

## Volatile Profile of Sparkling Wines Produced with the Addition of Mannoproteins or Lees before Second Fermentation Performed with Free and Immobilized Yeasts

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Heat map and hierarchical cluster analysis showed that sparkling wines produced with immobilized yeasts contained mostly higher levels of compounds that impart positive notes to wines. Fisher ratio pointed out nine volatile compounds as the main components responsible for differentiation of samples treated with mannoproteins/lees and others. Principal component analysis (PCA) showed differences between the sparkling wines produced with and without adjuvants (commercial mannoproteins or lees). Sparkling wines aged without adjuvants presented higher levels of decanoic and dodecanoic acids (fatty/metallic aroma), while the use of mannoproteins or lees resulted in higher content of some fruity esters, nerolidol and  $\beta$ -damascenone. Therefore, the combination of immobilized yeast and aging on mannoproteins or lees seems to be the most promising treatment to obtain the volatile profile that positively influence wine quality.

**Keywords:** sparkling wine, immobilized yeast, traditional method, champenoise, aging, volatile compounds

### Introduction

Sparkling wines produced by the traditional method are prepared through double fermentation followed by aging of the bottled wine in contact with the lees. The first fermentation transforms grape must into base wine, which undergoes alcoholic fermentation in the bottle after the addition of a suspension of yeasts and sugar (*liqueur de tirage*).<sup>1</sup> After second alcoholic fermentation, when the atmospheric pressure is greater than 4 atm at 20 °C and the alcoholic content is between 10 and 13%,<sup>2</sup> the aging on yeast lees from 8 to 24 months takes place. After aging, gravity conveys the lees sediment to the bottle neck. This process, known as *remuage*, has traditionally been carried out with the bottle on *pupitres*, by manually turning the bottle around 1/8 of a turn for around 15 days. Bottle inclination is gradually increased until they stay perpendicular to the floor. The elimination of yeast lees (*dégorgement*) is performed inserting the bottle neck in a calcium chloride or glycol solution to freeze the sediment, which is ejected by the pressure within the bottle.<sup>1</sup>

Although some wineries use automated systems that simultaneously move all bottles during *remuage*, this step is laborious and time consuming (2 weeks).<sup>3</sup> Immobilized yeasts have been used to simplify *remuage* and *dégorgement* procedures. During second fermentation and aging, the immobilized yeasts remain agglomerated and readily flocculate, unlike free yeasts, which are dispersed in the bottle and sediment slowly.<sup>4</sup>

Despite this advantage of immobilized yeasts compared to free yeasts, wineries have not used that technology, as they are afraid of compromising both yeast autolysis and the release of volatile compounds during autolysis/aging. One of the risks would be the sorption of volatile compounds on immobilized yeasts during aging due to the higher surface provided with the immobilization process. In this stage, cytoplasmic and cell wall compounds of yeasts are released due to enzymatic action, including mannoproteins.<sup>3</sup> Mannoproteins may have positive effect mainly on foam stability of sparkling wines<sup>5</sup> and on aroma of white wines.<sup>6</sup> Lees recovered from previous Charmat or traditional processes are rich in mannoproteins and may be used during vinification with the purpose of improving wine aroma. A successful recycling of lees

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in the vinification process is an interesting option for wineries, as this solid waste would not be incorrectly disposed in the environment and would add value to the final product. However, there is only one study reported in the literature about the use of such material in sparkling wines production, in which lees recovered from the first fermentation of traditional sparkling wines were included into the *liqueur de tirage* for the second fermentation of Bombino grapes base wine.<sup>7</sup> Higher concentration of fruity esters, in particular ethyl hexanoate and ethyl octanoate, besides the improvement in wine complexity (structure, body and aromatic persistence) were the main effects related to this practice.

The addition of commercial products rich in mannoproteins along with the *liqueur de tirage* to perform the second fermentation and aging of traditional sparkling wines produced with free yeasts has also been proposed to improve the perception of fruity<sup>8</sup> and floral aroma.<sup>9</sup> In still wines, the use of free yeast strains with a higher concentration of mannoproteins resulted in higher concentration of positive aroma compounds, such as terpenes and C13-norisoprenoids associated with the fresh, fruity, and floral characteristics of white wines.<sup>6</sup> Furthermore, mannoproteins added before bottling may be responsible to reduce the undesirable browning of white wines.<sup>10</sup> They may also delay or prevent tannin polymerization, leading to a reduction of astringency of red wines.<sup>11</sup> Still in red wines, mannoproteins may improve color stability, structure, roundness and help provide a full-bodied wine when added during fermentation.<sup>12</sup>

Headspace-solid phase microextraction (HS-SPME) and gas chromatography with mass spectrometric detection (GC-MS) are well-established techniques for extraction and determination of volatile compounds of wines, respectively.<sup>13-18</sup> SPME integrates extraction and concentration into a single solvent-free step, followed by thermal desorption of analytes in the injection port of the GC-MS. Additional advantages of these combined techniques include selectivity, sensitivity, absence of solvent, and the use of small amounts of sample.<sup>19</sup> Data obtained from different groups of samples may be evaluated using chemometric tools, including cluster analysis. This tool allows displaying subgroups of samples with homogeneous/similar characteristics; however, it is not possible to visualize which characteristics were responsible for each sub-cluster of samples. A heat map overcomes this drawback since it allows to simultaneously visualize the samples grouping after hierarchical cluster analysis (HCA) and the variables encoded with different colors. High and low data values appear on the heat map with high and low intensity colors, respectively, as for example from red to yellow.<sup>20</sup> Another

chemometric approach that may be used to evaluate volatile profile of sets of samples is the combination of Fisher ratio (FR) and principal component analysis (PCA).<sup>15-18</sup> FR is used to determine the features which best describe the data in terms of discriminative power between predefined classes and also to reduce the dimension of the original variables before a multivariate analysis. From the compounds with the highest values of FR, PCA may be used to provide the visualization of the samples and variables in a two-dimensional plane organized according to the differences/similarities between the data.<sup>16,18</sup>

Therefore, the goal of this study was to verify through statistical tools (heat map, HCA, FR and PCA), for the first time, if the use of mannoproteins during autolysis overcomes the drawback that yeast immobilization might cause on volatile profile of sparkling wines obtained by the traditional method. For this purpose, volatile compounds were evaluated in sparkling wines produced with free and immobilized yeasts, in addition to commercial mannoproteins and lees recovered from other sparkling wines previously produced.

## Experimental

### Reagents and supplies

All products used to process base wine were purchased from Amazon Group, Monte Belo do Sul-RS, Brazil, including silica (Solisil 30), gelatin (Lik-gel), *Saccharomyces cerevisiae* (Maurivin PDM strain), bentonite (Maxibent Plus) and cellulose fibers (Coacel). *S. cerevisiae* marketed in encapsulated (or immobilized) and free forms were used in this study to perform the second fermentation of the traditional sparkling wine vinification. ProElif<sup>®</sup> (Proenol, Canelas, Portugal) is a product resulting from immobilization of *S. cerevisiae* in calcium alginate obtained as dehydrated spheres with 2 mm of diameter. EC1118<sup>®21</sup> (Proenol) is a free form of *S. cerevisiae* isolated from the Champagne region and its use has been validated by the Comité Interprofessionnel du Vin de Champagne (CIVC).

A commercial product rich in mannoproteins named Biolees<sup>®</sup> (Laffort, Bordeaux, France) was evaluated. This preparation of yeast cell walls has a high sapid peptide content and is indicated for fining red wines.<sup>22</sup>

Standard compounds purchased from Aldrich (Steinheim, Germany) included: ethyl hexanoate, ethyl lactate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, ethyl 9-decenoate, diethyl butanedioate (diethyl succinate), ethyl phenylacetate, isoamyl acetate, 2-phenethyl acetate, hexyl acetate, 1-hexanol, 2-ethyl-1-hexanol, 1-dodecanol,

2-phenylethanol, hexanoic acid, octanoic acid, nonanoic acid, decanoic acid, dodecanoic acid,  $\alpha$ -terpineol, linalool, nerolidol and  $\beta$ -damascenone. The purity of all listed compounds was higher than 98%. Individual stock solutions of each compound (10 mg L<sup>-1</sup>) were prepared in double-distilled ethanol purchased from Nuclear (São Paulo-SP, Brazil) and diluted in a wine model solution prepared as previously reported.<sup>15</sup>

The StableFlex SPME fiber (2 cm × 50/30  $\mu$ m divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)), was purchased from Supelco (Bellefonte, PA, USA) and conditioned according to the manufacturer's recommendations prior to its first use. Sodium chloride (NaCl) of analytical grade was purchased from Nuclear and oven dried at 150 °C for 2 h before use. Twenty milliliter headspace vials with Teflon septa were purchased from Supelco.

#### Production of base wines

*Vitis vinifera* grapes of the cultivars Chardonnay, Pinot Noir, Viognier and Riesling were separately vinified (20 kg of each cultivar) in a winery located in Caxias do Sul, Serra Gaúcha region, Brazil. Grapes (18 to 20 Bx) were harvested from three vineyards located in three cities of Serra Gaúcha region: (i) Caxias do Sul (latitude: 29°10'4"S, longitude: 51°10'46"W; average altitude of 782 m); (ii) Monte Belo do Sul (latitude: 29°17'29"S, longitude: 51°39'10"W; average altitude of 438 m); and (iii) Nova Prata (latitude: 28°76'37"S, longitude: 51°36'30"W, average altitude of 699 m). Grapes were pressed to obtain the must, which was sulfited with 5 g hL<sup>-1</sup> of SO<sub>2</sub> and clarified using silica (40 g hL<sup>-1</sup>) and gelatine (4 g hL<sup>-1</sup>). *S. cerevisiae* (20 g hL<sup>-1</sup>) was used during the first fermentation in a stainless steel tank (25 L) at 15 ± 2 °C. Malolactic fermentation occurred spontaneously and the conversion of malic acid into lactic acid was monitored through paper chromatography. The stabilization of base wine occurred in cold storage (0 °C) for seven days followed by the clarification with bentonite (40 g hL<sup>-1</sup>) and filtration with cellulose fiber.

An *assemblage* of four grapes cultivars was obtained using 30, 30, 30 and 10% of Chardonnay, Pinot Noir, Viognier and Riesling base wines, respectively. These cultivars are commonly used by Brazilian wineries to prepare base wines for the elaboration of sparkling wines via traditional method. The physicochemical characteristics of this *assemblage* were according to limits established by Brazilian law for total acidity (55 to 130 mequiv L<sup>-1</sup>),<sup>23</sup> total SO<sub>2</sub> (maximum of 350 mg L<sup>-1</sup>)<sup>23</sup> and alcohol content (8.6 to 14%),<sup>24</sup> as shown in Table S1 (Supplementary Information (SI) section).

#### Immobilization of *Saccharomyces cerevisiae* used in the second fermentation

The immobilization of *S. cerevisiae* used in the second fermentation was performed according to Callone *et al.*<sup>25</sup> with slight modifications and is described as follows. *S. cerevisiae* (EC1118®; free form) was diluted in distilled water (1:10, m/v) at 25 °C and sodium alginate (2% m/v, Cromato, São Paulo-SP, Brazil) was used for the immobilization of the yeast. The solution of sodium alginate (20 mL) and hydrated yeast (20 mL; 2 g of lyophilized yeast was hydrated in 20 mL of distilled water) was dripped through a stainless steel needle (12.7 × 0.33 mm<sup>2</sup>) in 500 mL of a 0.1 mol L<sup>-1</sup> calcium chloride solution (Labsynth, São Paulo-SP, Brasil). This was performed by a peristaltic pump (P-1, Pharmacia Biotech, Uppsala, Sweden) with a flow rate of 2.5 mL min<sup>-1</sup>. The Erlenmeyer flask containing the calcium chloride solution was kept on an orbital shaker (Certomat MO, Braun Biotech, Göttingen, Germany) at 60 rpm for 30 min. The immobilized yeast beads were washed four times with distilled water (10 °C) and were stored in sealed polyethylene plastic bags and kept at 4 °C until use, which occurred in a maximum of 24 h.

#### Obtaining fine lees from previous sparkling wines production

Lees (160 g) recovered from the second fermentation of Charmat and traditional sparkling wines previously produced by the same winery that provided the base wine were centrifuged using 3000 g of force (4K15, Sigma Laboratory, Osterode am Harz, Germany) for 15 min to separate fine (size < 20  $\mu$ m) from gross lees. Gross lees comprise grape insoluble compounds, yeast cell mass, precipitated tannins/coloring matter/colloids, and particles formed from fining treatments. Fine lees included essentially dead yeast cells, therefore, rich in mannoproteins, which remain on top of the centrifugation vial (supernatant) and were recovered using a Pasteur pipette.

#### Second fermentation in bottles and aging on fine lees

Sucrose (Cristal, Aparecida de Goiânia-GO, Brazil; 30 g L<sup>-1</sup>) and diammonium phosphate ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>; 30 g L<sup>-1</sup>) were added to the *assemblage* base wine. Two types of alcoholic fermentation activators purchased from Laffort were also added to base wine: thiozote SP® (contains ammonium sulfate, diammonium phosphate and hydrochlorate thiamine) and BioActiv® (formulation based on yeast cellular envelopes (yeast hulls), inert support elements (cellulose), and inactivated yeast for vinification). In addition, a winemaking adjuvant named BioArom®

(30 g hL<sup>-1</sup>; Laffort) was used to prevent oxidation during lees maturation.

Base wines were bottled (750 mL) and nine types of sparkling wines were produced through the traditional method as detailed in Table 1. Three forms of *S. cerevisiae* were employed in the second fermentation: experimentally immobilized, commercial immobilized (ProElif<sup>®</sup>) and commercial free (EC1118<sup>®</sup>). The use of commercial mannoproteins and lees obtained from previous Charmat or traditional processes were also evaluated. Sparkling wines were produced in triplicate, totaling 27 bottles.

The type/amount of yeast and winemaking coadjutant (mannoproteins or fine lees) added in each bottle of the different treatments (T1 to T9) are shown in Table 1. The amount of the commercial yeast (EC1118<sup>®</sup>) used to produce sparkling wines (T5 to T8) followed manufacturer's recommendation. EC1118<sup>®</sup> (0.15 g *per* bottle) was hydrated in 1.5 mL of distilled water before addition to each bottle of base wine (T5 to T8 samples), and ProElif<sup>®</sup> (0.8 g *per* bottle) was used without water dilution in T9. The amount of experimentally immobilized yeast (T1 to T4) was higher (5.40 g *per* bottle) than that of commercial yeast (T5 to T9), since the weight of sodium alginate/calcium chloride and water volume used for the immobilization were also considered, when calculating the mass of the whole mixture added to the bottle (1.5 mL of hydrated yeast + 3.9 g of hydrated calcium alginate). However, the amount of yeast cells was the same for all treatments (10<sup>10</sup> colony forming units (CFU)), regardless of the type of yeast (free or immobilized). The technical data sheet provided by the yeast manufacturer was followed to estimate the amount of CFU based on the dry weight of lyophilized yeast. In

the encapsulation procedure, the calcium ion from calcium chloride replaces the sodium of sodium alginate, forming a gelatinous net of the immobilized yeast.

Bottles were closed with metal crown caps and kept in horizontal position for nine months at 20 ± 2 °C to allow second fermentation, which was followed by aging on lees. *Remuage* was carried out with the bottles on a *pupitre* for 10 days to enable flocculation of free yeasts of the sparkling wines (T5 to T8 samples shown in Table 1). The sedimentation of lees on the neck of sparkling wine bottles occurred immediately after the bottle was turned upside down, for both types of yeasts employed, experimentally immobilized (T1 to T4) and commercial immobilized yeasts (T9). The *dégorgement* was performed by keeping the bottle in the freezer (−18 °C) for 45 min. In this temperature/time, only the sediment of bottle neck was frozen, allowing the ejection of the sediment by the pressure within the bottle.

#### Determination of volatile profile

Samples were degassed in ultrasound (UltraCleaner 1400, Unique, Indaiatuba-SP, Brazil) for 30 min using ice to keep the water bath at 4 ± 2 °C. Extraction of volatile compounds were performed with HS-SPME, according to a previously optimized method.<sup>26</sup> In brief, sodium chloride (0.6 g) was added to 2 mL of sample placed in 20 mL glass HS-SPME vials and samples remained for 30 min at 40 °C before HS-SPME was carried out without sample agitation.

The analyses of wine volatile compounds were performed using gas chromatography coupled to a quadrupole mass spectrometric detector (GC-qMS) (QP2010, Shimadzu

**Table 1.** Description of the treatments followed to produce sparkling wines by the traditional method from a base wine made from an *assemblage* of different grape varieties (Chardonnay 30%, Pinot Noir 30%, Viognier 30% and Riesling 10% grapes) using free or immobilized yeast and commercial mannoproteins or lees obtained from previous Charmat/traditional processes

Treatment	<i>S. cerevisiae</i>	Amount / (g <i>per</i> bottle) <sup>a</sup>	Adjuvant	Amount / (g <i>per</i> bottle) <sup>a</sup>
T1			–	–
T2	experimentally immobilized yeasts <sup>b</sup>	5.40 <sup>c</sup>	commercial mannoproteins <sup>d</sup>	0.03
T3			lees (traditional method) <sup>e</sup>	0.06
T4			lees (Charmat method) <sup>e</sup>	0.06
T5			–	–
T6	free yeasts (commercial EC1118 <sup>®</sup> )	0.15 <sup>c</sup>	commercial mannoproteins <sup>d</sup>	0.03
T7			lees (traditional method) <sup>e</sup>	0.06
T8			lees (Charmat method) <sup>e</sup>	0.06
T9	immobilized (commercial ProElif <sup>®</sup> )	0.80 <sup>c</sup>	–	–

<sup>a</sup>Bottles of 750 mL; <sup>b</sup>yeasts were immobilized as described in Experimental section; <sup>c</sup>the amount of yeast cells was the same in all treatments (10<sup>10</sup> colony forming units). Treatments T1 to T8 contained 0.15 g of dry *S. cerevisiae* that were hydrated with distilled water (1:10, m/v) at 25 °C for 30 min, totaling 1.5 mL of hydrated yeast, as recommended in the technical data sheet of EC1118<sup>®</sup>.<sup>21</sup> T1 to T4 also contained 3.9 g of hydrated calcium alginate; <sup>d</sup>Biolees<sup>®</sup> (Laffort); <sup>e</sup>fine lees recovered from traditional/Charmat sparkling wine previously produced. ProElif<sup>®</sup> (Proenol) is a product resulting from immobilization of *S. cerevisiae* in calcium alginate obtained as dehydrated spheres with 2 mm of diameter.

Scientific Instruments, Kyoto, Japan). Linear temperature programmed retention indices (LTPRI) of volatile compounds were calculated in two different chromatographic stationary phases: a 5% diphenyl-95% dimethyl polysiloxane (DB-5) and a polyethylene glycol (DB-Wax).<sup>27</sup> Area percentage of each volatile compound of base and sparkling wines was calculated considering the sum of chromatographic areas of all detected peaks as 100%, with the exception of the solvent and spurious peaks. Criteria for peak detection were chromatographic area percentage higher than 0.01% and a minimum spectral similarity of 85%. A compound was considered tentatively identified, if it met the detection criteria and when the difference between the experimentally determined LTPRI and the LTPRI reported in literature was smaller than 19 for polar and non-polar columns. Both columns were purchased from Agilent (J & W Scientific Inc., Folsom, CA, USA) and their dimensions are as follows: 30 m × 0.25 mm × 0.25 μm. The oven was kept at 45 °C for 5 min and it was heated up to 180 °C at a rate of 3 °C min<sup>-1</sup>, reaching a final temperature of 240 °C at 20 °C min<sup>-1</sup>. Injector and detector temperatures were kept at 250 °C, while helium (analytical purity 99.999%, Linde Gases, Canoas-RS, Brazil) was employed as carrier gas at 1.0 mL min<sup>-1</sup>. Analyses were performed in splitless mode, with a liner that is designed for SPME, and purge time of 1 min. The MS parameters included electron ionization at 70 eV, 1250 V in the electron multiplier, and a mass range (*m/z*) of 45-450 in scan mode.

#### Chemometric analysis

Heat map and HCA were performed using XLSTAT2017<sup>28</sup> (Addinsoft, Paris, France) for Microsoft Excel. FR were calculated according to the approach previously applied by Welke *et al.*,<sup>16</sup> using Excel software, considering the ratio between the variances of chromatographic area percentages of a compound verified in the different classes of samples and within each class. PCA was performed with the compounds that present the highest FR using the STATISTICA software<sup>29</sup> (version 7.1, Statsoft, Tulsa, OK, USA).

## Results and Discussion

Thirty-six volatile compounds were identified (24 and 12 compounds were positively and tentatively identified, respectively) in the headspace of base and in the corresponding sparkling wines produced with the addition of mannoproteins or fine lees before second fermentation performed with free or immobilized yeasts. Table 2 shows the LTPRI (experimentally acquired and from literature)

obtained in polar and nonpolar chromatographic columns and the odor described in literature for these volatile compounds. In this table, the compounds are separated according to chemical classes and are listed in increasing order of LTPRI of the polar column. Esters were present in higher number (18), followed by higher alcohols (8), fatty acids (4), terpenes (4), C13-norisoprenoid (1) and ketone (1).

Percentage of the chromatographic area of compounds found in each sample analyzed is shown in Table S2 (SI section). These data were obtained with a polar column and were used in the statistical analysis, since the shape of the chromatographic peaks of compounds such as hexanoic acid, 2-phenylethanol and diethyl succinate had the ideal Gaussian shape only in a polar column, as shown in Figure S1a (SI section). In contrast, these peaks presented tail or inadequate shape (Figure S1b, SI section) when a nonpolar column was employed, even with different run temperature programs aiming to achieve a better chromatographic performance (results are not shown regarding different temperature programs).

The heat map (Figure 1) obtained using the percentage of the chromatographic areas of each volatile compound (Table S2, SI section) in base wine and in distinct sparkling wines (T1 to T9, according to Table 1) helps visualization of different contributions of each compound for each sample. According to Figure 1, red, orange and yellow colors represent higher, medium and lower percentage of chromatographic area, respectively.

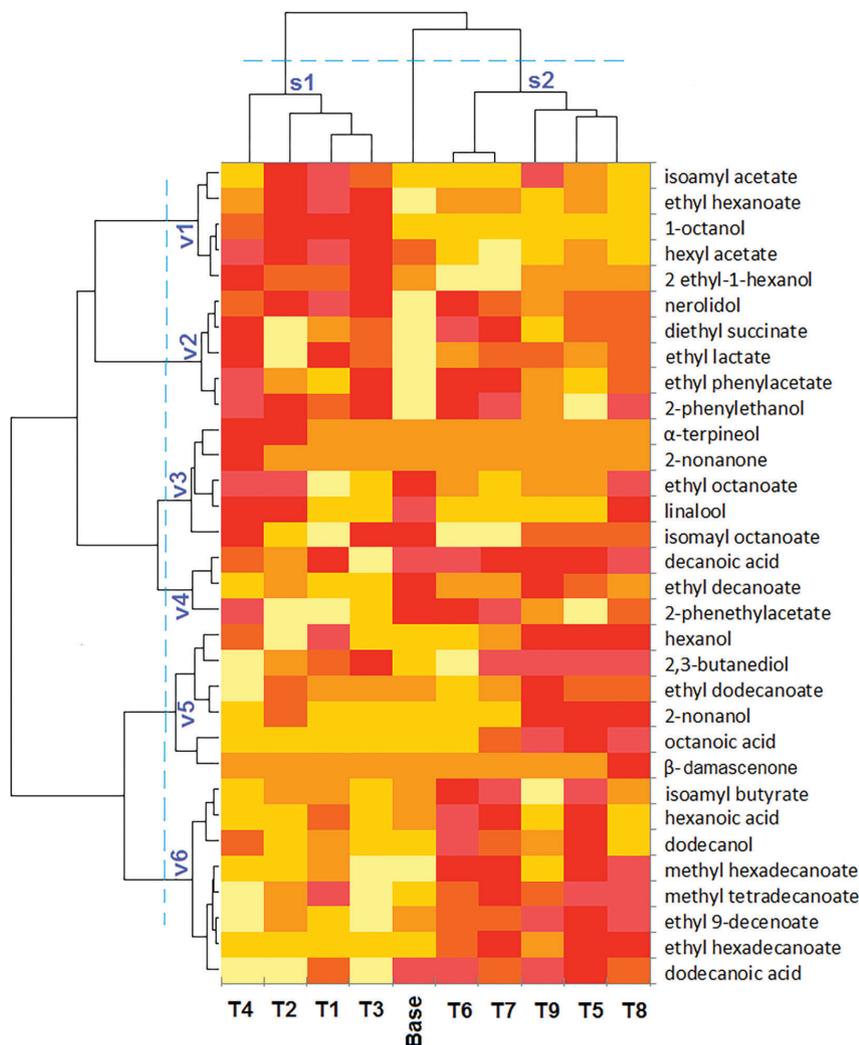
Six major clusters were responsible for the grouping of volatiles by the HCA (vertical axis). These clusters are indicated as v1 to v6 from the dashed line of Figure 1. These clusters grouped the compounds that presented higher chromatographic area (represented by red/deep pink color) mainly in the following samples: T1 to T4 (cluster v1), T1 to T7 (cluster v2), base wine, T2, T4 and T8 (cluster v3), base, T5, T6, T7 and T9 (cluster v4), T5, T8 and T9 (cluster v5), and T5 to T8 (cluster v6).

The separation of base wine from the sparkling wine samples (T1 to T9) is clear in Figure 1, as well as the grouping of the sparkling wines produced with experimentally immobilized yeasts (T1 to T4, cluster s1 in horizontal axis of heat map) from those vinified with free yeasts (T5 to T8, cluster s2 in horizontal axis of heat map). Sparkling wine produced with commercial immobilized *S. cerevisiae* (T9) was grouped in the cluster of T5 to T8 samples, which were fermented with free commercial yeasts. Therefore, it seems that the use of commercial immobilized or free yeasts did not result in differences in chromatographic volatile profile to a point that could be visualized in Figure 1.

**Table 2.** Tentatively and/or positively identified compounds in base wine and in the corresponding sparkling wines produced with the addition of mannoproteins or lees before second fermentation performed with free or immobilized yeasts

No.	Compound	CAS No.	LTPRI <sub>exp</sub> <sup>a</sup>	LTPRI <sub>lit</sub> <sup>b</sup>	LTPRI <sub>exp</sub> <sup>c</sup>	LTPRI <sub>lit</sub> <sup>d,8</sup>	Odor <sup>e</sup>
			DB-Wax		DB-5		
Fatty acids							
1	hexanoic acid <sup>f</sup>	142-62-1	1858	1861 <sup>13</sup>	985	967	pungent, rancid, wax <sup>18</sup>
2	octanoic acid <sup>f</sup>	124-07-2	2079	2086 <sup>13</sup>	NF	–	rotten fruit <sup>13</sup>
3	decanoic acid <sup>f</sup>	334-48-5	2285	2282 <sup>30</sup>	1378	1364	fatty <sup>13,30</sup>
4	dodecanoic acid <sup>f</sup>	143-07-7	2496	2477 <sup>31</sup>	1564	1565	metallic <sup>37</sup>
Higher or fusel alcohols							
5	1-hexanol <sup>f</sup>	111-27-3	1357	1363 <sup>13</sup>	879	863	cut grass, <sup>13</sup> green grass <sup>37</sup>
6	2-ethyl-1-hexanol <sup>f</sup>	104-76-7	1495	1490 <sup>31</sup>	1030	1030	fruity, sweet <sup>14</sup>
7	2-nonanol	628-99-9	1525	1530 <sup>32</sup>	1103	1097	fruity, green <sup>37</sup>
8	2,3-butanediol <sup>f</sup>	513-85-9	1546	1545 <sup>13</sup>	780 <sup>g</sup>	785	cream, <sup>13</sup> fruity, floral <sup>18</sup>
9	1-octanol	111-87-5	1563	1563 <sup>13</sup>	1073	1063	fruity, sweet <sup>18</sup>
10	2-phenylethanol <sup>f</sup>	60-12-8	1945	1946 <sup>13</sup>	1114	1106	rose, <sup>13</sup> floral <sup>18</sup>
11	1-dodecanol <sup>f</sup>	12-53-8	1972	1981 <sup>13</sup>	1476	1469	unpleasant <sup>37</sup>
12	tridecanol	112-70-9	NF	–	1577	1575	NF
Ketones							
13	2-nonanone	821-55-6	1385	1386 <sup>31</sup>	1094	1087	fruity <sup>38</sup>
C13-Norisoprenoids							
14	β-damascenone <sup>f</sup>	23696-85-7	1830	1831 <sup>13</sup>	1387	1383	honey, sweet <sup>13</sup>
Esters							
15	isoamyl acetate <sup>f</sup>	123-92-2	1120	1125 <sup>13</sup>	884	869	banana <sup>13,18</sup>
16	ethyl hexanoate <sup>f</sup>	123-66-0	1237	1235 <sup>30</sup>	1001	997	fruity, sweet <sup>18</sup>
17	isoamyl butyrate	106-27-4	1268	1255 <sup>30</sup>	NF	–	floral, fruity <sup>30</sup>
18	hexyl acetate <sup>f</sup>	142-92-7	1270	1269 <sup>5</sup>	1015	1007	fruity <sup>13</sup>
19	ethyl lactate <sup>f</sup>	97-64-3	1340	1334 <sup>30</sup>	814 <sup>g</sup>	821	fruity <sup>30</sup>
20	ethyl octanoate <sup>f</sup>	106-32-1	1440	1444 <sup>6</sup>	1200	1196	ripe fruit, <sup>13</sup> fruity, <sup>30,34</sup> sweet <sup>18</sup>
21	ethyl decanoate <sup>f</sup>	110-38-3	1644	1648 <sup>13</sup>	1398	1395	fruity, <sup>13</sup> grape <sup>2</sup>
22	isoamyl octanoate	2035-99-6	1662	1670 <sup>13</sup>	1449	1442	oily <sup>13</sup>
23	diethyl succinate or diethyl butanedioate <sup>f</sup>	123-25-1	1685	1687 <sup>13</sup>	1183	1176	floral, <sup>13</sup> fruity, sweet <sup>30</sup>
24	ethyl 9-decenoate <sup>f</sup>	67233-91-4	1694	1691 <sup>33</sup>	NF	–	rose <sup>13</sup>
25	ethyl phenylacetate <sup>f</sup>	101-97-3	1771	1779 <sup>13</sup>	1259	1254	rose, fruity <sup>13</sup>
26	2-phenethyl acetate <sup>e</sup>	103-45-7	1830	1830 <sup>34</sup>	1247	1243	flowery <sup>34</sup>
27	ethyl-4-decenoate	76649-16-6	NF	–	1390	1380	NF
28	ethyl dodecanoate <sup>f</sup>	106-33-2	1852	1856 <sup>13</sup>	1596	1594	sweet, fruity <sup>30</sup>
29	methyl tetradecanoate	124-10-7	2014	2009 <sup>33</sup>	1727	1722	waxy <sup>39</sup>
30	methyl hexadecanoate	628-97-7	2206	2207 <sup>33</sup>	1928	1921	fruity <sup>18</sup>
31	ethyl hexadecanoate	123-66-0	2285	2288 <sup>13</sup>	1995	1992	fruity, apple, wine-like <sup>37</sup>
32	methyl linoleate	112-63-0	NF	–	2103	2095	NF
Terpenes							
33	linalool <sup>f</sup>	78-70-6	1565	1569 <sup>34</sup>	1101	1095	flowery <sup>34</sup>
34	nerolidol <sup>f</sup>	40716-66-3	2001	1999 <sup>35</sup>	1567	1561	apple, rose <sup>39</sup>
35	α-terpineol <sup>f</sup>	98-55-5	1699	1719 <sup>13</sup>	1189	1186	floral <sup>13</sup>
36	geranyl acetone	689-67-8	NF	–	1456	1453	NF

<sup>a</sup>Experimental retention index (RI) calculated using *n*-alkanes (C8-C24) analyzed in polar (DB-Wax) column of a GC-MS; <sup>b</sup>literature RI obtained in a polar column: Gürbüz *et al.*,<sup>13</sup> Fan and Qian,<sup>30</sup> Umano *et al.*,<sup>31</sup> Werkhoff *et al.*,<sup>32</sup> Bosch-Fusté *et al.*,<sup>33</sup> Escudero *et al.*,<sup>34</sup> and Zhao *et al.*,<sup>35</sup> <sup>c</sup>experimental RI calculated using *n*-alkanes (C8-C24) analyzed in nonpolar (DB-5) column of a GC-MS; <sup>d</sup>literature RI obtained in a nonpolar column as described by Adams,<sup>36</sup> <sup>e</sup>odor description as reported by Tao and Li,<sup>14</sup> Nicolli *et al.*,<sup>18</sup> Li *et al.*,<sup>37</sup> Qian and Wang,<sup>38</sup> Choi,<sup>39</sup> and Peinado *et al.*,<sup>40</sup> <sup>f</sup>positively identified compounds using standards; <sup>g</sup>LTPRI values were extrapolated from the linear relation between retention times of *n*-alkanes and their predetermined LTPRI. CAS: Chemical Abstracts Service; LTPRI<sub>exp</sub> and LTPRI<sub>lit</sub>: experimental and literature linear temperature programmed retention indices, respectively; DB-Wax: polyethylene glycol stationary phase; DB-5: 5% diphenyl-95% dimethyl polysiloxane stationary phase; NF: in the LTPRI<sub>exp</sub> column, it means that this compound was not detected in this specific stationary phase; in the odor column, it means that the odor of this specific compound was not found in scientific literature.



**Figure 1.** Heat map obtained using the percentage of the chromatographic areas of each volatile compound (Table S2, SI section) in base wine and in distinct sparkling wines (T1 to T9, according to Table 1). Red, orange and yellow colors represent higher, medium and lower percentage of the chromatographic area, respectively. Clusters related to the grouping of volatiles (v) and samples (s) were designated v1 to v6 (vertical axis) and s1 and s2 (horizontal axis) according to the blue dashed line. For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article. Samples in cluster s1 are the wines produced with experimentally immobilized yeasts (T1 to T4) and the ones in the cluster s2 are the wines vinified with free yeasts (T5 to T8).

Even though an olfactometric analysis would be necessary for a precise definition of the influence of volatile compounds on the aroma, some considerations were done based on literature information.<sup>13,34,37</sup> In addition, discussions were corroborated by previous study of this research group,<sup>18</sup> where sensory and GC-olfactometry were employed to find the possible aromatic contributions of several volatile compounds to different types of wines. This approach provides a simpler and faster data treatment that may render useful results in a preliminary data evaluation and can be of importance especially for quality control of process and products in the sparkling wine industry.<sup>16,26</sup>

Among the clusters of Figure 1, the sparkling wines produced with immobilized yeasts (T1 to T4) may be

highlighted because they presented the highest levels of compounds that positively influence the aroma, mainly isoamyl acetate (No. **15**, banana),<sup>18</sup> ethyl hexanoate (No. **16**, fruity/sweet),<sup>18</sup> 1-octanol (No. **9**, fruity/sweet),<sup>18</sup> hexyl acetate (No. **18**, fruity),<sup>13</sup> and 2-ethyl-1-hexanol (No. **6**, fruity/sweet).<sup>14</sup> The number used in the text references the compounds listed in all tables. Furthermore, all compounds that could impart a negative aroma to base and sparkling wines (see Tables 2 and S2, SI section) were almost always found in lower chromatographic percentages when immobilized yeasts were used (T1 to T4, Figure 1), including hexanoic acid (No. **1**, Table 2, pungent/rancid/wax),<sup>18</sup> octanoic acid (No. **2**, rotten fruit),<sup>13</sup> decanoic acid (No. **3**, fatty),<sup>13</sup> dodecanoic acid (No. **4**, metallic),<sup>37</sup> 1-hexanol (No. **5**, cut grass),<sup>13</sup> 1-dodecanol (No. **11**,

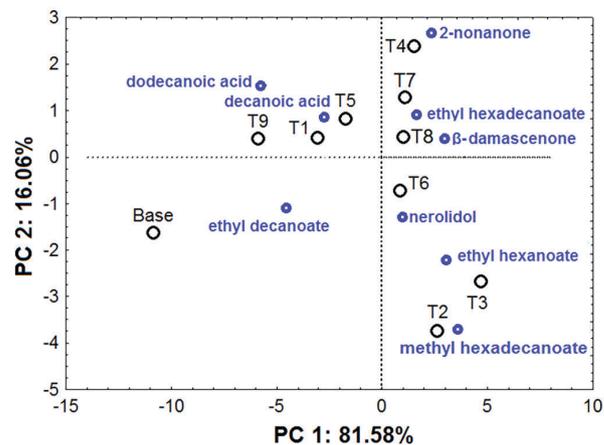
unpleasant),<sup>37</sup> isoamyl octanoate (No. **22**, oily)<sup>13</sup> and methyl tetradecanoate (No. **29**, waxy).<sup>39</sup>

The use of immobilized yeast may have impaired the amino acid catabolism through the Ehrlich pathway and this might have positively influenced wine quality. Amino acids are initially transaminated to  $\alpha$ -keto acids, which are decarboxylated, followed by oxidation or reduction reactions resulting in the formation of acids or higher alcohols, respectively.<sup>41</sup> As mentioned before, a trend for lower levels of all acids was identified in the sparkling wines (Table 2) produced with immobilized yeasts (Figure 1). Additionally, the same trend was observed for two alcohols (hexanol and 1-dodecanol) and two higher molecular weight methyl esters (isoamyl octanoate and methyl tetradecanoate), which are known for negative contribution to odor. These latter compounds were probably enzymatically produced during fermentation through the reaction between an alcohol (ethanol or higher alcohol) and an acid.

FR were calculated taking into account the percentage chromatographic area of the 32 volatile compounds found in base and sparkling wines by GC-qMS using a polar column (Table 2). The compounds with the highest FR were those that contributed the most to differentiate samples according to the presence and contribution of some volatile compounds. Values of FR are shown in Table S2 (SI section). The compounds that presented FR corresponding to at least 15% of the FR value of the most discriminant compound (2-nonanone, No. **13**, FR 331.63) were used in a PCA. This approach has been successfully applied to differentiate Chardonnay sparkling wines,<sup>16</sup> Moscatel sparkling wines<sup>17</sup> and Merlot wines produced following different canopy managements<sup>18</sup> in previous studies. The following volatiles, presented in descending order of FR, were used in the PCA: 2-nonanone (No. **13**, FR 331.63), ethyl hexadecanoate (No. **31**, FR 120.22),  $\beta$ -damascenone (No. **14**, FR 72.77), ethyl decanoate (No. **21**, FR 64.33), methyl hexadecanoate (No. **30**, FR 60.90), nerolidol (No. **34**, FR 60.00), decanoic acid (No. **3**, FR 57.75), dodecanoic acid (No. **4**, FR 55.85), and ethyl hexanoate (No. **16**, FR 50.44).

The main components (PC) were determined using the Kaiser rule, which selects PC with eigenvalues greater than one.<sup>42</sup> The two PC that presented eigenvalues greater than 1 and explained 97.64% of the variation in the volatile composition of the base and sparkling wines (T1 to T9), are shown in Figure 2.

Table S3 (SI section) shows the eigenvalues, variances and loadings of each variable related to the two PC. The variables with the highest loadings are those that contributed most to explain each PC and were highlighted



**Figure 2.** Principal component analysis (PCA) of the nine volatile compounds of the base and sparkling wines (T1 to T9) with the highest Fisher ratios according to Table S2 (SI section).

in bold in Table S3 (SI section). PC1 explains 81.58% of the data variability and is mainly related to two esters (ethyl hexanoate, No. **16**, and ethyl decanoate, No. **21**) and to two acids (decanoic, No. **3**, and dodecanoic, No. **4**). PC2 explains 16.06% of the data and relates to 2-nonanone (No. **13**), ethyl hexadecanoate (No. **31**),  $\beta$ -damascenone (No. **14**), nerolidol (No. **34**) and methyl hexadecanoate (No. **30**). All compounds that are important to locate sparkling wines of T4, T7, T8, T6, T3 and T2 on the right side of the PCA graph impart positive aroma to the wine.

A closer look at the compounds that characterize the samples according to the PCA (Figure 2) shows that ethyl decanoate (No. **21**) is the main compound related to base wine. The concentration of this ester (fruity aroma) decreased during the production of the sparkling wines (T1 to T9). This may be visualized in Figure 1 and Table S2 (SI section). Zhang *et al.*<sup>43</sup> suggested that the activity of esterases increases during fermentation resulting in the hydrolysis of ethyl decanoate and therefore, decreasing its concentration.

Even though it was not possible to verify the differences/similarities between the sparkling wines produced without (T1, T5 and T9) or with some adjuvant (T2, T3, T4, T6, T7 and T8), using HCA and the heat map (dealing with all the tentatively identified compounds), the PCA made only with the volatiles with higher FR helped to verify these distinct groups (Figure 2). According to this approach, sparkling wines aged without the use of commercial mannoproteins or lees (T1, T5 and T9) differed from the other samples due to the higher levels of decanoic (No. **3**) and dodecanoic acid (No. **4**). These acids may compromise the quality of the aroma of the sparkling wines, as they are known for their fatty<sup>13</sup> and metallic<sup>37</sup> notes, respectively.

Aging on adjuvants resulted in the presence of compounds that may positively contribute to the wine

aroma and were found in some particular samples, such as 2-nonanone (No. **13**, fruity aroma), which was found only in T4 sparkling wine and  $\beta$ -damascenone (No. **14**, honey/sweet) detected only in T8 sample. T7 and T8 sparkling wines are located near each other in PCA plot due to the higher levels of ethyl hexadecanoate (No. **31**, fruity, apple, wine-like) that was found in higher percentage in these samples. These distinct characteristics shown in PC2 make clear the distinction between samples T4, T7, T8, and T2, T3 and T6. Samples T2, T3 and T6 are associated with higher levels of nerolidol (No. **34**, apple/rose), ethyl hexanoate (No. **16**, fruity/sweet) and methyl hexadecanoate (No. **30**, fruity), which can positively influence the aroma. The use of mannoproteins and fine lees increased the levels of fruity esters such as ethyl hexanoate (No. **16**, fruit/sweet), methyl (No. **30**, fruity) and ethyl hexadecanoate (No. **31**, fruity, apple, wine-like) probably due to the esterification of fatty acids released by yeasts during fermentation or autolysis.

The presence of 2-nonanone (fruity aroma) may be also attributed to the oxidation of fatty acids.<sup>44</sup> Pérez-Magariño *et al.*<sup>8</sup> have reported that the fruity aroma was better perceived in the sensory analysis of monovarietal sparkling wines Verdejo, Godello, Tempranillo and Garnacha produced with free yeasts and aged with commercial free yeast products rich in mannoproteins, which is in accordance with the results of the present study. Nerolidol (No. **34**, T2, T3 and T6) and  $\beta$ -damascenone (No. **14**, T8) released from glycosidic precursors may also be related to the aged sparkling wines on mannoproteins/lees (T2, T3, T4, T6, T7 and T8). Compounds belonging to these classes (terpenes and C-13 norisoprenoids, respectively) were present in higher concentration in still Albariño wines produced using yeasts containing higher levels of mannoproteins.<sup>6</sup>

## Conclusions

Heat map and HCA helped to show that lower levels of fatty acids that negatively contribute to wine aroma were found in sparkling wines produced with immobilized yeasts (T1 to T4) than those produced using free yeast (T5 to T8). FR calculation followed by PCA revealed that the use of commercial mannoproteins (T2 and T6) or fine lees (T3, T4, T7 and T8) resulted in higher levels of positive aroma compounds, such as fruity esters. The use of lees recovered from previous Charmat and traditional processes might be a way to take advantage of this material that is discarded by the wineries and can be used to improve the aromatic profile of sparkling wines. Therefore, the combination of immobilized yeast and aging on mannoproteins (T2) or lees

(T3 and T4) seems to be the most promising treatment to obtain a better quality for volatiles of these sparkling wines.

## Supplementary Information

Supplementary data (physicochemical parameters of base wines, chromatographic data and PCA loadings) are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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## References

- Ribéreau-Gayon, P.; Dubourdieu, D.; Donéche, B.; Lonvaud, A.; *Handbook of Enology: The Microbiology of Wine and Vinifications*, vol. 1, 2<sup>nd</sup> ed.; John Wiley & Sons: Chichester, 2006.
- Brasil; *Decreto No. 8.198 de 20 de fevereiro de 2014*. Available at [http://www.planalto.gov.br/ccivil\\_03/\\_ato2011-2014/2014/decreto/d8198.htm](http://www.planalto.gov.br/ccivil_03/_ato2011-2014/2014/decreto/d8198.htm), accessed in March 2018.
- Moreno-Arribas, M. V.; Polo, M. C.; *Wine Chemistry and Biochemistry*; Springer: New York, 2009.
- Torresi, S.; Frangipane, M. T.; Anelli, G.; *Food Chem.* **2011**, *129*, 1232.
- Martínez-Lapuente, L.; Guadalupe, Z.; Ayestarán, B.; Pérez-Magariño, S.; *Food Chem.* **2015**, *174*, 330.
- Juega, M.; Nunez, Y. P.; Carrascosa, A. V.; Martínez-Rodríguez, A. J.; *J. Food Sci.* **2012**, *77*, 499.
- la Gatta, B.; Picariello, G.; Rutigliano, M.; Lopriore, G.; Petrella, G.; Rusco, G.; Tremonte, P.; Di Luccia, A.; *Eur. Food Res. Technol.* **2016**, *242*, 1307.
- Pérez-Magariño, S.; Martínez-Lapuente, L.; Bueno-Herrera, M.; Ortega-Heras, M.; Guadalupe, Z.; Ayestarán, B.; *J. Agric. Food Chem.* **2015**, *63*, 5670.
- Rodríguez-Nogales, J. M.; Fernández-Fernández, E.; Gómez, M.; Vila-Crespo, J.; *J. Food Sci.* **2012**, *77*, 1005.
- Ribeiro, T.; Fernandes, C.; Nunes, F. M.; Filipe-Ribeiro, L.; Cosme, F.; *Food Chem.* **2014**, *159*, 47.
- Rodrigues, A.; Ricardo-Da-Silva, J. M.; Lucas, C.; Laureano, O.; *Food Chem.* **2012**, *131*, 907.

12. Guadalupe, Z.; Martínez, L.; Ayestarán, B.; *Am. J. Enol. Vitic.* **2010**, *61*, 191.
13. Gürbüz, O.; Rouseff, J. M.; Rouseff, R. L.; *J. Agric. Food Chem.* **2006**, *54*, 3990.
14. Tao, Y.; Li, H.; *Nat. Sci.* **2009**, *1*, 176. DOI: 10.4236/health.2009.13028.
15. Welke, J. E.; Zanus, M.; Lazzarotto, M.; Zini, C. A.; *Food Res. Int.* **2014**, *59*, 85.
16. Welke, J. E.; Zanus, M.; Lazzarotto, M.; Pulgati, F. H.; Zini, C. A.; *Food Chem.* **2014**, *164*, 427.
17. Nicolli, K. P.; Welke, J. E.; Closs, M.; Caramão, E. B.; Costa, G.; Manfroi, V.; Zini, C. A.; *J. Braz. Chem. Soc.* **2015**, *26*, 1411.
18. Nicolli, K. P.; Biasoto, A. C. T.; Souza-Silva, E. A.; Guerra, C. C.; Santos, H. P.; Welke, J. E.; Zini, C. A.; *Food Chem.* **2018**, *243*, 103.
19. Arthur, C.; Pawliszyn, J.; *Anal. Chem.* **1990**, *62*, 2145.
20. Sengupta, A.; *Advances in Multivariate Statistical Methods*, vol. 4; World Scientific Publishing: Singapore, 2009.
21. <https://www.proenol.com/web/produtos/leveduras-encapsuladas/prolif-1-detail>, accessed in March 2018.
22. [http://vinestovintages.ca/LaffortTechnicalInfo/PDS\\_Biolees.pdf](http://vinestovintages.ca/LaffortTechnicalInfo/PDS_Biolees.pdf), accessed in March 2018.
23. Brasil; *Portaria No. 229, de 25 de outubro de 1988*. Available at <http://www.agricultura.gov.br/assuntos/vigilancia-agropecuaria/vegetal/bebidas-arquivos/portaria-no-229-de-25-de-outubro-de-1988.pdf/view>, accessed in March 2018.
24. Brasil; *Lei No. 10.970, de 12 de novembro de 2004*. Available at [http://www.planalto.gov.br/ccivil\\_03/\\_ato2004-2006/2004/lei/110.970.htm](http://www.planalto.gov.br/ccivil_03/_ato2004-2006/2004/lei/110.970.htm), accessed in March 2018.
25. Callone, E.; Camprostrini, R.; Carturan, G.; Cavazza, A.; Guzzon, R.; *J. Mater. Chem.* **2008**, *18*, 4839.
26. Soares, R. D.; Welke, J. E.; Nicolli, K. P.; Zanus, M.; Caramão, E. B.; Manfroi, V.; Zini, C. A.; *Food Chem.* **2015**, *183*, 291.
27. Dettmer-Wilde, K.; Engelwald, W.; *Practical Gas Chromatography: A Comprehensive Reference*; Springer: Berlin, 2014.
28. Addinsoft; *XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel*; Addinsoft, Paris, France, 2017.
29. Statsoft, Inc.; *Statistica for Windows*, version 7.1; Statsoft Inc., Tulsa, OK, USA, 2005.
30. Fan, W.; Qian, M. C.; *J. Agric. Food Chem.* **2006**, *54*, 2695.
31. Umamo, K.; Nakahara, K.; Shoji, A.; Shibamoto, T.; *J. Agric. Food Chem.* **1999**, *47*, 3702.
32. Werkhoff, P.; Güntert, M.; Krammer, P.; Sommer, M.; Kaulen, J.; *J. Agric. Food Chem.* **1998**, *46*, 1076.
33. Bosch-Fusté, J.; Riu-Aumatell, M.; Guadayol, J. M.; Caixach, J.; López-Tamames, E.; Buxaderas, S.; *Food Chem.* **2007**, *105*, 428.
34. Escudero, A.; Gogorza, B.; Melús, M. A.; Ortín, N.; Cacho, J.; Ferreira, V.; *J. Agric. Food Chem.* **2004**, *52*, 11.
35. Zhao, Y.; Xu, Y.; Li, J.; Fan, W.; Jiang, W.; *J. Food. Sci.* **2009**, *74*, 90.
36. Adams, R. P.; *Identification of Essential Oil Component by Gas Chromatography/Mass Spectrometry*, 4<sup>th</sup> ed.; Allured Publishing Corporation: Carol Stream, 2007.
37. Li, H.; Tao, Y.-S.; Wang, H.; Zhang, L.; *Eur. Food Res. Technol.* **2008**, *227*, 287.
38. Qian, M.; Wang, Y.; *J. Food Sci.* **2005**, *70*, 13.
39. Choi, H. S.; *J. Agric. Food Chem.* **2003**, *51*, 2687.
40. Peinado, R. A.; Moreno, J.; Medina, M.; Mauricio, J. C.; *Biotechnol. Lett.* **2004**, *26*, 757.
41. Nedović, V.; Gibson, B.; Mantzouridou, T. F.; Bugarski, B.; Djordjević, V.; Kalušević, A.; Paraskevopoulou, A.; Sandell, M.; Šmogrovičová, D.; Yilmaztekin, M.; *Yeast* **2015**, *32*, 173.
42. Kaiser, H. F.; *Psychometrika* **1958**, *23*, 187.
43. Zhang, M.; Pan, Q.; Yan, G.; Duan, C.; *Food Chem.* **2011**, *125*, 743.
44. Pérez-Serradilla, J. A.; Luque de Castro, M. D.; *Food Chem.* **2008**, *111*, 447.

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