed (SB) with a crown seal and constituted the CO_2 overpressure condition.

The fermentation was followed by changes in the endogenous pressure, measured with an internal aphrometer (Oenotilus, Station Oenotechnique de Champagne, Epernay, France) and changes in wine composition were monitored at three sampling points. The first point corresponds to the initial base wine (BW), the second in the middle (OB1, SB1) and the third to the end of the process (OB2, SB2). These two last points were considered when the pressure of endogenous CO_2

reached 3 and 6 bar (SB1 and SB2, respectively), which correspond to the consumption of 12 and 24 g/L of sucrose in the non-pressure condition (OB1 and OB2, respectively). For counting the viable yeast cells, the content of three bottles randomly selected, was homogenized. After appropriate dilutions with Ringer solution, aliquots were plated with Sabouraud-chloramphenicol agar medium and incubated at 28 °C for 48 h. All analyses were carried out in triplicate.

2.3. Oenological parameters

Total acidity, volatile acidity, pH, ethanol content (% v/v), free and total sulphur dioxide were analysed by Infrared Spectroscopy (FT-IR) in a Winescan 120 FOSS, (Rellingen Germany), according to the International Methods of Analysis of Wines and Musts (OIV, 2018). The Total Phenol Index considered as A_{280}, the chromatic parameters A_{420}, A_{520}, A_{620} and the CIELab space coordinates were measured according to OIV (2018), in a UV-Vis spectrophotometer Lambda 25 (Perkin –Elmer, Massachusetts, USA). Sugar, malic acid, lactic acid, ammonium, free amine nitrogen and yeast available nitrogen (YAN) were analysed by enzymatic method using a multi-parametric analyzer Lisa 200 (Hycel Diagnostics, Technology Diffusion Iberica, Barcelona, Spain). The foam characteristics (maximum foam height (HM) and the plate height (HS) or foam persistence) were measured using the Mosalux procedure. For it, 100 mL of degasified wine is placed in a graduate glass tube (40 mm diameter and 440 mm of height) having a porous disk (16-40 μm diameter) located at the bottom, through which CO_2 gas is injected at 7 L/hour. One photoelectric cell provides a signal that is transmitted to a computer equipped with a specific software for the data processing. A graph of the foam height evolution in millimetres versus the elapsed time in seconds is obtained which shows HM and HS.

2.4. Volatilome analysis

Several hundred of compounds constitute the wine volatilome and they are grouped in major and minor volatile compounds, according to their content (Martínez-García et al., 2017).

2.4.1. Major volatile compounds and polyols

The major volatiles were present at a concentration of 10 mg/L or higher and they were analysed following the gas-chromatographic method described by Peinado, Moreno, Munoz, Medina, & Moreno, 2004 , which consists of a direct injection of 1 μL of the mixture made with 1 mL of an internal standard solution (4-methyl-2-pentanol at 1 g/L) and 10 mL of wine sample. The Gas-Chromatograph (GC) used was an Agilent 6890 from Palo Alto, (CA) provided with a CP-WAX 57 CB capillary column (60 m, 0.25 mm i.d., 0.4 μm film thickness) from Varian (Palo Alto, CA), and a Flame Ionization Detector (FID). The identification and quantification of each compound was made using a calibration table obtained from standard solutions of pure compounds analysed by the same procedure as wine samples. The identification of compounds was confirmed using an Agilent 7890A GC, coupled to a MSD5975C Mass Detector from Agilent Technologies (Wilmington, DE, USA), equipped with the same column and using identical chromatographic conditions.

2.4.2. Minor volatile compounds

This fraction of the volatilome groups the compounds with content below 10 mg/L and was analysed by liquid-solid extraction using the Stir Bar Sorptive Extraction technique, followed by Thermal Desorption – Gas Chromatography – Mass Spectrometry (SBSE-TD-GC-MS) analytical platform, as was previously reported by Martínez et al., (2017) and López de Lerma et al. (2018). For their extraction, the wine sample (1 mL) was placed in a 10 mL vial and diluted in a proportion 1:10 with an hydroalcoholic solution (12%, v/v ethanol and pH 3.5), previously added with 0.1 mL of an internal standard solution (0.446 mg/L of ethyl nonanoate in ethanol). Then, a Stir Bar 'Twister' (0.5 mm film thickness and 10 mm length, from Gerstel GmbH, Mülheim an der Rühr, Germany) coated with polydimethylsiloxane (PDMS) was used to stir the samples at 1200 rpm for 100 min at 20 °C. After removal from the wine sample, the Twister was rinsed with distilled water, dried with a cellulose tissue and transferred into thermal desorption tube for GC-MS analysis.

The analytical platform SBSE-TD-GC-MS, consisted of: a multipurpose sampler (MPS), and a Thermo Desorption Unity (TDU) from Gerstel that was coupled to and Agilent 7890A- MSD 5975C system. The GC was fitted with an HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25

μm film thickness) and the operating conditions for GC and MSD were described by Lopez de Lerma, et al., (2018).

Compounds were identified through the comparison of their mass spectrum with those of the NIST08 and Wiley7N collection and by comparing the mass spectrum of samples with those obtained from the standard solutions of pure compounds. Furthermore, the identification was also confirmed using the linear retention index (LRI) obtained for each compound in the samples and those reported in the NIST webbook of Chemistry (NIST, 2018), using Van Den Dool & Kratz, 1963, method.

Each compound was quantified by their calibration curve, obtained with standard solutions of pure compounds subjected to the same analytical conditions as wine samples. Target and qualifiers ions used for this purpose were selected using the Chemstation software (Agilent Technologies, Palo Alto, CA). The quantification limits obtained for the compounds with content levels close to 1 μg/L, showed values among 0.13-1.07 μg/L.

2.5. Data statistical analysis

All data showed are the result of three biological replicates, analysed in triplicate. One-way analysis of variance (ANOVA) and Kruskall Wallis test to establish Homogeneous Groups (HG) at a significance level $p \leq 0.05$ and the Multiple Variable Analysis (MVA) were performed using Statgraphics Plus v. 2, de STSC, Inc. (Rockville, MD, USA) software. An online resource (http://www.metaboanalyst.ca/) was used to build a Heatmap based on the Euclidean distance and to carry out a Principal Component Analysis (PCA), in view to establish differences between the wine samples and the effect of CO_2 overpressure. Data were previously Normalized according the root square and Pareto scaling, to avoid the differences introduced by the measure unities used.

3. Results and discussion

3.1. Evolution of endogenous CO_2 pressure

Figure 1 shows the pressure evolution of endogenous CO_2 gas, generated by the flor yeast G1 during the second fermentation in sealed bottles. This pressure comes from the fermentative activity and reveals that this yeast reached 3.6 bar at 10 days and 6.4 bar at 28 days, which are the middle and the end points of this process, respectively. After that, the pressure shows no significant changes. The live yeast populations were 2.9 10^6 in the middle and 2.9 10^4 cells/mL at the end of the fermentation in sealed bottles, while they were 3.7 10^6 and 7.1 10^5 cells/mL in open bottles at the same stages. This reveals a negative effect of the CO_2 pressure on cell viability for this yeast and a slightly slower kinetic of formation, compared to others *S. cerevisiae* strains (Di Gianvito, et al., 2018; Martínez-García et al., 2017).

Another effect was observed during the second fermentation. The yeast formed thick floccules with fast sedimentation and less adhesion to the walls of the bottle that favoured the greater wine clarification.

3.2. Enological features

Table 1 shows the average and standard deviation values for base wine (BW) and wines obtained in the middle (OB1, SB1) and at the end (OB2, SB2) of the second fermentation in open and sealed bottles. An ANOVA established five homogeneous groups (HG) only for N amine, four HG for A520, tonality, H*, HM and YAN at $p \leq 0.05$ significant level. The remaining parameters showed only 3 or less HG for all the samples.

Changes observed among base wine and wines after the second fermentation are similar to those obtained with other *S. cerevisiae* strains by applying the traditional method (Martinez-Rodriguez & Polo, 2000; Pozo-Bayon, et al., 2009). In this way, the increases in ethanol and glycerol and the decreases in sugar content are related to the second alcoholic fermentation itself. The changes found for nitrogen fractions are explained by yeast up-taking during the first stages of process, while the increases observed at the end, are a consequence of the release to the wine, when cell death occurs and autolysis begins (Martinez-Rodriguez & Polo, 2000).

Absorbance measurement at 280 nm (A280) is considered as a total polyphenol index (TPI) of wines and showed a slight, but significant, decrease with respect to the BW, which can be interpreted to a loss of compounds with conjugated double bonds **(Table 1**), including furanic derivatives (Serra-Cayuela, Castellari, Bosch-Fuste, Riu-Aumatell, Buxaderas, & Lopez-Tamames, 2013). A420 is a measure of browning for the white wines and it is related to the formation of yellowish-brown pigments from phenols - ortho-quinones polymerization (Bosch-Fuste, Riu-Aumatell, Guadayol, Calxach, Lopez-Tamamaes, & Buxaderas, 2007) and A520 is related to reddish-brown pigments. This latter and A620 values decrease during the second fermentation, impacting on the increase of tonality (A420/A520). Lastly, changes in the CIELab space are considered typical for these special white wines.

Regarding to the foam properties, there were observed higher values in persistence (HS) and foamability (HM) for the BW and minor values for wines fermented in open bottles (OB). Pozo-Bayón et al. (2009) describe a decrease in HM and an increase in HS during the second fermentation and Puig-Pujol et al., 2013, attribute the HS decrease to the bentonite used to facilitate the riddling process. Nevertheless, no bentonite was added in this study, thus the decrease in HS should be explained by the presence of C8, C10 and C18 fatty acids (Torresi et al., 2011).

Table 1 shows that the total acidity decreases and the values for pH and volatile acidity increase slightly from the base wine to the end of fermentation, particularly in the last sampling point. The malic and lactic acid content remain constant from the base wine (BW) to the middle of fermentation in the two studied conditions. Nevertheless, malic acid decreases at the end of fermentation while lactic acid increases its content, compared with other experiments carried out with *S. cerevisiae* P29 strain, under the same conditions (Martinez-García et al., 2017). This should be explained by the effect of malolactic fermentation. By comparing the results for P29 and those here exposed for G1, higher values were obtained for this later in lactic acid, A420, A520, A620 and colour intensity (CI). Therefore, the opposite trend was obtained for P29 in malic acid, tonality, HM and HS values. Lastly, only six parameters showed a clear dependence with the CO_2 over-pressure (N amine, A620,

tonality, a*, H*, HM), either in open or sealed bottles. Volatile acidity, lactic acid, A520 and YAN showed a dependence only in some sampling points. Similar results were obtained for P29 yeast strain, especially for the parameters: A520, tonality, a*, HM, N amine.

3.3. Wine volatilome

Supplementary table 1 shows the CAS number, the LRI calculated (LRIc) and reported in the literature (LRIr) for the 53 metabolites quantified. All of them are considered wine volatile metabolites, exception made for the polyols 2,3-butanediol and glycerol, which are semi-volatile and non-volatile compounds respectively. From them, ten were classified as major and forty as minor volatile compounds. This number of compounds is higher than those quantified for P29 strain under similar conditions (Martinez-Garcia, et al., 2017).

A tentative identification of metabolites was carried out considering a similarity value equal or higher than 75% among the mass spectrum obtained for each chromatographic peak in the samples and the mass spectral libraries (NIST and Willey). A difference below 15 between LRIc and LRIr was also used as identification criteria. (Soares, Welke, Nicolli, Zanus, Caramao, Manfroi, et al., 2015). According to this last criterion, all those compounds with LRIs differences higher than 15, were subjected to a definitive confirmation through the standard addition method and the mass-spectrum of their respective pure standards (Martinez-Garcia et al., 2017). Regarding the minor volatile compounds, similarity values for mass spectrum ranged from 80 to 90 and the maximum LRI difference was ±4 units, exception made for phenylethyl phenyl acetate with a value of 12.55.

Table 2 lists the concentration of volatile metabolites classified by chemical families, their odor descriptors, perception threshold (OPT) and the odorant series (OS) in which they are grouped. There were quantified 7 higher alcohols, 5 carbonyl compounds (4 aldehydes and 1 ketone), 5 carboxylic acids, 19 esters, 3 lactones, 3 terpenoids, 4 volatile phenols, 4 furanoids and 2 polyols. The results obtained by a non-parametric ANOVA at $p \leq 0.05$ level, carried out with the data matrix, showed that only 5 metabolites (2-ethylhexan-2-ol (15); decanal (18); dodecanoic acid (22); ethyl hexadecanoate (39) and the 5-(hydroxymethyl)-2-furaldehyde (53) established five HG, in a clear correspondence to

the five sample types analysed. Likewise 9 esters (numbered 24, 26, 27, 30, 31, 32,36, 37, 38), 3 terpenoids (43, 44, 45), 2 alcohols (1 and 2), 2 carbonyl compounds (7 and 16), 2 carboxylic acids (20 and 21), 2 volatile phenols (46 and 49), 2 furanic compounds (51 and 52) and a polyol (11) established four HG. Another 18 volatiles, 5 major (3, 4, 9, 10, 12) and 13 minor compounds (14, 18, 19, 23, 25, 28, 29, 34, 35, 40, 41, 42 and 48) established three HG, while 5 compounds exhibited only two HG and only 4-ethyl-2-methoxyphenol (47) showed no significant differences among the five samples.

Table 2 shows that alcohols exhibit higher values, which increase in relation to BW from 50 to 74 mg/L in OB1, OB2 and SB1, except to SB2 that decreases in 38.4 mg/L. Similar trend was observed for P29 strain under the same experimental conditions, although the increases for BW-SB1 (14 mg/L) and BW-SB2 (-36.3 mg/L) were lower (Martínez-García et al., 2017). These changes are mainly caused by the four major higher alcohols, such as propan-1-ol (2), 2-methylpropan-1-ol (3), isoamyl alcohols (4) and 2-phenylethan-1-ol (5), being the two latter the greatest contributors to the wine aroma with OAV >1. The formation of higher alcohols during the alcoholic fermentation is related to keto-acids pool, through the sugar and amino acids metabolism, via Erlich pathway (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). However, changes during the second fermentation in bottle, when yeasts are subjected to stress conditions, are in accordance with the results obtained by Coelho, Coimbra, Nogueira, & Rocha, 2009. This is explained by complex balances among the intracellular synthesis and extracellular adsorption-desorption processes in the cell wall (Pozo-Bayon et al., 2010). Low increases in methanol (1) content are explained by the cold-active pectinolytic activity of yeasts (Merin, Mendoza, Farias, & Ines Morata de Ambrosini, 2011). The C6-alcohols such as hexan-1-ol (14) and 2-ethylhexan-1-ol (15) have as precursors the unsaturated fatty acids (linoleic and linolenic acids) in an enzymatic pathway involving lipoxygenase and hydroperoxide lyase (Carlin, Vrhovsek, Franceschi, Lotti, Bontempo, Camin, et al., 2016). Furthermore, hexan-1-ol is involved in the formation of the corresponding ester, hexyl acetate, a product of yeast metabolism, being expected to decrease throughout the second fermentation, as

evidences the high correlation coefficient (r = -0.697) obtained for these compounds. However, the increase observed suggests that other precursors such as hexanoic acid are involved in its formation (Martinez-Garcia, et al., 2017). In relation to pressure effect, the percentage decrease obtained between the OB2-SB2 samples for this chemical family was 22.7%. Martínez-García, et al. (2017) obtained a percentage of - 8.13% for P29 strain when compare the alcohol content under similar conditions.

The carbonyl compounds family (6, 7, 16, 17 and 18) reached maximum increments from 70.8 to 80.5 mg/L in the middle of fermentation (SB1 and OB1) compared to BW, while SB2 registered a decrease of 4.75 mg/L. Acetaldehyde showed the highest content among this family and, together with nonanal and decanal are the main contributors to the aroma of G1 wines. It shows OAV = 8 for SB2 wines, close to the obtained for others non-veil forming yeasts and according to their contribution to the sparkling wine aroma. This value is four times lower than those obtained for the "Fino" type sherry wines, that are subjected to biological aging under flor veil growing conditions (Zea, Moyano, Moreno, Cortes & Medina, 2001; Zea, Moreno & Medina, 2007). This aldehyde is an important by-product released by yeasts during fermentation and constitutes a precursor of acetoin (7) and 2,3-butanediol (11, 12). Thereby, the decreases observed for this compound at the end of second fermentation may be attributed to its high chemical reactivity. Similar trend was obtained by Martínez-García et al. (2017) in a previous study with P29 yeast strain and the different content observed for these compounds is attributed to the yeast strains, in accordance to Regodon Mateos, Perez-Nevado, & Ramirez Fernandez, 2006. Others aldehydes such as benzaldehyde (16) and nonanal (17) increase along this study, while decanal (18) drastically decreases. This trend was described by Welke et al., (2014), for the aldehydes 3-phenyl-2-propenal, nonanal, undecanal, phenylacetaldehyde and hexanal, when comparing sparkling wines with their respective base wines. Nevertheless, the decreases observed in acetaldehyde and acetoin content for OB2 and SB2, have been described for the first time, whereas the percentage decrease registered between the same conditions was -24.8% in carbonyl compounds.

Medium and long chain organic acids (19, 20, 21, 22 and 23) are metabolites whose content are dependent on yeast strain and increase under stress conditions (Martinez-Garcia et al., 2017; Torrens et al., 2008). This explains why the number of compounds identified for G1 is higher than those identified for P29 yeast, although the total content of acid is greater in this latter. Acids C6, C8 and C10 highlight by its rise during second fermentation, reaching higher values in SB2 samples and the total content in acids increased in 18.9 mg/L, compared to BW. C8 and C10, jointly with C12 acid, show an important contribution to the aroma, but it is difficult to establish a comparison with other yeasts, due to the different wine-making conditions and aging time. Similar trends are described by Welke et al., 2014 for butanoic acid and other medium chain fatty acids. Changes observed for dodecanoic acid may be explained by adsorption-desorption processes of the cell walls and by enzymatic or chemical hydrolysis of their esters, linked to yeast autolysis phenomena. Related to CO_2 pressure, an increase of 7.8% was registered when comparing OB2 with SB2 samples.

Esters family are strongly related with organic acids and are synthetized either enzymatically during alcoholic fermentation by yeasts or by chemical esterification during ageing at the low pH of wines (Ruiz-Moreno, et al., 2017). Both processes explain the increases obtained for the final wines, whose total content are twice those of the BW. Compared to other studies carried out with other yeasts in the same experimental conditions, both qualitative and quantitative differences were observed. Thus, ethyl lactate (9), ethyl hexanoate (29) and phenylethyl phenyl acetate (38) were only identified in G1 wines, whereas 2-methylpropyl acetate, ethyl-2-methylbutanoate and ethyl 3-methylbutanoate are characteristic of P29 wines (Martínez-García et al., 2017). In addition, the total content of ester in the final G1 wines is lower than P29 wines, in which the average values were 41.7 ± 1.7 and 42.8 ± 4.1 mg/L for OB2 and SB2, respectively. Muñoz-Redondo et al. (2017), using a commercial yeast strain and Pedro Ximénez grape variety obtained a total esters content of 2.218 mg/L after 11-12 weeks of the second fermentation. Ethyl esters of fatty acids (EEFA) are the most important group among esters and 11 were quantified in this study, being three considered as major

(ethyl acetate, ethyl lactate, diethyl succinate) and eight as minor volatile compounds (ethyl propanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl-2-methyloctanoate, ethyl decanoate, ethyl dodecanoate, ethyl tetradecanoate and ethyl hexadecanoate). The ethyl acetate diminished during the second fermentation while ethyl esters of lactic acid and succinic acid showed a marked increase. Changes observed in OB2 and SB2 samples for ethyl lactate and lactic acid are similar and could be explained by the malolactic fermentation. The increase for ethyl succinate in OB1 and SB1 samples, are explained as a consequence of alcoholic fermentation itself. These changes are a consequence of enzymatic or chemical synthesis and hydrolysis process (Welke et al., 2014a). Other factors involved are: temperature, aeration, skin contact, yeast strain or the ageing in contact with lees (Ruiz-Moreno, et al., 2017). According to pressure effect, Martinez-Garcia, et al.(2017) found that ethyl dodecanoate (36), ethyl tetradecanoate (37) and ethyl butanoate (27) were the main contributors to differentiate the wines obtained through a second fermentation with and without CO_2 over-pressure. Ethyl isobutanoate (26) decreases along the second fermentation, reaching higher values at 6 bar of pressure. These results are in accordance to Muñoz-Redondo et al., (2017) and the study made by Martinez-Garcia, et al. (2017) with P29 strain, which suggests this compound as a possible marker of the CO_2 over-pressure. The presence of ethyl heptanoate (31) is associated by some authors to the effect of maceration processes during the elaboration of base wine (Ruiz-Moreno, et al., 2017). Lastly, the acetates are an important esters family formed during fermentation from an alcohol and acetyl-CoA, in a reaction catalyzed by alcohol acetyltransferases. In addition to ethyl acetate (8), there were quantified methyl acetate (24), 3-metylbutyl acetate (28), hexyl acetate (30), 2-phenylethyl acetate (34) and phenylethyl phenyl acetate (38), being compound 28 the most important contributor to wine aroma. In general terms, these compounds trend to decrease in their content during the second fermentation, which may be explained by chemical hydrolysis (Ruiz-Moreno, et al., 2017) and also by the incorporation of the acetate compounds in yeast metabolism (Munoz-Redondo et al., 2017). Related to CO_2 pressure dependence, the acetates here exposed experimented changes with this factor, being observed higher values under pressure

conditions. Nevertheless, the changes in the total content of esters between the conditions at the end of the fermentation (OB2-SB2) resulted in a decrease of 30.1%.

Lactones are formed through intramolecular esterification of hydroxyacids, during the alcoholic fermentation by yeasts or during wine subsequent evolution as a consequence of some chemical processes (Cotea, Zanozaga, & Cotea, 2014). Generally, their content increases during wine maturation and aging, contributing to candy floss, sweet fruits, coconut and butter odour. This trend has been observed for all the lactones (γ-crotonolactone, γ-butyrolactone and decalactone) in G1 and P29 yeasts. However, the γ-crotonolactone content was higher in this later (Martinez-Garcia et al., 2017), being the total content in these compounds for P29 OB2 and SB2 samples of 73 ± 7and 70 ± 12 mg/L, respectively. The γ-crotonolactone was also the main contributor to the aroma of G1wines, followed by γ-butyrolactone. With respect to the CO_2 pressure, a significant decrease ($p < 0.05$) is obtained in the total lactone content for the SB samples, being around 10 mg /L lower than the OBs. No similar trend was obtained in wines for P29 yeast (Martínez-García, et al., 2017).

Monoterpenes in free form are important contributors to the aroma of Muscat grape varieties and have floral and citrus odours. However, the glyosidic combinations, mainly formed by hydroxy-terpenes, have no odour, unless the corresponding aglycone is released through the action of the β-glycosidases. In this respect, literature describes increasing amounts of monoterpenols and sesquiterpenols during the second fermentation and attributes to yeast effect the differences observed (Carlin, et al., 2016). In this work, all terpenic compounds quantified increase, but only changes in the isomeric forms of nerolidol (44,45) may be attributed to the G1 β-glycosidase activity, because limonene (43) is not an hydroxy-terpene, not exceeding their OAV 0.01 units. Lopez de Lerma et al., (2018) obtain lower contents with yeast strains P29 and QA23 (1.5 ± 0.1 and 1.65 ± 0.05 μg/L, respectively) in sparkling wines at 32 months of aging time. On the other hand, no compounds of this family were identified by Martínez-García, et al. (2017) in the study of P29 strain at the end of the second fermentation. Lastly, the significant differences between OB and SB samples, show the

possible influence that CO_2 has on the enzymatic activity, being obtained an increase of 25 % between the OB2-SB2 samples.

According to Cotea, et al. (2014), about 20 volatile phenols have been identified in wines, that often contribute to the "spicy", "floral" and "clove-like" odors. Nevertheless, only 4 have been quantified (46, 47, 48 and 49) in this study that are twice fold those identified by Martinez-Garcia et al., 2017) in P29 strain. Despite this, the total content of these compounds for P29 in OB2 and SB2 bottles (0.44 and 0.46 mg/L, respectively), is close to those values shown for G1 in table 2. Higher contents have been described by other authors in wines obtained with QA23 yeast strain (0.75 mg/L) after 32 months of aging time (López de Lerma et al., 2018). Considering the contribution to the wine aroma (OAV > 1), highlight guayacol (46) a monomethyl ester of pyrocatechin with a resin smell whose content increases along the second fermentation. On the other hand, ethyl-phenols are formed during the fermentation in low amounts by *Saccharomyces* and *Brettanomyces* genus from hydroxycinnamic acids through the action of vinylphenol reductase. In this respect, only the 1,3-dimethoxy-2-hidroxybencene (49) showed a decrease at the final stage in the two studied conditions, having quantified in 2.2% the difference in content between OB2- SB2 samples for this chemical family.

The last chemical family quantified is the furanic compounds (50, 51, 52, 53) mainly formed throughout the Maillard reactions, which have brown color and caramel odors. According to Serra-Cayuela et al., (2013) its formation is affected by several factors (sugar, nitrogen compounds and alcohol, low pH and high temperature values). From them, only furan-2-carbaldehyde (51) with OAV >1, contributes to the final aroma with its toasted nuances. In relation to this, the contents of these compounds are lower in G1 wines (Table 2) than the obtained in the study of Martínez- García, et al. (2017) for P29 (5.2 ± 1.1 mg/L for OB2 and 4.8 ± 0.9 mg/L for SB2 samples). However, the content showed by other authors in wines with a long aging time and different yeast strain ranged from 650 to 732 μg/L (López de Lerma et al., 2018). With respect to base wine, the increase shown for these compounds in **table 2** is directly related with increases for the absorbance values at 280 nm

during this stage (**Table1**). Nevertheless, a general decrease quantified by -17.4% between OB2-SB2 samples, is observed for this family due to the CO_2 pressure effect.

3.4. Statistical Analysis

3.4.1. Volatile metabolites Heatmap

The heat map **(Supplementary Figure 1)**, built with the content of the wine volatiles, provides a comprehensive and easy to understand overview about the effects of the second fermentation and to the endogenous CO_2 overpressure. The square Euclidean distance and Ward's method were used as grouping rule and as a measure of the proximity among samples. According to this, low and high content are shown in blue and red colours, respectively. In this regard, most of the volatiles quantified showed a general increase, compared with the base wine (BW). However, decanal (18), ethyl butanoate (27), isoamyl acetate (28), hexyl acetate (30), ethyl octanoate (32) and 2-phenylethyl acetate (34), decreased their content.

3.4.2. Volatilome based footprintings

Two footprinting types (**Figure. 2. a, b**), based on the volatilome data matrix, are obtained by a Multiple Variable Analysis (MVA). **Figure 2a** shows a 10-vertex polygonal shape obtained by grouping the volatile compounds into ten chemical families listed in table 2: 1. Alcohols; 2. Aldehydes; 3. Ketones; 4. Carboxylic acids; 5. Esters; 6. Lactones; 7. Polyols; 8. Terpenes; 9. Volatile phenols and 10. Furanic compounds. **Figure 2b** has an eight-vertex polygonal shape obtained from the OAV for each volatile, grouped into eight odorant series (OS) as it is shown in **Table 2**: I. Chemical; II. Fruity; III. Floral; IV. Fatty; V. Balsamic; VI. Vegetal; VII. Empyreumatic; VIII. Spicy. These footprintings provide a graphical and useful way to associate the cause and the effects studied in this work. One-way analysis of variance was also made to identify differences among each family or odorant series from the group of samples. Data set were previously scaled and normalized.

3.4.2.1. Footprints from chemical families

Figure 2a shows five footprints, being the smallest and the most regular polygon for the base wines (BW). In contrast, wines at the end of the second fermentation without pressure (OB2) have higher content in most of the chemical families, except to carboxylic acids (4) and terpenes (8). Whereas, wines obtained under pressure (SB1, SB2) have higher levels of these latter chemical families and lower levels for alcohols (1) and aldehydes (2) in SB2 samples. These results confirm the CO_2 pressure effects in the chemical families previously described by Martínez-García et al., 2017.

3.4.2.2. Footprints from odorant series

The contribution of each volatile compound to the wine aroma can be evaluated qualitatively by its odour descriptor and quantitatively by its odorant activity value (OAV) that is defined as the ratio Concentration/OPT (Lopez de Lerma, et al., 2018). Thus, each compound is associated to one or several aroma descriptors, being possible to group those compounds with similar descriptor into the same odorant series (OS). This criterion provides a most objective odorant profile compared to sensorial analysis.

In this study, OAVs were calculated for all the compounds quantified, except to ethyl-2-methyloctanoate (33) and phenylethyl phenylacetate (38), whose odour descriptor and threshold (OPT) was not available in the wine literature. **Supplementary table 2** shows the average contents of OAVs obtained by odorant series in each wine.

Figure 2b, states that BW footprint differs significantly ($p<0.05$) from the remaining samples by their higher values in Fruity (II), Fatty (IV), Vegetal (VI) and Empyreumatic (VII) series and low levels in Chemical (I), Balsamic (V) and Spicy (VIII) series. Samples taken in the middle of the second fermentation under pressure (SB1), increased their Fruity, Fatty, Balsamic, Vegetal and Spicy series, compared to their homologous in open bottle (OB1), which only have higher values for Empyreumatic series. However, the opposite trend was observed at the end of fermentation (OB2, SB2). Differences due to CO_2 pressure are obtained for Chemical, Fruity and Fatty series that show a

decrease in their values. In general, these results are in agreement with those obtained by Martinez-Garcia et al. (2017) for the P29 strain.

3.4.3. Principal Component Analysis (PCA)

The PCA is a multivariate statistical tool widely used for an automatically reduction of the data set dimension. This unsupervised recognition method permits the feature extraction that helps to establish differences among the samples from a high dimensional data matrix. In order to study the effect of endogenous CO_2 pressure on the volatilome, a PCA was built by using as variables the content of volatiles that showed significant changes among the open or sealed bottles (OB, SB) during the second fermentation. In this way, only seven volatile metabolites were discarded for PCA because they showed not significant differences ($p \geq 0.05$) according the Kruskall-Wallis test among the four samples (OB1, OB2, SB1, SB2). These were isobutanol (3), 2-phenylethan-1-ol (5), diethyl succinate (10), nonanal (17), isoamyl acetate (28), ethyl-2-methyloctanoate (33) and 4-ethyl-2-methoxyphenol (47). The remaining 45 volatiles showed significant differences and the two PCs obtained for these explained the 86.4% of the total cumulative variance (71.7 % for PC1 and 14.8 % for PC2). **Figure 3A** plots the sample scores and **Figure 3B** the variable loadings for each PC.

Components PC1 and PC2 define the plane plotted in **Fig. 3A**, where the samples are clearly grouped. Samples on the left have negative scores for PC1 and corresponds to the middle of the second fermentation, while samples to the right have positive scores in PC1 and correspond to the end of this process. In the same way, samples subjected to CO_2 overpressure are located on the top of PC2 and those no subjected to pressure on the bottom. **Figure 3B** shows that PC1 correlates positively with ethyl lactate (9) and 2-methoxy phenol (46) (with loadings 0.689 and 0.157, respectively) and negatively with volatiles numbered 36 (-0.307), 37 (-0.261) and 38 (-0.235). PC2 correlates with volatiles with positive loadings 31 (0.259), 11 (0.227), 8 (0.219), 18 (0.209), 36 (0.183) and negatively with 38 (-0.411), 26 (-0.295), 37 (-0.273) and 41 (-0.240). To sum up, nine volatile metabolites are the most affected by the CO_2 over-pressure during the second fermentation in sealed bottles and five volatiles are mainly dependent on the fermentation stage.

4. Conclusions

A *Saccharomyces cerevisiae* flor yeast strain, has been used for 'in bottle' second fermentation, the first step of the foam-forming process in the sparkling wine-making. Significant changes caused by CO_2 over-pressure, were obtained in nine chemical families (higher alcohols, aldehydes, ketones, carboxylic acids, esters, lactones, polyols, terpenes, and furanic compounds) at the end of second fermentation (P = 6 bar) and only six (carboxylic acids, lactones, polyols, terpenes, volatile phenols and furanic compounds) in the middle of this process (P = 3 bar). Furthermore, the average Odor Activity Values for eight odorant series provided an aroma footprint that reveals the influence of CO_2 overpressure and the fermentation stage on the wine aroma. Finally, a Principal Component Analysis, established that nine volatiles were the main contributors of CO_2 pressure effect and five of the fermentation stages.

The use of high ethanol-tolerant flor yeasts is a suitable strategy to obtain new sparkling wines with characteristic volatilome and odorant profiles. Further researches are required to provide evidence of the effectiveness and feasibility of these yeasts related to the formation of aggregates and flocculation processes along the on lees aging period.

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6. Appendix of supplementary data

7. References

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Table 1. *Composition of base wine (BW) and wines in the middle and at the end of the second fermentation in open (OB1, OB2) or sealed bottles (SB1, SB2).*

Parameter	*BW*	*OB1*	*OB2*	*SB1*	*SB2*
Ethanol (% v/v)	10.23^{a}±0.02	10.65^{b}±0.02	11.4^{c}±0.2	10.76^{b}±0.04	11.4^{c}±0.1
Reducing sugars (g/L)	0.33^{a}±0.03	12.7^{c}±0.3	0.30^{a}±0.00	10.5^{b}±0.2	0.30^{a}±0.00
Propane-1.2.3-triol (Glycerol) (mg/L)	4020^{a}±656	4810^{b}±22	4818^{b}±197	4493^{ab}±164	4227^{ab}±297
Volatile acidity (g/L)	0.23^{b}±0.02	0.19^{a}±0.01	0.28^{c}±0.00	0.20^{b}±0.00	0.28^{c}±0.02
Total acidity (g/L)	5.5^{b}±0.1	5.3^{b}±0.1	4.4^{a}±0.1	5.2^{b}±0.1	4.1^{a}±0.5
pH	3.29^{a}±0.01	3.29^{a}±0.01	3.37^{b}±0.01	3.30^{a}±0.01	3.37^{b}±0.01
Malic acid (g/L)	1.8^{b}±0.1	1.90^{b}±0.00	0.10^{a}±0.00	1.90^{b}±0.00	0.2^{a}±0.2
Lactic acid (g/L)	0.1^{a}±0.0	0.1^{a}±0.0	1.2^{c}±0.1	0.1^{a}±0.0	1.1^{b}±0.1
Total SO_2 (mg/L)	94.0^{b}±0.2	88^{a}±2	85^{a}±3	87^{a}±2	88^{a}±4
Free SO_2 (mg/L)	4.6^{a}±0.7	4.00^{a}±0.00	7^{b}±1	7^{ab}±3	10^{b}±4
TPI (AU 280 nm)	5.41^{c}±0.00	5.15^{a}±0.02	5.33^{b}±0.03	5.17^{a}±0.02	5.35^{b}±0.02
A420 (AU 420 nm)	0.157^{c}±0.001	0.049^{a}±0.001	0.063^{b}±0.003	0.052^{a}±0.001	0.065^{b}±0.004
A520 (AU 520 nm)	0.125^{d}±0.005	0.007^{a}±0.001	0.015^{c}±0.002	0.008^{b}±0.001	0.017^{c}±0.003
A620 (AU 620 nm)	0.017^{c}±0.001	0.001^{c}±0.000	0.005^{b}±0.002	0.001^{a}±0.000	0.007^{b}±0.003
Color intensity (A420+A520+A620)	0.40^{c}±0.06	0.057^{a}±0.001	0.08^{b}±0.01	0.062^{a}±0.001	0.09^{b}±0.01
Tonality (A420/A520)	1.3^{a}±0.1	7.4^{d}±0.6	4.2^{b}±0.4	6.3^{c}±0.5	4.0^{b}±0.5
a*	-0.62^{ab}±0.01	-0.64^{ab}±0.03	-0.60^{b}±0.05	-0.63^{ab}±0.04	-0.66^{a}±0.01
b*	2.55^{a}±0.01	3.74^{b}±0.01	4.3^{c}±0.1	3.8^{b}±0.1	4.4^{c}±0.1
L*	89.76^{a}±0.04	99.5^{c}±0.1	98.8^{b}±0.2	99.40^{c}±0.00	98.7^{b}±0.3
C*	2.64^{a}±0.03	3.80^{b}±0.01	4.4^{c}±0.1	3.9^{b}±0.1	4.4^{c}±0.1
H*	103.54^{d}±0.02	99.7^{c}±0.5	98^{a}±1	99.4^{bc}±0.5	98.6^{ab}±0.2
HM (Maximum height, mm)	33^{d}±1	13^{b}±2	11^{a}±1	17^{c}±3	17^{c}±2
HS (High stability, mm)	24^{b}±1	9^{a}±1	9^{a}±1	10^{a}±1	11^{a}±1
YAN (mg/L)	27^{d}±1	13.0^{a}±0.0	17^{c}±1	15^{b}±2	19^{c}±1
N amine (mg/L)	25^{e}±1	12.0^{a}±0.0	15^{c}±1	13^{b}±1	17^{d}±1
N ammoniacal (mg/L)	2^{b}±1	1.0^{a}±0.0	2^{ab}±1	2^{ab}±1	1.3^{ab}±0.6

Results are the average values of three batches of samples analyzed by triplicate. BW - base wine; OB - fermentation in open bottle; SB - fermentation under CO_2 overpressure. Number 1 or 2 refer to samples in the middle and at the end of the second fermentation respectively.
Letters a, b, L, C and H correspond to the Cielab color coordinates. HM and HS are parameters used for foam properties. TPI: Total Phenol Index. YAN: Yeast Assimilable Nitrogen. $^{a.\ b.\ c.\ e}$ Different letters in the same row indicate statistical differences of the normalized and scaled data at 0.05 level according to Kruskall Wallis' least significant difference method. ns - non-significant.

Table 2. *Average concentrations ± standard deviation of major and minor aroma compounds (mg/L) in the base wine and sparkling wines in the middle and at the end of the second fermentation in open (OB1, OB2) and sealed bottles (SB1, SB2).*

No	Compound name	BW	OB1	OB2	SB1	SB2	Odor descriptor	OPT (mg/L)	OS
Σ Alcohols		*419^{b}±10*	*477^{c}±10*	*493^{c}±17*	*489^{c}±10*	*381^{a}±31*			
1	Methanol	32^{a}±3	48.1^{c}±0.4	51^{cd}±1	57^{d}±1	39^{b}±6	[I] Chemical, medicinal, pungent fruity	668[I]	***1***
2	Propan-1-ol	11.6^{a}±0.3	16^{c}±1	19^{d}±1	16^{c}±1	13^{b}±2	[I]Solvent, ripe fruit	830[I]	***1,2***
3	2-methylpropan-1-ol	20^{b}±1	21.9^{c}±0.1	23^{c}±1	22^{c}±1	18^{a}±2	[I]Like wine, nail polish	40[I]	***1***
4	**Isoamyl alcohols†**	**306^{b}±8**	**317^{bc}±6**	**330^{c}±11**	**323^{bc}±9**	**258^{a}±18**	[I]Like wine, nail polish, whisky, ripe fruit	30[I]	***1,2***
5	2-Phenylethan-1-ol	49^{a}±4	70^{b}±6	69^{b}±5	70^{b}±2	50^{a}±13	[I]Rose talc, honey	10[I]	***3***
14	Hexan-1-ol	1.2^{a}±0.2	1.6^{b}±0.1	1.78^{bc}±0.04	1.9^{a}±0.04	2.0^{b}±0.1	[I]Grass just cut	2.5[I]	***6***
15	2-Ethylhexan-1-ol	0.24^{a}±0.02	2.21^{e}±0.02	0.5^{b}±0.1	1.3^{d}±0.2	0.8^{c}±0.1	[I]Citrus, fresh floral	8[I]	***2,3***
Σ Aldehydes and ketones		*123^{a}±2*	*203^{c}±2*	*157^{b}±14*	*193.4^{c}±0.4*	*118^{a}±15*			
6	**Acetaldehyde**	**87^{a}±1**	**137^{b}±2**	**120^{b}±13**	**132^{b}±1**	**87^{a}±16**	[I]Pungent, stewed apple	10[I]	***1,2***
7	**Acetoin**	19^{a}±1	**66^{d}±4**	**36^{c}±2**	**61^{d}±1**	**31^{b}±2**	[I]Sour yogurt, sour milk	30[I]	***4***
16	Benzaldehyde *(μg/L)*	8^{a}±1	28^{d}±3	23^{cd}±1	12^{b}±2	20.7^{c}±0.2	[III]Bitter almond, walnut, smoky	2[III]	***2,7***
17	**Nonanal**	**0.087^{a}±0.004**	**0.12^{b}±0.01**	**0.14^{c}±0.01**	**0.13^{bc}±0.01**	**0.12^{b}±0.01**	[I]Citrus, fatty, green, slightly pungent	0.01[I]	***1,2,4,6***
18	**Decanal**	**15.9^{e}±0.1**	**0.09^{a}±0.01**	**0.14^{c}±0.01**	**0.17^{d}±0.01**	**0.13^{b}±0.01**	[VI]Grassy, Orange peel	0.0075[V]	***2,6***
Σ Acids		*14.3^{a}±0.1*	*21^{b}±1*	*31^{c}±2*	*31^{c}±1*	*33.2^{d}±0.2*			
19	Hexanoic acid	0.14^{a}±0.01	0.21^{b}±0.01	0.19^{b}±0.01	0.26^{c}±0.01	0.27^{c}±0.02	[III]Cheese, rancid	0.42[III]	***4***
20	**Octanoic acid**	**12.4^{a}±0.1**	**18^{b}±1**	**27^{cd}±2**	**26^{c}±1**	**28.7^{d}±0.1**	[I]Fatty, waxy, rancid, oily	0.5[I]	***4***
21	**Decanoic acid**	0.96^{a}±0.04	**2.1^{b}±0.1**	**2.9^{c}±0.2**	**3.1^{d}±0.2**	**3.3^{d}±0.1**	[I]Unpleasant, rancid, sour	1[I]	***4***
22	**Dodecanoic acid**	n.f.^{a}	**0.155^{d}±0.003**	**0.122^{c}±0.003**	**0.177^{e}±0.003**	**0.117^{b}±0.003**	[II]Waxy, soapy	10[II]	***4***
23	Tetradecanoic acid	0.83^{a}±0.01	0.91^{c}±0.01	0.88^{b}±0.02	0.865^{b}±0.004	0.857^{b}±0.002	[I]Waxy, fatty, soapy	10[I]	***4***
Σ Esters		*33^{a}±1*	*58^{b}±3*	*87^{c}±5*	*57^{b}±1*	*61^{b}±12*			
8	**Ethyl acetate**	**21^{b}±1**	**15^{a}±2**	**20^{b}±1**	**18.4^{b}±0.4**	**14^{a}±2**	[I]Pineapple, varnish, balsamic	7.5[I]	***1,2,5***

No	Compound name	BW	OB1	OB2	SB1	SB2	Odor descriptor	OPT (mg/L)	OS
9	Ethyl lactate	n.f.[a]	n.f.[a]	48[c]±4	n.f.[a]	29[b]±9	[VI]Sweet, fruity, lactic, yogurt, buttery	100	***2,4,7***
10	Diethyl succinate	n.f.[a]	37[c]±3	12.2[b]±0.4	32[c]±1	11[b]±2	[I]Overripe melon, lavender	100[I]	***2,4,7***
24	Methyl acetate	0.10[a]±0.01	0.16[b]±0.01	0.23[d]±0.01	0.146[b]±0.004	0.20[c]±0.02	[I]Sweet, ether	0.7[I]	***2,3***
25	Ethyl propanoate	0.16[c]±0.01	0.13[b]±0.01	0.17[c]±0.01	0.0750[a]±0.0004	0.13[b]±0.02	[I]Sweet fruity, grape, pineapple	5.5[I]	***2***
26	Ethyl isobutanoate *(μg/L)*	3[d]±1	1.7[b]±0.2	1.27[a]±0.04	1.1[a]±0.1	2.4[c]±0.2	[I]Fruity, apple, strawberry	0.015[I]	***2***
27	**Ethyl butanoate**	**2.21[d]±0.02**	**0.195[a]±0.004**	**0.30[b]±0.02**	**0.203[a]±0.004**	**0.33[c]±0.02**	[I]Fruity, sweet, tutti frutti, apple	0.02[I]	***2***
28	**3-Methylbutyl acetate**	**5[c]±1**	**2.31[a]±0.04**	**2.4[ab]±0.2**	**2.2[a]±0.1**	**2.7[b]±0.2**	[I]Sweet, fruity, banana, solvent	0.03[I]	***2,7***
29	**Ethyl hexanoate**	n.f.[a]	**0.77[b]±0.01**	**0.87[c]±0.04**	**0.80[b]±0.02**	**0.86[c]±0.04**	[III]Green apple	0.014[II]	***2***
30	Hexyl acetate	0.29[d]±0.01	0.103[ab]±0.004	0.10[a]±0.01	0.121[c]±0.002	0.11[bc]±0.01	[I]Green, fruity, sweet, fatty, fresh, apple and pear	1.5[I]	***2,4,6,7***
31	**Ethyl heptanoate**	n.f.[a]	**0.006[c]±0.001**	**0.006[cd]±0.001**	**0.008[d]±0.001**	**0.004[b]±0.001**	[I]Fruity, pineapple, sweet, banana	0.002[I]	***2,7***
32	**Ethyl octanoate**	**2.817[d]±0.003**	**1.625[b]±0.03**	**1.67[b]±0.03**	**1.9[c]±0.1**	**1.4[a]±0.1**	[I]Pineapple, pear, soapy	0.005[I]	***2,4***
33	Ethyl-2-methyloctanoate *(μg/L)*	3.7[a]±0.4	4.6[ab]±0.4	4.3[b]±0.3	5.1[b]±0.4	5[b]±1	n.f.	n.f.	***n.f***
34	**2-Phenylethyl acetate**	**0.75[c]±0.02**	**0.43[b]±0.01**	**0.406[a]±0.003**	**0.48[c]±0.01**	**0.50[c]±0.03**	[I]Fruity, rose, sweet, honey	0.25[I]	***2,3,7***
35	**Ethyl decanoate**	**0.65[c]±0.02**	**0.59[c]±0.04**	**0.40[b]±0.04**	**0.68[c]±0.03**	**0.28[a]±0.04**	[I]Sweet, fruity, nuts and dried fruit	0.2[I]	***2,7***
36	Ethyl dodecanoate *(μg/L)*	0.8[b]±0.1	15[d]±1	1.5[c]±0.4	14[d]±1	0.5[a]±0.1	[I]Sweet	1.5[I]	***7***
37	Ethyl tetradecanoate *(μg/L)*	n.f.[a]	1.5[d]±0.2	n.f.[a]	0.4[c]±0.1	0.2[b]±0.1	[I]Sweet fruit, butter, fatty odor	2[I]	***2,4***
38	Phenylethyl phenylacetate *(μg/L)*	n.f.[a]	2.3[d]±0.2	n.f.[a]	0.551[c]±0.004	0.49[b]±0.03	n.f.	n.f.	***n.f***
39	Ethyl hexadecanoate *(μg/L)*	1.1[a]±0.1	7.4[e]±0.2	4.2[b]±0.1	6.1[d]±0.3	4.6[c]±0.2	[I]Fatty, rancid, fruity, sweet	1.5[I]	***2,4,7***
Σ Lactones		*29[a]±2*	*43.9[c]±0.1*	*57[d]±4*	*33[b]±3*	*46[c]±4*			
4	**5-H-furan-2-**	**29[a]±2**	**36[b]±1**	**52[c]±4**	**28[a]±3**	**38[b]±4**	[I]Toasty,	1[I]	***7***

No	Compound name	BW	OB1	OB2	SB1	SB2	Odor descriptor	OPT (mg/L)	OS
0	**one (γ-crotonolactone)**						caramel		
41	**Dihydrifuran-2(3H)-one (γ-butyrolactone)**	n.f.^{a}	**8^{c}±1**	**6^{b}±1**	**5.6^{b}±0.2**	**8.1^{c}±0.3**	[III]Toasted, burned	1[III]	*7*
42	γ-Decalactone *(μg/L)*	n.f.^{a}	4.5^{b}±0.4	4.9^{b}±0.2	6.1^{c}±0.4	5.6^{c}±0.2	[I]Peach, milky	0.01[I]	***2,4***
Σ *Terpenes*		*0.009^{a}±0.001*	*0.026^{c}±0.001*	*0.020^{b}±0.001*	*0.029^{d}±0.001*	*0.025^{c}±0.001*			
43	1-Metil-4-(1-metilentenil)-ciclohexeno (Limonene) *(μg/L)*	9^{d}±1	3.6^{b}±0.5	1.8^{a}±0.5	5^{c}±1	2.3^{a}±0.2	[VII]Citrus, sweet, herbal	0.2[VII]	***2,6,7***
44	3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol (E-Nerolidol) *(μg/L)*	n.f.^{a}	13.10^{c}±0.04	10.1720^{b}±0.003	13.6^{d}±0.2	13^{c}±1	[IV,VI] Rose, green apple, citrus, waxy	1[IV,VI]	***2,3,4***
45	3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol (Z-Nerolidol) *(μg/L)*	n.f.^{a}	9.6^{c}±0.1	8.04^{b}±0.02	9.9^{d}±0.1	9.5^{c}±0.3	[VI] Waxy, floral	1[IV]	***3,4***
Σ *Volatile Phenols*		*0.20^{a}±0.03*	*0.25^{b}±0.01*	*0.5^{d}±0.1*	*0.35^{c}±0.01*	*0.47^{d}±0.02*			
46	**2-methoxifenol *(Guayacol)***	**0.049^{a}±0.003**	**0.06^{b}±0.01**	**0.13^{d}±0.02**	**0.073^{c}±0.004**	**0.14^{d}±0.01**	[VI]Smoky, sweet, medicinal	0.001 [VI]	***5,7***
47	4-Ethyl-2-methoxyphenol *(μg/L)*	n.f.^{ns}	7.8^{ns}±0.3	7.6^{ns}±0.1	7.6^{ns}±0.1	7.6^{ns}±0.1	[VI]Medicinal, wood, clove, smoked	0.033 [VI]	***5,7,8***
48	**4-Ethenyl-2-methoxyphenol**	**0.15^{a}±0.02**	**0.16^{a}±0.01**	**0.29^{c}±0.03**	**0.24^{b}±0.01**	**0.262^{bc}±0.004**	[I]Spices, clove, peanut, woody	0.04[I]	***8***
49	1,3-Dimethoxy-2-hydroxybenzene	n.f.^{a}	0.019^{b}±0.002	0.03^{c}±0.01	0.028^{c}±0.002	0.06^{d}±0.01	[I]Medicine, phenol, smoky	0.57[I]	***1,7***
Σ *Furanic compounds*		*1.19^{a}±0.03*	*3.9^{d}±0.1*	*3.8^{d}±0.2*	*3.3^{c}±0.1*	*3.17^{b}±0.03*			
50	(Furan-2-yl)methanol	0.31^{a}±0.02	1.6^{c}±0.1	1.7$^{c±}$0.2	1.25^{b}±0.03	1.32^{b}±0.02	[I]Alcoholic, chemical, caramel, bread, coffee	15[I]	***1,7***
51	**Furan-2-carbaldehyde**	**0.88^{a}±0.01**	**1.56^{d}±0.01**	**1.2^{b}±0.1**	**1.4^{c}±0.1**	**1.13^{b}±0.03**	[I]Solvent, toasted bread	0.77[I]	***1,7***
52	5-Methyl-2-furaldehyde	n.f.^{a}	0.31^{c}±0.01	0.37^{d}±0.04	0.24^{b}±0.01	0.37^{d}±0.02	[I]Toasted	1.1[I]	*7*
53	5-	n.f.^{a}	0.49^{d}±0.02	0.56^{e}±0.01	0.436^{c}±0.00	0.37^{b}±0.03	[I]Rancid,	100[I]	***4,7***

Nº	Compound name	BW	OB1	OB2	SB1	SB2	Odor descriptor	OPT (mg/L)	OS
3	(hydroxymethyl)-2-furaldehyde				2		toasted		
Σ	*Polyols*	*172^{b}±7*	*126^{a}±7*	*262^{d}±11*	*221^{c}±3*	*200^{bc}±33*			
11	2.3-Butanediol (levo)	142^{b}±5	100^{a}±4	216^{d}±10	185^{c}±1	164^{b}±29	[I]Buttery, creamy	668[I]	***4***
12	2.3-Butanediol (meso)	29^{ab}±2	25^{a}±6	46^{c}±2	36^{b}±3	36^{b}±4	[I]Buttery, creamy	668[I]	***4***

[a. b. c. e] Different letters in the same row indicate statistical differences of the normalized and scaled data at 0.05 level according to Kruskall Wallis' least significant difference method; n.f.= not found; ns= non-significant. Identification of wine samples: BW- base wine; OB-fermentation in open bottle; SB-fermentation under CO_2 overpressure; sampling time: 1- Half and 2-End of fermentation. † Isoamyl alcohols = 2-methylbutanol + 3-methylbutanol. In bold, the concentrations of compounds having the Odour Activity Value (OAV) >1. **OPT**: *Odor Perception Threshold*. **OAV**: Odor Activity Value. **OS**: *Odorant Series.* ***1. Chemical. 2. Fruity. 3. Floral. 4. Fatty. 5. Balsamic. 6. Vegetal. 7. Empyreumatic. 8. Spicy***. I- Martínez-García R. et al. (2017). II- Zea et al. (2007). III- López de Lerma et al. (2012). IV-Zea et al. (2001). V- Culleré et al. (2011). VI- Welke et al. (2014b). VII- http://www.leffingwell.com/odorthre.htm.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Use of a flor yeast strain for the second fermentation of sparkling wines: effect of endogenous CO_2 over-pressure on the volatilome

Running Title: Use of a flor yeast strain for the diversification of sparkling wines

by

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Highlights

1. Use of flor yeast in sparkling wine production for the first time
2. CO_2 effect on twenty-six variables and fifty-three volatiles are studied
3. A heatmap visualizes the changes caused by CO_2 and second fermentation
4. CO_2 pressure affects nine chemical families at the end of second fermentation
5. CO_2 pressure affects six chemical families in the middle of second fermentation
6. Aroma profiles based on Odorant series show differences due to CO_2 effect
7. PCA highlights differences in several volatile metabolites by the CO_2