Development of vinegar obtained from lemon juice: optimization and chemical characterization of the process

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ABSTRACT

A research on the production of lemon vinegar directly from lemon juice was conducted. For the alcoholic fermentation, two different strains of *Saccharomyces cerevisiae*, as well as two types of cultures (submerged culture and yeasts immobilized in alginate beads) were used. A microbiological control was also implemented during this stage. Acetic fermentation variables were optimized for a semi-continuous process. The whole process was monitored by means of volatile (SBSE/GC-MS), polyphenolic composition (UPLC-DAD), and sensory analysis was also conducted for the optimization procedure. The best conditions for alcoholic fermentation were obtained when *Saccharomyces cerevisiae var. bayanus* strain was used under a submerged culture, where a content of 13.53 mL ethanol/100mL of wine was obtained. With regards to acetic fermentation, the use of an unloading volume of 33.33% of the total volume produced lemon vinegar in 20-24 h with an acidity of 13.29 g/100 mL of vinegar (expressed as acetic acid). The chemical monitoring of the process demonstrated that the majority of volatile compounds and polyphenols studied decreased during alcoholic and acetic fermentations. Multivariate statistical analysis such as principal component analysis allowed the classification of the samples according to the matrix (juice, wine and vinegar).

KEYWORDS: lemon, vinegar, optimization, volatile compounds, polyphenols

1. INTRODUCTION

In recent years, new vinegar varieties have been developed to diversify the offer and cover the new requirements from consumers. Attending to its official definition, vinegar is a suitable for human intake liquid obtained from double fermentation (alcoholic and acetic) based on agricultural raw materials that contain starch and/or sugars (Codex Alimentarius, 1987). Therefore, any fruit juice could be susceptible to be transformed into vinegar.

Nowadays, the market exhibits a wide catalogue of commercial vinegars according to their raw material. Wine vinegar is the most important kind of vinegar and it is mainly produced in Europe (Italy, Spain). Cider and malt vinegars are common in Anglo-Saxon countries, whereas USA and some European countries produce alcohol and cereal vinegars. In addition, vinegars from rice or honey are usually produced in Japan or China (Llaguno & Polo, 1991). Usually, when any fruit other than grapes or apples are used in the production of vinegar, they are ingredients added to white wine vinegar to achieve the characteristic taste and flavor of this product (Solieri & Giudici, 2008). There are a few references where fruits are employed as raw material for vinegar production: orange (Cejudo-Bastante, Castro-Mejías, Natera-Marín, García-Barroso, & Durán-Guerrero, 2016; Cejudo-Bastante, Durán-Guerrero, García-Barroso, & Castro-Mejías, 2017b); tomato (Cejudo-Bastante, Durán-Guerrero, García-Barroso, & Castro-Mejías, 2017a); orange, mango, cherry and banana (Coelho, Genisheva, Oliveira, Teixeira, & Domingues, 2017); apple, persimmon, kiwifruit and cereal (Ren et al., 2017); pineapple (Roda et al., 2017); persimmon and strawberry (Ubeda, Callejón, et al., 2011); strawberry (Ubeda et al., 2012); persimmon (Ubeda et al., 2011) but more information on this field is still needed.

Lemon (*Citrus limon*) is the fruit from a species of evergreen tree from the flowering plant family Rutaceae, which is cultivated in several countries all over the world. It grows mainly in warm regions with mild winters, such as Mediterranean countries, south of California or Argentina (Allegrone, Belliardo, & Cabella, 2006). According to FAO (2012), China, Argentina, India and Spain, are the world main fresh lemon exporting countries, and just in Europe 20% of annual losses are produced due to the quality levels fixed by sellers (FAO, 2012). Therefore, the development of new products from lemon juice such as lemon vinegar could be a new way to exploit this valuable fruit.

Citrus genus fruit contains a wide variety of chemical compounds such as polyphenols and

volatile compounds and most of them have been widely studied due to their alimentary use, their general industrial applications, and their employment in the cosmetic or pharmaceutical industries (Patil, Jayaprakasha, Chidambara Murthy, & Vikram, 2009). Both, lemon's polyphenolic and aromatic profile can be modified by several reactions which take place during alcoholic and acetic fermentations, such as oxidation reactions (Bacigalupi et al., 2013).

This research studies the production of lemon vinegar directly from lemon juice. For the alcoholic fermentation, two different strains of *Saccharomyces cerevisiae*, as well as two types of cultures (submerged culture and yeasts immobilized in alginate beads) were used. A microbiological control was also implemented during this stage to ensure proper development of the alcoholic fermentation. Moreover, the variables of acetic fermentation were also optimized for a semi-continuous process. The whole process to produce lemon vinegar was monitored by studying its volatile and polyphenolic composition.

2. MATERIALS AND METHODS

2.1. Lemon juice samples

Lemon juice obtained from *Citrus limon* (Eureka) fruits was supplied by a local company (Sainberg La Ibense, S.A.) in a proper sanitary state for human intake. Its initial sugar content was equivalent to 7.46 ºBrix or 4.16 ºBaumé, with a density of 1.028 g/mL (at 20 ºC) and 2.27 pH.

2.2. Wine making

2.2.1. Preparation of the yeast cultures

Two different types of commercial active dry yeasts were used: *Saccharomyces cerevisiae* AWRI 796 and *Saccharomyces cerevisiae var. bayanus* (Maurivin™, Toowoomba, Australia). For each yeast strain, two types of cultures were employed: submerged culture and immobilized culture in alginate beads.

For the submerged culture, dry yeasts were re-hydrated according to supplier's instructions and then, the required amount to obtain a population of 10^6 cfu/mL was added to the medium.

Alginate beads were prepared in each case as a 1:1 solution of 2.5 g/100mL sodium alginate and re-hydrated yeast (5 g in 500 mL), which was pumped onto a 2 g/100mL $CaCl_2$ solution. Then, alginate beads were washed with a 0.85 g/100mL NaCl solution before they were used.

2.2.2. Alcoholic fermentation

Laboratory-scale alcoholic fermentations were carried out using 3L-glass vessels covered by mosquito nets to avoid contamination from insects and 0.16 g/L of potassium metabisulfite, (Agrovin, Ciudad Real, Spain) were added to prevent the growth of undesirable microorganisms. As well, 0.35 g/L of diammonium phosphate (Actimax Plus +, Agrovin) were added as the fermentation nutrient and 2 g/100 mL of pectolytic enzymes (Enartis Zym RS, Trecate, Italy) were also added to the matrix as a clarifying agent. Sugar content was increased two times (before starting and during the fermentation) up to 10 ºBaumé using commercial sugar suitable for human intake in order to obtain a suitable alcoholic degree. Finally, the pH level of the juice was set at 3.14 by adding $CaCO_3$ (Sigma-Aldrich, Stainheim, Germany) to ensure the proper growth of yeasts. Alcoholic fermentation was promoted by means of the two different types of commercial active dry yeasts (*S. cerevisiae* and *S. cerevisiae var. bayanus*). Each fermenter was filled with 2.5 L of lemon juice and both strains of yeast were tested in the two types of culture: submerged and immobilized in alginate beads. After reaching ideal conditions, pilot scale fermentations were carried out employing 40 L of lemon juice in stainless steel tanks. The temperature was maintained below 24 ºC both for laboratory-scale and pilot fermentations and all the experiments were carried out in duplicate. The lemon wine obtained was then

filtered by means of a plate and frame filter press with six CKP-V16 plates (Cordenons, Milan, Italy) and stored at 4 ºC until used.

2.2.3. Monitoring the yeasts inoculated into the alcoholic fermentation

The genetic characterization of dry yeast strains was carried out. After the rehydration of the yeasts, they were grown in YPD culture medium (20 g/L bacteriological peptone, 10 g/L yeast extract, 20 g/L glucose) for 24 h at 30 °C. DNA extraction was performed following the protocol described by Silva, da Bernardi, Schaker, Menegotto, & Valente (2012), with several modifications. Cells were spinned down by centrifugation for 5 min at 15000xg and then, the pellets were re-suspended in 1 mL of sterile water, and incubate at 90 °C for 5 min. After the incubation period, the samples were homogenized by vortex for 10 s and the tubes were frozen at –20 °C for 24 h. The samples were allowed to thaw at room temperature and centrifuged at 40000xg for 15 min. The pellets were re-suspended in 120 µL of SDS (Sodium Dodecyl Sulfate, Thermo Fisher Scientific, MA, USA) and incubated at 65 °C for 5 min. Then, 200 µL of cold 5 mol/L potassium acetate (Panreac, Barcelona, Spain) were immediately added and briefly mixed. The samples were incubated –20 °C for 10 min. After this step, the pellets were centrifuged at 4 °C for 5 min at 55000xg. The supernatants were transferred to a clean tube with 500 µL isopropanol (Sigma-Aldrich, Steinheim, Germany) and incubated at 4 ºC for 5 min. The tubes were then centrifuged for 2 min at 55000xg and washed with 50 µL of ethanol solution (70 mL/100mL). The DNA pellets were dried for 30 min with a Speed-Vac apparatus (Eppendorf, Hamburg, Germany). Finally, the pellets were rehydrated in 50 µL of free DNA and RNA sterile water.

The identification of the yeasts was performed by microsatellite multiplex PCR technique described by Vaudano & García-Moruño (2008). The amplified PCR products were separated by electrophoresis on 2 g/100 mL agarose gels in 1 x TBE buffer and 0.5 µg/mL

of ethidium bromide (Sigma-Aldrich). The image of the gel was digitalized in a Molecular Imager apparatus (Gel-Doc XR, Bio-Rad, CA, USA) and analyzed using Quantity One 1-D software (Bio-Rad). Hyperladder^{TM} 50pb molecular weight marker (Bioline, London, UK) was used.

The sampling was carried out during the fermentation process at laboratory-scale. The wine samples used for the molecular analysis were taken at the beginning, in the middle and at the end of the alcoholic fermentation. The method described by Rodríguez, Infante, Molina, Rebordinos, & Cantoral (2011) was applied to extract the DNA directly from the fermenter samples, without previously isolating the yeast colonies. Also, the microsatellite multiplex PCR technique was applied to monitor the dry yeast strains inoculated in the fermentation sample.

2.3. Vinegar making

Two hundred mL of unfiltered Sherry vinegar were centrifuged at 10000xg for 5 min at 4 ºC to obtain the acetic acid bacteria to start the culture. This was then dissolved in 2.5 L lemon wine and was aerated (1 L/h·L), under controlled temperature (29 ºC) in an 8 L Frings Acetator (Heinrich Frings, Bonn, Germany) to allow the growth of acetic acid bacteria. When the acetic fermentation started, the aeration flow was set at 7.5 L/h·L and 0.40 g/L of diammonium phosphate (Actimax Plus +; Agrovin, Ciudad Real, Spain) were added as nutrient. At this point, the matrix volume was increased to 6 L by adding lemon wine. When the alcohol content of the matrix was 0.3 mL/100mL, the unloading step started and 4 L of vinegar were unloaded (end of cycle). Then, the loading step of the lemon wine up to the final volume started at around 0.25 L/h in order to avoid a sharp modification of the alcohol content and the temperature of the medium. This starts a new cycle. The maximum allowable ethanol content was set at 5 mL/100mL. Alcohol content to determine the loading/unloading steps in the reactor was measured by means of an

alcohol sensor (Alkosens; Heinrich Frings, Bonn, Germany) and all the procedures were controlled by a computer application (Acetomat S7; Siemens AG, Munich, Germany).

2.4. Analysis of phenolic compounds

UPLC separation and identification of phenolic compounds were performed in duplicate using a Waters Acquity UPLC system (Waters Corps. Milford, MA, USA), equipped with a diode array detector (DAD) employing the method proposed by Schwarz, Rodríguez, Guillén, & Barroso (2009). All the samples were previously filtered through 0.45 and 0.22 μm nylon filters, from Scharlab (Barcelona, Spain).

The identification of each compound was carried out by comparing retention times and UV-Vis spectra with those provided for commercial standards (all of them purchased from Sigma-Aldrich). Calibration curves were constructed using five concentration levels. Unknown compounds were quantified as gallic acid (compounds A-E). Calibration curves for caffeic acid and quercetin were used for the quantification of caffeic acid and quercetin derivatives, respectively. Flavonol A and B were quantified as quercetin. Benzoic acids were quantified by DAD chromatograms obtained at 280 nm, whereas a wavelength of 320 nm was used for narirutin, hesperidin, neohesperidin and hydroxycinnamic acid derivatives.

2.5. Analysis of volatile compounds

Commercial 10 mm long x 0.5 mm thick polydimethylsiloxane stir bars (supplied by Gerstel, Mülheim a/d Ruhr, Germany) were used to stir bar sorptive extractions (SBSE). For each SBSE analysis, a volume of 10 mL of sample was pipetted and placed into a 100-mL Erlenmeyer flask with 3 g of NaCl (Scharlau, Barcelona, Spain) and 50 μL of a solution of 4-methyl-2-pentanol (Sigma-Aldrich, Steinheim, Germany) (0.22894 g/L in Milli-Q water containing 80 g/L of acetic acid). The Erlenmeyer flask was placed on a 15-position magnetic stirrer (Gerstel). Later, the stir bar was rotated at 1800 rpm at 25 ºC for

60 min. After extracting it from the vinegar sample, and in order to eliminate NaCl and any remains of sample attached, the stir bar was placed for a few seconds in distilled water and then gently dried with a lint-free tissue. Then, it was transferred into a glass thermal desorption tube. The thermal desorption and subsequent chromatographic analysis by GC/MS was performed following the method proposed by Guerrero, Marín, Mejías, & Barroso (2006).

Peak identification was carried out using the Wiley 7N Edition Library (Wiley Registry of Mass Spectral Data, 7th Edition, 2000) by analogy of mass spectra and confirmed by standard retention times when available, or by the retention data found in the literature. The linear retention indices (LRI) of all the compounds were calculated on a DB-Wax polar column and compared to those found in the literature. Semi-quantitative data were obtained by measuring the relative molecular ion peak area in relation to that of 4-methyl-2-pentanol, the internal standard. All the analyses were performed in duplicate.

2.6. Sensory analysis

Samples were submitted to sensory analysis. Twenty mL of the samples were presented in black wine glasses to avoid the influence of color and covered with a glass top in order to minimize possible losses in aroma intensity. The evaluation sessions were carried out in a standard tasting room (UNE 87-004) at 22 ºC and the panel of 15 judges had been trained for general and specific aspects of sensory evaluation.

Lemon wine samples were submitted to descriptive analysis. The samples' attributes such as fruity, citrus, floral, sweetness, and general impression were studied using a structured six-level scale, from 0 (absence) to 5 (very intense).

Lemon vinegar samples were submitted to triangle tests in order to check possible differences derived from different operational conditions. Significant differences (α=0.05) were defined using the critical number of correct answers proposed by Roessler, Pangborn,

Sidel, & Stone (1978).

2.7. Statistical analysis

Data were submitted to Analysis of Variance (ANOVA) and Principal Component Analysis (PCA) by means of the statistical computer package Statgraphics Centurion XVII (Statpoint Technologies, Inc., VA, USA).

3. RESULTS AND DISCUSSION

3.1. Optimization of alcoholic fermentation

Two types of cultures (submerged culture and immobilized culture in alginate beads) and two different types of commercial yeasts (*Saccharomyces cerevisiae* and *Saccharomyces cerevisiae var. bayanus*) were employed. Alcoholic fermentation started after 2 days from inoculation in all cases and yeasts consumed all sugar after 4 days from the beginning of the fermentation. At that moment, 10 additional ºBaumé were added in order to reach a higher alcoholic degree. This extra sugar content was consumed after 11 days from the beginning of the fermentation. The final wines presented a sugar residual content between 2.3 and 4.3 ºBaumé (Table 2). As can be seen, submerged culture produced wines with higher alcohol content than immobilized culture in alginate beads for both type of yeasts (the differences were significant at $p<0.05$). Normally, in this type of culture the nutrients and substrates that are required for the correct development of yeasts have more difficulties to penetrate the beads, so a low metabolic activity of microorganisms was shown and a, subsequently, low final alcoholic content was obtained (Oliveira et al., 2011).

In addition, sensory analysis showed higher values ($p<0.05$) for the descriptors fruity, citrus and general impression in the wines obtained from *S. cerevisiae* var *bayanus* yeast (4.6, 3.9, and 4.1, respectively) compared to those obtained from the regular variety (3.5, 2.9, and 3.2, respectively). Therefore, *S. cerevisiae var. bayanus* yeast in submerged culture was employed for the pilot scale fermentation. Forty L of lemon juice were

fermented in duplicate in a 15 day period, where the fermentation started 24 h after the inoculation of the yeasts. The lemon wine obtained presented 13.53 mL/100mL mean alcohol content. As can be seen, a higher alcohol value was obtained when higher volumes of lemon juice were employed.

3.2. Microbiological control of the alcoholic fermentation

After analyzing the band pattern obtained for each active dry strain (Fig. 2), several bands were identified. When the amplification was carried out by means of single locus primers (YOR267 locus, Fig. 2A, lines a) they could not be differentiated. When the band patterns obtained by the other two singleplex loci versus the multiplex PCR were compared, it could be seen that the best identification of the two yeast strain was achieved by co-amplification of the 3 microsatellites (Fig. 2B).

After characterizing both LSA, the multiplex microsatellite PCR (data not shown) was applied to each of the samples taken at the beginning, middle and end of the alcoholic fermentation process of the lemon juice. To consider that the inoculated yeast strain was responsible for the process, the same criterion as Cordero-Bueso et al. (2017) was followed, that is, to consider a positive result when the pattern obtained for the sample taken directly from the lemon wine was identical to that of the inoculated yeast strain. The analysis revealed that each yeast carried out the fermentation of the lemon juice satisfactorily.

3.3. Optimization of acetic fermentation

Acetic fermentation was performed in submerged culture. In order to reduce fermentation time, the right option was to keep the bacteria population in the phase of exponential growth. Therefore, two different unloading volumes were tested (2 and 4 L) and a total of 8 consecutive cycles for each set of conditions were performed. When 2 L of unloading were employed, uniform loading/unloading cycles were obtained with a 20-24 h length. In

addition, in order to check the similarity between the vinegars obtained from each complete cycle, triangle tests were performed. The results showed that vinegars were indistinguishable between cycles (only 6 judges could tell the difference out of 15 total judges, 5% significance level, Table 3). On the other hand, when 4 L of unloading were employed, no uniform cycles could be obtained as their duration varied between 68 h and 91 h. A large unloading volume would remove a large amount of bacteria population from the medium and the process would be slowed down. Although cycles were not uniform, vinegars obtained from the different cycles were also indistinguishable according to triangle tests (5 judges could tell the difference out of 15 total judges, 5% significance level, Table 3). Finally, in order to compare both processes, triangle tests were also performed to samples obtained from the last cycle with 2 L and 4 L of unloading. In this case, samples were correctly differentiated with a significance level of 5% (10 judges could tell the difference out of 15 total judges, Table 3). Therefore, the unloading volume can significantly affect the organoleptic profile of the vinegar produced. In view of these results, 2 L of unloading were employed for the production of vinegar, and the mean acidity obtained was 13.29 g acetic acid/100 mL of vinegar and 0.3 mL ethanol/100 mL of vinegar.

3.4. Study of volatile composition during the process

Thirty nine volatile compounds, all of them distinctive from citrus matrices (Cejudo-Bastante et al., 2016) have been correctly identified and determined in lemon juice, lemon wine and lemon vinegar samples (Table 4). As can be seen, significant differences (ANOVA, $p<0.05$) were obtained according to the matrix (Table 4). On the one hand, in general terms most of the compounds decreased their concentration levels or even disappeared in the transformation process from lemon juice to lemon vinegar. This is the logical behavior of primary aromas derived from the original fruit (Cejudo-Bastante et al.,

2016). Despite of their decrease, some of these compounds such as linalool, fenchol or 4-terpineol were still detected in lemon vinegar thanks to their high content in the initial lemon juice. On the other hand, the amount of some compounds, such as some alcohols and esters, increased significantly during alcoholic fermentation and then decreased during acetic fermentation. These results match those from previous researches (Cejudo-Bastante et al., 2016; Cejudo-Bastante et al., 2017a) on the production of tomato and orange vinegars. In addition to these changes, some compounds such as isovaleric acid, hexanoic acid, diethyl succinate, 2-phenylethyl acetate, or isoamyl acetate, increased their concentrations as lemon juice was transformed into lemon vinegar. This may derive from the metabolism of acetic acid bacteria that produces compounds other than acetic acid (Nieto et al., 1993). Other authors also detected that other acids and acetates had increased their content during the production of tomato vinegar (Cejudo-Bastante et al., 2017a).

The data obtained were submitted to multivariate statistical study (principal component analysis, PCA). They revealed the existence of 4 PCs that could explain 95.6% of the variability (eigenvalues > 1). The first 2 PCs would explain 89.2% of the variability. Fig. 3 represents the distribution of the samples onto the plane formed by the first two PCs. As can be seen, PC1 separated juice samples from wine and vinegar samples, whereas PC2 was able to separate wine samples from the rest of the samples. Compounds that contributed more to PC1 (all of them negatively) were mainly terpenes (β-myrcene, α-terpinene, d-limonene, *p*-cymene, terpinolene, and β-bisabolene) and alcohols (β-terpineol, *cis* and *trans*-geraniol) that come from lemon juice. PC2 was mainly affected by compounds formed during the alcoholic fermentation (ethyl esters, alcohols and benzyl compounds).

3.5. Study of the polyphenolic composition during the process

Twenty polyphenolic and furanic compounds were studied in juice, wine and vinegar

317 samples. Those compounds that could not be identified were labeled as compound A, B,
318 etc. As can be seen in Table 5, the majority of the compounds showed significant content
319 differences when transformed from lemon juice into lemon vinegar according to ANOVA
320 ($p < 0.05$). Only three compounds presented similar concentrations for the three studied
321 matrices (compound A, kaempferol-3-glucoside and quercetin-3-glucoside) and just a few
322 compounds (narirutin, neohesperidin, vanillic acid, compound B, caffeic acid derivative B,
323 compound E and quercetin derivative B) decreased their concentrations gradually under
324 both the alcoholic and the acetic fermentations. The majority of the compounds decreased
325 their concentrations during the alcoholic fermentation but they were not affected by the
326 acetic fermentation (Table 5). This is in line with a previous study in which it was
327 observed that the submerged culture acetic fermentation did not significantly affect the
328 polyphenolic composition in the production process of orange vinegars (Cejudo-Bastante
329 et al., 2017b). Only two compounds (caffeic acid derivative A and tyrosol) increased their
330 concentrations when transformed from lemon juices into lemon vinegar. Other authors
331 have reported general increases in the polyphenolic composition during wine production.
332 This would be derived from the contact with the solid parts of the grapes (Darias-Martín,
333 Rodríguez, Díaz, & Lamuela-Raventós, 2000). In our case, no maceration with lemon
334 peels took place, therefore the above mentioned extraction of polyphenols could not occur.
335 The increase of tyrosol can be explained from the expected degradation of tyrosine during
336 the alcoholic fermentation produced by yeast metabolism (Cejudo-Bastante et al., 2017a),
337 whereas the increase in caffeic acid derivative could be explained on the basis of possible
338 condensation or esterification reactions that may have occurred during both fermentation
339 processes.
340 Polyphenolic data were also submitted to PCA. The results showed that 2 PCs explained
341 95.4% of the variability (eigenvalues > 1). Fig. 4 shows the plot with the samples on the

plane defined by PC1 and PC2 and the contributions from the studied variables. Compounds such as vanillic acid, flavonol A, compound E and quercetin derivatives B and C contributed more significantly in PC1, whereas PC2 was affected mainly by caffeic acid derivative A, tyrosol, kaempferol-3-glucoside and quercetin-3-glucoside (Fig. 4). As can be seen, juice samples were placed in a PC1 positive-value area (mainly due to the contribution of characteristic compounds from juice), while vinegar samples had negative values in PC1 and wine samples were plotted at an intermediate position. On the other hand, PC2 (mainly formed by compounds derived from alcoholic fermentation) had the samples divided into two groups: lemon juice samples with PC2 negative values and wine samples with PC2 positive values. It can also be observed that generally, vinegar samples were gradually placed at a greater distance from wine samples as the number of cycles increased (Fig. 4).

4. CONCLUSIONS

It has been proven that lemon is an appropriate raw material to produce vinegar. The best conditions for alcoholic fermentation were obtained when *Saccharomyces cerevisiae var. bayanus* was applied to a submerged culture. Regarding acetic fermentation, 2 L unloading volume produced lemon vinegar in 20-24 h. The chemical monitoring of the process demonstrated that the majority of volatile compounds and polyphenols studied decreased during alcoholic and acetic fermentations. Although this loss of volatile compounds and polyphenols is naturally to be expected after two fermentation processes, further research could be conducted aiming at preserving as much as possible the initial chemical composition of the raw material. The development of lemon vinegar could be a new way to exploit this valuable fruit and it could also lead to diversifying the vinegar offer in the market.

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FIGURE LEGENDS

Fig. 1. Electrophoretic patterns of strain *S. cerevisiae* AWRI 796 (1) and *S. cerevisiae var. bayanus* (2) obtained after PCR (A): singleplex locus YOR267 (lines a), SCPTSY7 (lines b), SC8132X (lines c) and multiplex (B). M: Hyperladder 50pb

Fig. 2. PCA on volatile compounds. Distribution of all samples onto the plane defined by the first two PCs. J: lemon juice; W: lemon wine; V: lemon vinegar

Fig. 3. PCA on polyphenolic compounds. Scatterplot of the samples onto the plane defined by the first two PCs. J: lemon juice; W: lemon wine; V: lemon vinegar

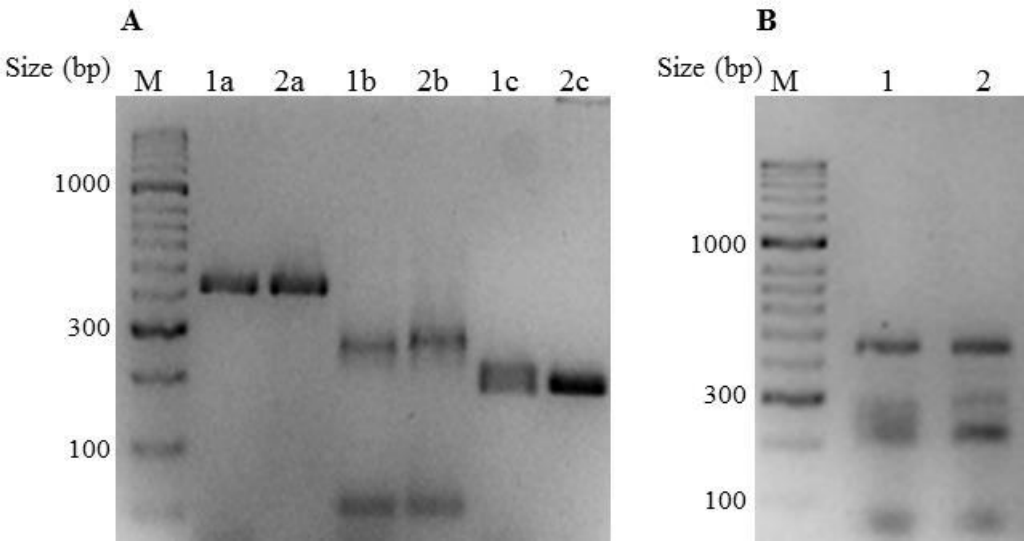


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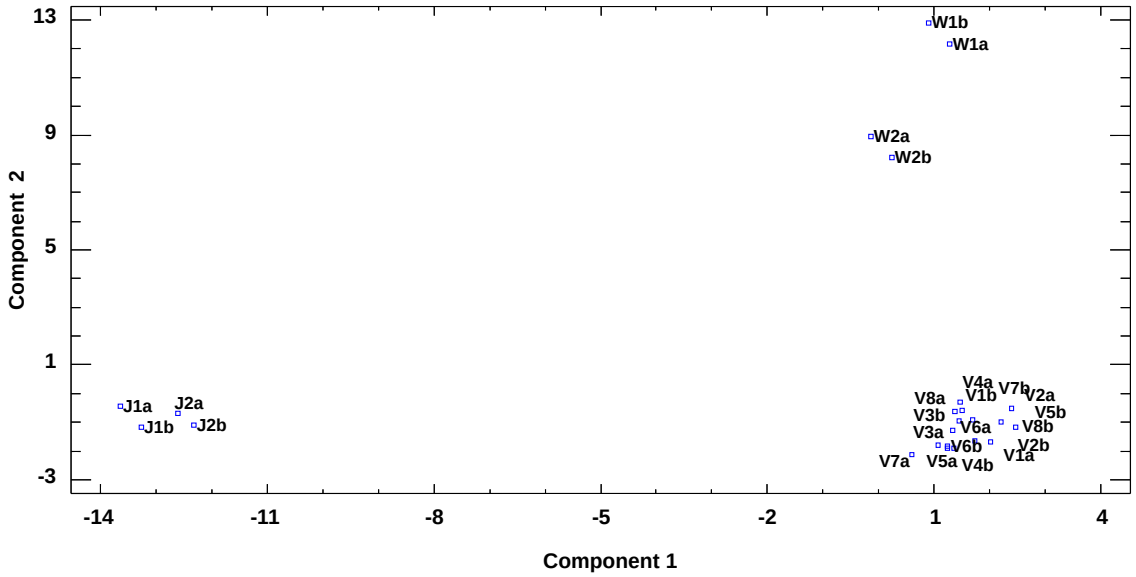


Figure 2. PCA on volatile compounds. Distribution of all samples onto the plane defined by the first two PCs. J: lemon juice; W: lemon wine; V: lemon vinegar.

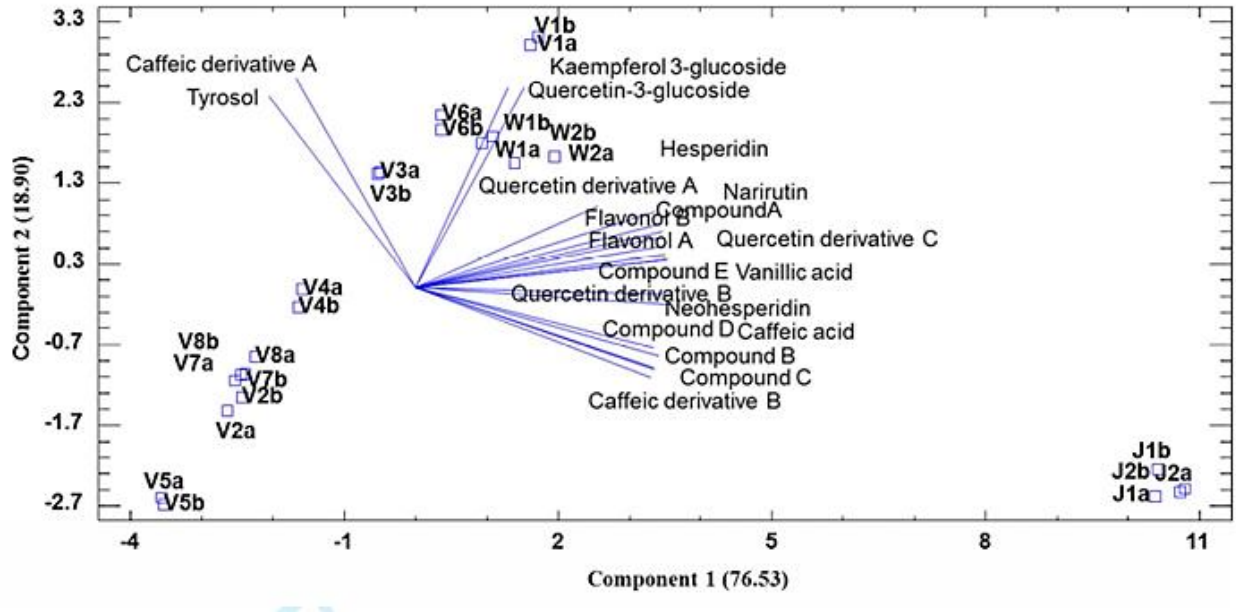


Figure 3. PCA on polyphenolic compounds. Scatterplot of the samples onto the plane defined by the first two PCs. J: lemon juice; W: lemon wine; V: lemon vinegar

Table 1. Obtained parameters for the different experiments of alcoholic fermentation.

Exp.	Yeast	Culture	T (ºC)	Brix	Be	Density* (g/cm^3)	% Ethanol (v/v)
1	*S. cerevisiae*	Submerged	21.1	6.25	3.49	1.02283	5.38
2	*S. cerevisiae*	Submerged	21.1	4.18	2.34	1.01455	5.39
3	*S. cerevisiae*	Immobilized	21.1	5.04	2.81	1.01796	3.75
4	*S. cerevisiae*	Immobilized	21	5.3	2.96	1.01902	4.48
5	*S. cerevisiae var. bayanus*	Submerged	21.2	7.65	4.27	1.02849	6.06
6	*S. cerevisiae var. bayanus*	Submerged	21.1	5.1	2.85	1.01819	6.06
7	*S. cerevisiae var. bayanus*	Immobilized	20.9	5.04	2.82	1.01798	3.43
8	*S. cerevisiae var. bayanus*	Immobilized	21	6.07	3.39	1.02209	3.15

* Measured at 20.0ºC

Table 2. Mean relative areas for the studied volatile compounds in the different matrices during the production of lemon vinegar. Analysis of variance.

Rt (min)	Compound	Lemon Juice	Lemon Wine	Lemon Vinegar	ANOVA	
					F ratio	p-Value
20.25	Isoamyl acetate	ND^{a}	32.7^{ab}	61.7^{b}	5.23	0.0169*
21.61	β-Myrcene	0.66^{a}	ND^{b}	ND^{b}	58088	0.0000*
22.25	α-Terpinene	0.34^{a}	0.10^{b}	ND^{c}	315	0.0000*
23.35	D-Limonene	25.3^{a}	ND^{b}	ND^{b}	16308	0.0000*
24.54	Isoamyl alcohol	ND^{a}	130^{b}	51.0^{c}	75.6	0.0000*
25.86	*p*-Cimene	0.35^{a}	0.08^{b}	0.04^{c}	398	0.0000*
26.37	Terpinolene	1.00^{a}	ND^{b}	ND^{b}	515157	0.0000*
30.36	Nonanal	0.04^{4}	0.25^{b}	0.15^{b}	3.78	0.0439*
31.73	Ethyl octanoate	ND^{a}	15.8^{b}	0.35^{a}	2373	0.0000*
33.73	2-Ethyl-1-hexanol	0.16^{a}	1.06^{b}	0.43^{c}	81.8	0.0000*
35.24	Benzaldehyde	ND^{a}	ND^{a}	0.53^{b}	30.7	0.0000*
35.24	Camphor	0.33^{a}	ND^{b}	ND^{b}	4705	0.0000*
35.9	Linalol	6.39^{a}	4.99^{b}	1.67^{c}	117	0.0000*
37.53	Fenchol	6.90^{a}	7.38^{a}	1.76^{b}	134	0.0000*
38.49	4-Terpineol	26.2^{a}	25.5^{a}	10.2^{b}	110	0.0000*
39.11	Butyrolactone	ND^{a}	0.79^{b}	0.31^{c}	48.4	0.0000*
39.14	β-Terpineol	0.38^{a}	ND^{b}	ND^{c}	82.8	0.0000*
39.97	Ethyl decanoate	ND^{a}	8.58^{b}	ND^{a}	4973	0.0000*
39.97	Safranal	2.32^{a}	6.64^{b}	2.49^{a}	76.2	0.0000*
40.17	1-Nonanol	0.35^{a}	0.38^{b}	ND^{c}	45464	0.0000*
40.17	Isovaleric acid	ND^{a}	ND^{a}	2.19^{b}	12.2	0.0005*
40.59	Diethyl succinate	ND^{a}	3.41^{b}	5.09^{b}	18.5	0.0001*
42.04	Borneol	1.00^{a}	3.78^{b}	1.85^{c}	53.4	0.0000*
42.05	α-Terpineol	61.7^{a}	69.9^{a}	43.0^{c}	21.5	0.0000*
42.58	Neryl acetate	1.98^{a}	ND^{b}	ND^{b}	5672	0.0000*
42.80	β-Bisabolene	0.33^{a}	ND^{b}	ND^{b}	1715	0.0000*
43.57	Geranyl acetate	1.22^{a}	ND^{b}	ND^{b}	7487	0.0000*
43.84	β-Citronellol	0.84^{a}	12.9^{b}	1.44^{a}	318	0.0000*

45.22	*Cis*-geraniol	6.42[a]	2.25[b]	ND[c]	1168	0.0000*
46.82	2-Phenylethyl acetate	ND[a]	ND[a]	31.9[b]	6.94	0.0063*
46.37	*Trans*-carveol	0.34[a]	0.28[b]	ND[c]	603	0.0000*
46.37	Hexanoic acid	ND[a]	1.75[b]	1.45[b]	23.4	0.0000*
46.82	Ethyl laurate	ND[a]	1.61[b]	ND[a]	246424	0.0000*
46.82	Geraniol	8.58[a]	ND[b]	0.65[c]	1184	0.0000*
47.45	Butyl butyrate	ND[a]	1.17[b]	0.54[c]	68.9	0.0000*
48.97	2-Phenylethanol	0.29[a]	50.9[b]	37.5[c]	27.8	0.0000*
53.79	Octanoic acid	0.33[a]	28.6[b]	19.2[c]	23.6	0.0000*
58.06	*p*-Vinylguaiacol	ND[a]	2.05[b]	0.59[c]	50.9	0.0000*
60.38	Decanoic acid	ND[a]	31.6[b]	0.58[a]	145	0.0000*

Different letters in different columns indicate significant differences according to Tukey's test ($p < 0.05$).
* Values are significant at $p<0.05$; ND: Not detected

Table 3. Mean concentrations (mg/L) for the studied phenolic compounds in the different matrices during the production of lemon vinegar. Analysis of variance.

Rt (min)	Compound	Lemon Juice	Lemon Wine	Lemon Vinegar	ANOVA	
					F ratio	p-Value
0.989	Compound A	1.62	1.21	0.88	3.42	0.0564
1.061	Compound B	1.12[a]	0.34[b]	0.23[c]	313	0.0000*
1.235	Compound C	12.2[a]	1.02[b]	0.78[b]	1758	0.0000*
1.309	Compound D	1.11[a]	0.39[b]	0.27[b]	85.6	0.0000*
1.54	Caffeic acid derivative A	1.25[a]	10.1[b]	7.92[b]	19.4	0.0000*
1.607	Tyrosol	ND[a]	5.35[b]	4.26[b]	34.5	0.0000*
1.816	Vanillic acid	18.4[a]	12.6[b]	9.40[c]	29.4	0.0000*
1.816	Caffeic acid	2.36[a]	0.39[b]	0.33[b]	1047	0.0000*
2.385	Caffeic acid derivative B	10.76[a]	2.68[b]	2.08[c]	502	0.0000*
2.565	Flavonol A	1.81[a]	1.12[b]	0.92[b]	26.2	0.0000*
2.607	Compound E	11.5[a]	6.30[b]	4.74[c]	62.1	0.0000*
3.043	Flavonol B	10.2[a]	7.08[b]	5.70[b]	18.5	0.0001*
3.389	Quercetin derivative A	1.52[a]	1.10[b]	0.85[b]	14.8	0.0002*
3.487	Quercetin derivative B	1.79[a]	1.11[b]	0.80[c]	29.3	0.0000*
3.606	Quercetin derivative C	20.8[a]	14.0[b]	11.06[b]	22.7	0.0000*
4.547	Narirutin	27.0[a]	19.2[b]	14.9[c]	18.5	0.0001*
4.843	Kaempferol-3-Glucoside	0.78	0.99	0.77	0.64	0.5376
4.894	Quercetin-3-Glucoside	0.57	0.57	0.53	0.07	0.9289
5.029	Hesperidin	0.93[a]	0.67[b]	0.54[b]	11.9	0.0006*
5.093	Neohesperidin	7.05[a]	3.66[b]	2.73[c]	76.7	0.0000*

Different letters in different columns indicate significant differences according to Tukey's test ($p < 0.05$).

* Values are significant at $p<0.05$; ND: Not detected