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Effect of processing Verdejo grape must by UHPH using non-*Saccharomyces* yeasts in the absence of SO₂†

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Ultra-High Pressure Homogenization (UHPH) is an emerging non-thermal technology that can eliminate wild microorganisms from grape juice facilitating the use of non-competitive non-*Saccharomyces* yeast in fermentation to modulate the sensory profile. The use of UHPH processing in must from Verdejo variety grapes (*Vitis vinifera* L.) produces a more varietal profile reducing the contents of fermentative fruity varietal esters (–25–50%) and enhancing the release of volatile thiols (+25–75%). The aromatic profile of UHPH wines is clearly separated of controls by the aroma PCA. Additionally, the inactivation of oxidative enzymes by UHPH preserves a better color in wines with a paler yellow color and lower b* values. A better implantation of some non-*Saccharomyces* yeasts such as *Lachancea thermotolerans* can help to reduce the pH in wines from warm areas. Improved varietal aroma, paler wine color and, depending on the strain fermented with, lower pH help control the impact of global warming on wines.

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Sustainability spotlight

UHPH is a sustainable and environmentally friendly technology with very low power consumption (50% lower than HHP), even lower compared with conventional thermal treatments. The small system volume demands very moderate requirements of the process and cleaning water. Additionally, the use of steam to sanitize or sterilize the system is unnecessary because it can be done by pressure, and just hot water at 70–80 °C as technical fluid is necessary. Furthermore, the low impact of this technology in sensory and nutritional quality favors the production of healthier and less processed foods. This technology can support the United Nations' Sustainable Development Goals (SDGs), especially Goal 2 (zero hunger) and Goal 13 (climate action).

1. Introduction

Ultra-High Pressure Homogenization (UHPH) is an emerging technology that can be considered non-thermal because of the gentle effect on sensitive molecules with impact on the sensory profile and micronutrients.^{1–4} The UHPH process is done by ultra-high pressure pumping at more than 200 MPa (normally 300 MPa) followed by subsequent instantaneous depressurization (<0.2 seconds) across a highly resistant valve.^{1,4} It has shown a protective effect on terpenes and other aroma compounds,^{5,6} anthocyanins⁷ and vitamins.⁸ Due to the extreme impact and shear forces in the valve the temperature can be very

high (75–150 °C), but with low thermal impact because of the very short residence time, and there is a very scarce formation of thermal markers such as HMF,⁵ furosine,⁹ or carcinogenic compounds such as ethyl carbamate, the formation of which may be enhanced by temperature.¹⁰

UHPH can control easily high microbial loads in liquid foods, and the inactivation of yeast populations of 6–4 log CFU mL^{–1} belonging to the *Saccharomyces cerevisiae* (Sc) species but also to other non-*Saccharomyces* yeasts^{7,11} and 4–3 log of aerobic bacteria in grape juices^{7,11} has been reported. Depending on the in-valve temperature also sporulate bacteria can be eliminated and it has been observed that under mild conditions (80–90 °C of in-valve temperature) the off-flavor producer, acidophilic and thermoresistant *Alicyclobacillus acidoterrestris* can be eliminated. Additionally, UHPH produces an effective control of enzymes,^{10,12} especially oxidative enzymes (PPOs), with inactivation higher than 90% and a control of browning.^{5,11,13} Therefore this technology can be used to produce wines with a low or null content of SO₂.^{4,5,7}

The use of non-*Saccharomyces* is a current hot topic in wine biotechnology because of their ability to modify and improve the sensory profile of wines enhancing wine quality, aroma,

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flavor, structure, freshness and color.^{14–18} Several species of non-*Saccharomyces* have deserved special attention because of their impact on wine quality among them: *Torulaspora delbrueckii* (Td) by its impact on aroma, foaming properties, and recently in bioprotection,^{19,20} *Metschnikowia pulcherrima* (Mp) by the expression of enzymatic activities, capacity to lower the ethanol, and bioprotective effect,^{21,22} *Lachancea thermotolerans* (Lt) by acidification and aroma improvement,^{23–25} and *Hanseniaspora vineae* (Hv) by aroma and impact on the body and structure.^{26,27} A major problem of many non-*Saccharomyces* yeasts is that they are weaker fermenters compared to Sc, and they have problems to compete and be prevalent in must fermentation. The use of UHPH is a way of eliminating all the competitive wild yeasts in grape juice promoting a good implantation of starters from non-*Saccharomyces* yeasts. Additionally, it can be done at low levels of SO₂ that is harmful for many non-*Saccharomyces*.

Furthermore, UHPH is a sustainable and environmentally friendly technology because of the low power consumption (50% lower than HHP) and the small system volume with low requirements of the process and cleaning water (<https://www.ypsicon.com/>). Additionally, the use of steam to sanitize or sterilize the system is unnecessary because it can be done by pressure, and just hot water at 70–80 °C as technical fluid is necessary.

The aim of this research is to study the effect of UHPH processing on grape must of Verdejo variety and the impact on the quality of the wines produced after fermentation by several non-*Saccharomyces* species.

2. Materials and methods

2.1. Grape juice

The must was obtained from Verdejo blanco grapes (*Vitis vinifera* L.; VIVC variety number: 12 949) from the DO Rueda in Valladolid, Spain. Grapes were mechanically harvested and pressed using a pneumatic press model Diemme 150 (Lugo, Ravenna, Italy) at less than 2 bar, and the yield was 60% of grape juice; after that the juice obtained was settled at low temperature (4 °C) for 2 days and processed by UHPH. No sulphites were added to the juice. The settling was performed without the use of pectolytic enzymes.

2.2. UHPH treatment

After settling the must was processed with a 60 L h⁻¹ UHPH device (Ypsicon Technologies, Barcelona, Spain) at 300 ± 3 MPa with an inlet temperature of 8 °C, reaching 92 °C in the UHPH valve and being refrigerated after valve depressurization and by additional continuous cooling at 15 °C. Processed juice was aseptically packed in sterilized 2 L plastic bags in the absence of O₂ and stored refrigerated at 4 °C until fermentation. Control unprocessed juice was also kept at 4 °C until fermentation in 5 L plastic bottles.

2.3. Fermentation and inoculation

UHPH juices and unprocessed controls were dosed at 200 mL in 250 mL ISO flasks and kept isothermally at 20 °C during

fermentation. Inocula of *Saccharomyces* and non-*Saccharomyces* yeasts were grown in YPD liquid media, and the populations were synchronized by two successive passes of 2% v/v in 24 h before the final inoculation in the fermenters. Inoculation volume was 2% v/v (4 mL), producing a final population of 7 log CFU mL⁻¹. The inocula were grown at 20 °C. Fermentation was performed in triplicate. All fermentation samples of non-*Saccharomyces* were inoculated again on the 8th day of fermentation with *S. cerevisiae* (Sc7VA) until total sugar depletion to ensure the dryness of wines.

The Sc and non-*Saccharomyces* yeasts used were *Saccharomyces cerevisiae* strain 7VA (Sc7VA) selected at the enotecUPM lab (Madrid, Spain), *Lachancea thermotolerans* L31 (L31) selected at the enotecUPM lab, *Torulaspora delbrueckii* BIODIVA™ (Td) Lallemand, *Hanseniaspora vineae* 205 (Hv) selected by the Professor Carrau team at the Universidad de la República (Uruguay), and *Metschnikowia pulcherrima* M29 (M29) selected at the enotecUPM lab. All of them were kept in YPD agar and refreshed by a pass in solid YPD agar and growth at 20 °C for 48 h previously to be synchronized in the liquid YPD.

All fermentation samples from days 2, 4 and 6 were monitored for inoculated and wild yeast populations by plate seeding according to Section 2.5.

2.4. Optical microscopy of the juice

Optical microscopy was performed on a drop of sediment of the control and UHPH juices using a BA310 LED microscope (Motic, San Antonio, USA) with a 60× achromatic objective and a digital camera FULLHD Moticam 1080INT. The sediment was obtained after centrifugation at 5000 rpm for 5 minutes of 3 mL of juice in an Eppendorf tube.

2.5. Yeast counts

Yeast counts were determined by plating in YPD agar for total yeasts and in lysine agar and CHROMagar™ Candida (Conda, Barcelona, Spain) for non-*Saccharomyces* counts. The identification was done according to the color and colony appearance in the selective and differential media. Plates were counted after 48 h at 25 °C in the dilution that had a count number in the range of 30–300 colonies.

2.6. Lactic acid and enological parameters

L-Lactic acid has been analyzed at the end of fermentation by enzymatic analysis using an automatic enzymatic analyzer Y25 (BioSystems, Barcelona, Spain). Previously samples were filtered with 0.45 μm membrane filters.

The major compounds at the end of fermentation, such as residual sugars, organic acids, and total acidity, were measured with a FTIR OenoFoss equipment (FOSS Iberia, Barcelona, Spain). The pH was measured using a CRISON micropH 2000 pHmeter (HACH LANGE, Barcelona, Spain).

2.7. Fermentative volatiles quantified by GC-FID

Fermentative volatiles were quantified by gas chromatography with a flame ionization detector (GC-FID) using an Agilent



Technologies 6850 gas chromatograph. The injector temperature was set at 250 °C, and the FID was at 300 °C. Compounds were separated on a DB-624 column (60 m × 250 μm × 1.40 μm) with a 1 : 10 split ratio. The quantification was performed using GC quality standards: acetaldehyde, methanol, 1-propanol, 1-butanol, 2-butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethyl acetate, 2-phenylethyl alcohol, diacetyl, ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl butyrate, 3-ethoxy-1-propanol, ethyl lactate, and hexanol (Fluka, Sigma-Aldrich Corp., Buchs SG, Switzerland). The compound 4-methyl-2-pentanol was added as the internal standard, also from Fluka. The temperature program for the oven was 40 °C for five minutes, and then an increase of 10 °C per minute up to 250 °C, which was kept for five minutes, with a total running and conditioning time of 40 minutes. The carrier gas was hydrogen with a column flow of 2.2 mL min⁻¹. One mL of sample was filtered with a 0.45 μm membrane and spiked with 100 μL of IS. The injection volume was 1 μL. The detection limit was set at 0.1 mg L⁻¹. The volatile compounds analyzed were calibrated using a five-point calibration curve with $r^2 > 0.999$, except 2,3-butanediol (0.991) and 2-phenylethyl alcohol (0.994).

2.8. Volatile compounds quantified by HS-SPME-GC-MS

Volatile compounds (VOCs) were determined by SPME-GC-MS by slightly modifying the method published by Tat *et al.*²⁸ The following compounds were analysed: ethyl hexanoate, hexyl acetate, ethyl-3-hexenoate, 3-hexen-1-ol acetate, 3-hexen-1-ol, (*E*)-, 3-ethoxy-1-propanol, 3-hexen-1-ol, (*Z*)-, ethyl octanoate, acetic acid, 3-methylbutyl hexanoate, ethyl-3-octenoate, 2-ethyl-1-hexanol, benzaldehyde, linalool, 1-octanol, 2-methyl-propanoic acid, γ-butyrolactone, butanoic acid, ethyl decanoate, 3-methylbutanoic acid, diethyl succinate, ethyl 9-decenoate, 3-methylthio-1-propanol, β-citronellol, ethyl phenylacetate, ethyl 4-hydroxybutanoate, β-damascenone, hexanoic acid, phenyl methanol (benzyl alcohol), octanoic acid, 4-vinyl-guaiacol, ethyl hexadecanoate, decanoic acid, ethyl 9-hexadecenoate, ethyl octadecanoate, and ethyl 9-octadecenoate. The instrument used was a GCMS-QP2020 NX GC-MS system (Shimadzu, Kyoto, Japan), equipped with a 2800T autosampler (HTA S.r.l., Brescia Italy). Samples were prepared as follows: 10 mL of filtered wine were introduced into 20 mL glass vials and mixed with 3 g of NaCl; 100 μL of ethyl heptanoate (0.106 g L⁻¹ in ethanol) was added as the internal standard and vials were sealed with PTFE/silicon septa. SPME was carried out using a 2 cm long 50/30 μm DVB/Carboxen/PDMS fiber (Supelco, Bellefonte, PA, USA), at 40 °C for 15 min. Vials were pre-conditioned in an autosampler for 15 min before micro-extraction, to allow the thermal equilibration of the samples. Injections were performed in splitless mode, with a splitless time of 60 s. The temperatures of the injector and the transfer line were 250 °C and 240 °C respectively, while the ion source was set at 200 °C. The carrier gas was helium at a linear flow rate of 36 cm s⁻¹. Compounds were separated on a J&W DB-Wax capillary column, with 30 m × 0.25 mm i.d. and 0.25 μm film thickness (Agilent Technologies Inc., Santa Clara, CA, USA), according to the gradient reported by Comuzzo *et al.* (2018).²⁹

Electron impact mass spectra were recorded at 70 eV and the identification of volatile compounds was carried out by comparison of their mass spectra and retention times with those of standard compounds, or by comparison of the mass spectrum with those reported in the mass spectrum libraries Wiley 6 and NIST 107. Linear retention indices were also calculated on the basis of the retention times of *n*-alkanes and compared with those reported in the literature. Semi-quantitative analysis was performed by the internal standard method, considering a response factor equal to 1.00.

2.9. Thiols quantified by LC-MS/MS

The volatile thiols 3-mercaptohexan-1-ol (3 MH), 4-mercaptopentan-2-one (4MMP), 4-mercapto-4-methylpentan-2-ol (4MMPOH), and 3-mercaptohexyl acetate (3MHA) were analysed by LC-MS/MS. Wines were extracted with modified Quechers using a mixture of ethyl acetate/acetonitrile (1 : 1) without the clean-up step. An aliquot of this extract was analyzed by liquid chromatography coupled to mass spectrometry (LC-MS/MS). The equipment used was an Agilent 1290 Chromatograph coupled to a triple quadrupole mass spectrometer (model 6460) also from Agilent. The ionization source was electrospray type (ESI) with a gas temperature of 300 °C at a flow of 5 L min⁻¹. The separation was carried out on a Phenomenex C18 column with the following dimensions (100 × 3 mm, 250 Å, and 2.6 μm) at a temperature of 25 °C and an injection volume of 5 μL. The mobile phases were as follows: (A) 0.1% formic acid in water and (B) acetonitrile at a flow rate of 0.5 mL min⁻¹. Detection was performed in MRM mode for each of the thiols.

2.10. Color

The DNA Phone Smart Analysis – Wine device (Parma, Italy) was used to determine the color parameters. The parameters included the CIELChuv coordinates for chroma (C) and hue (h), as well as the CIELab coordinates for lightness (L*), green-red (a*), and blue-yellow (b*). The samples were put into polymethyl methacrylate cuvettes with a 1 mm path length. The samples were filtered using 0.45 μm methyl-cellulose membranes before analysis.

2.11. Statistical analysis

Statistical analysis was performed using Statgraphics Centurion v19 software (Graphics Software Systems, Rockville, MD, United States) to calculate the mean, standard deviation, analysis of variance (ANOVA), and least significant difference (LSD). Significance was set at $p < 0.05$.

SPME-GC-MS data were elaborated by Principal Component Analysis (PCA) and One-Way ANOVA, using Statistica for Windows (version 8.0). Regarding PCA, factor loadings (FLs) were calculated by factor analysis, and the most relevant variables were selected for PCA, when marked FLs were higher than 0.7. Concerning One Way ANOVA, means and standard deviations were calculated, and significant differences were assessed by a Tukey HSD test at $p < 0.05$. Variances were homogeneous according to the Brown-Forsythe test.



3. Results and discussion

3.1. Results

3.1.1. Micro- and macroscopic effects of UHPH. The raw grape juice after settling shows a low colloidal turbidity with a yellow pale color that starts to show some browning in the beaker (Fig. 1A). The microscopic preparation shows abundant crystal fragments of tartrates together with colloidal particles from the cell walls of the grape pulp. The fragments were irregular in size and shape. The UHPH processed juice showed a thin colloidal structure with a more turbid appearance in the beaker and paler color (Fig. 1B). The microscopic aspect was thinner with practically not observable crystals or other big colloidal fragments. The size was much more regular with a homogeneous granulometry.

3.1.2. Yeast counts of *Saccharomyces* and non-*Saccharomyces* yeasts. The wild and inoculated populations of yeasts were counted by plating on days 2, 4 and 6 of fermentation. The average count of wild yeasts on the 2nd day of fermentation was 1.38×10^7 CFU mL⁻¹, in the range 1.83×10^6 – 3.13×10^7 (Table 1). Additionally, wild yeasts were not found in the UHPH must even on the 6th day of fermentation (Table 1). All the inoculated yeasts had populations of approximately 7 log on the 2nd day of fermentation in the control and UHPH processed juices except for Td which were closer to 6 log. The populations of all the non-*Saccharomyces* were higher than 5 log on day 6 of fermentation.

3.1.3. Lactic acid and enological parameters. *Lachancea thermotolerans* has been included because of its ability to transform sugars into lactic acid with high efficiency in some strains facilitating a good control of the pH. As can be observed the fermentative production of lactic acid is very scarce for Sc or most of the non-*Saccharomyces* ranging below 100 mg L⁻¹ (Fig. 2). However, Lt is able to produce contents of several g L⁻¹. In the must processed by UHPH and the control, the differences were non-significant with a value ranging from 1.7 to 1.9 g L⁻¹ with a clear impact on pH. The average pH of the fermentation samples with all the strains except Lt is 3.7, while fermentation managed with Lt produced a pH reduction of 0.2 units (Table 2), which means an approximate decrease of 0.1 pH units per 0.8–1.0 g L⁻¹ of lactic acid.

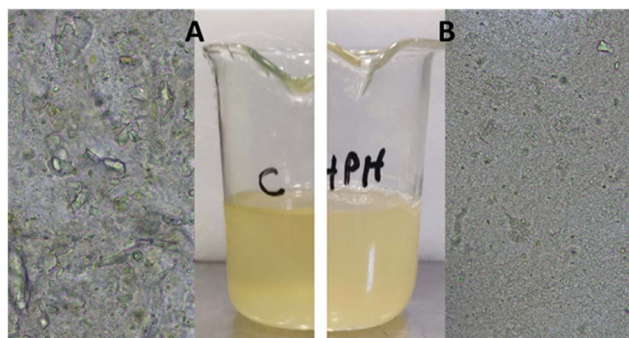


Fig. 1 Optical microscopic and macroscopic appearance of the unprocessed control must made from Verdejo grapes (*Vitis vinifera* L.) (A) and the UHPH processed (B).

Table 1 Yeast counts (CFU mL⁻¹) in controls and UHPH juices on days 2, 4 and 6 of fermentation. Inoc is the population inoculated of the corresponding yeasts *Saccharomyces cerevisiae* (Sc7VA), *Lachancea thermotolerans* (L31), *Torulaspota delbrueckii* (Td), *Hanseniaspora vineae* (Hv), and *Metschnikowia pulcherrima* (M29). Wild means the initial wild population of yeasts in the juice. nd non detected, zero counts

Yeasts/day of fermentation	2	4	6
Control Sc7VA-Inoc	3.93×10^7	2.43×10^7	5.83×10^6
UHPH Sc7VA-Inoc	2.23×10^7	8.33×10^6	4.00×10^5
Control Sc7VA-Wild	1.83×10^6	1.53×10^7	1.33×10^5
UHPH Sc7VA-Wild	nd	nd	nd
Control L31-Inoc	1.40×10^7	3.13×10^7	1.01×10^7
UHPH L31-Inoc	2.17×10^7	7.50×10^6	7.33×10^5
Control L31-Wild	1.37×10^7	1.99×10^7	5.80×10^6
UHPH L31-Wild	nd	nd	nd
Control Td-Inoc	5.33×10^6	1.39×10^7	2.00×10^6
UHPH Td-Inoc	1.50×10^6	5.43×10^7	1.87×10^7
Control Td-Wild	3.13×10^7	4.73×10^7	8.67×10^6
UHPH Td-Wild	nd	nd	nd
Hv-Inoc	3.29×10^7	1.27×10^7	7.47×10^6
U-Hv-Inoc	6.77×10^6	9.33×10^6	8.33×10^5
Hv-Wild	1.63×10^7	1.27×10^7	5.73×10^6
U-Hv-Wild	nd	nd	nd
Control M29-Inoc	4.67×10^7	6.67×10^6	6.00×10^6
UHPH M29-Inoc	2.05×10^7	1.73×10^7	1.00×10^7
Control M29-Wild	6.13×10^6	2.83×10^7	5.67×10^6
UHPH M29-Wild	nd	nd	nd

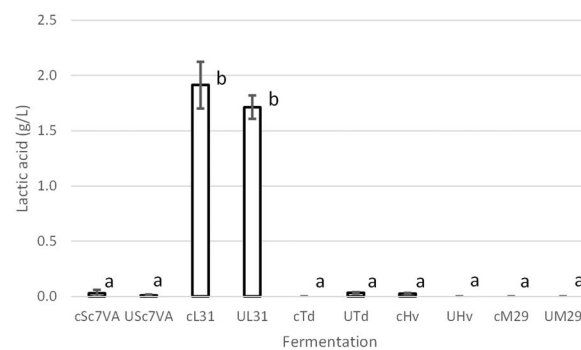


Fig. 2 Lactic acid production in control (c) and UHPH (U) must fermented by *Saccharomyces cerevisiae* (Sc7VA), *Lachancea thermotolerans* (L31), *Torulaspota delbrueckii* (Td), *Hanseniaspora vineae* (Hv), and *Metschnikowia pulcherrima* (M29). Lactic acid determined by enzymatic analysis. Values are means \pm sd (fermentation in triplicate) in g L⁻¹. Different letters mean significant differences ($p < 0.05$).

3.1.4. Fermentative volatiles by GC-FID. UHPH fermentation had less higher alcohols than controls, on average 374.23 mg L⁻¹ in controls and 260.53 in UHPH (data obtained from Table 3), which means a 30% lower content. Similarly, the UHPH fermentation showed lower contents of esters than controls, on average 36.79 mg L⁻¹ for control fermentation and 26.99 mg L⁻¹ for UHPH and this difference is 27% of reduction. The carbonyl compounds acetoin and diacetyl showed similar concentrations in control and UHPH wines, with a difference of just 3% on average (Table 3). However, the contents of



Table 2 Enological parameters in controls and UHPH must fermented by *Saccharomyces cerevisiae* (Sc7VA), *Lachancea thermotolerans* (L31), *Torulaspora delbrueckii* (Td), *Hanseniaspora vineae* (Hv), and *Metschnikowia pulcherrima* (M29). Compounds analyzed by FTIR, and values are means \pm sd (fermentation in triplicate) in g L^{-1} . Different letters mean significant differences ($p < 0.05$)

	Ethanol (% v/v)	Total acidity (g L^{-1}) ^a	pH	Volatile acidity (g L^{-1}) ^b	Residual sugars (g L^{-1})	Malic acid (g L^{-1})	TPI ^c	Glucose (g L^{-1})	Fructose (g L^{-1})
eSc7VA Control	12.9 \pm 0.0bc	4.2 \pm 0.1ab	3.7 \pm 0.0c	0.2 \pm 0.0a	1.5 \pm 0.2d	1.3 \pm 0.0 cd	12.7 \pm 1.8d	0.8 \pm 0.1a	0.9 \pm 0.3bcd
USc7VA UHPH	12.4 \pm 0.3ab	4.1 \pm 0.1ab	3.7 \pm 0.0bc	0.4 \pm 0.0c	1.5 \pm 0.1d	1.3 \pm 0.1bc	11.7 \pm 2.0cd	0.9 \pm 0.1a	0.8 \pm 0.1bc
eL31 Control	12.8 \pm 0.1bc	6.3 \pm 0.2f	3.5 \pm 0.0a	0.3 \pm 0.0b	1.1 \pm 0.1ab	1.1 \pm 0.1b	11.2 \pm 1.3bcd	0.8 \pm 0.1a	0.4 \pm 0.1a
UL31 UHPH	12.5 \pm 0.1ab	5.5 \pm 0.2e	3.5 \pm 0.0a	0.2 \pm 0.0a	1.0 \pm 0.3a	0.8 \pm 0.1a	5.4 \pm 3.7a	1.5 \pm 0.1d	0.4 \pm 0.3a
eTd Control	13.1 \pm 0.0c	4.4 \pm 0.1cd	3.7 \pm 0.0bc	0.3 \pm 0.0b	1.2 \pm 0.1abc	1.5 \pm 0.0e	9.8 \pm 1.5bcd	1.0 \pm 0.1a	0.8 \pm 0.1b
Utd UHPH	12.5 \pm 0.0ab	4.2 \pm 0.1abc	3.6 \pm 0.0b	0.2 \pm 0.0a	1.3 \pm 0.3bcd	1.3 \pm 0.1bc	8.5 \pm 2.4abc	1.4 \pm 0.2cd	1.0 \pm 0.2bcd
eHv Control	12.8 \pm 0.0bc	4.6 \pm 0.2d	3.7 \pm 0.0bc	0.4 \pm 0.0c	1.7 \pm 0.1e	1.3 \pm 0.1cd	9.8 \pm 1.7bcd	1.2 \pm 0.1b	1.1 \pm 0.1d
UHv UHPH	12.1 \pm 0.4a	4.0 \pm 0.1a	3.7 \pm 0.0b	0.4 \pm 0.0c	1.4 \pm 0.1cd	1.2 \pm 0.1bc	7.5 \pm 2.6ab	1.3 \pm 0.2bc	1.0 \pm 0.1bcd
eM29 Control	13.1 \pm 0.1c	4.5 \pm 0.2cd	3.7 \pm 0.0bc	0.3 \pm 0.0b	1.2 \pm 0.1abcd	1.4 \pm 0.1de	12.1 \pm 0.2cd	0.9 \pm 0.1a	0.8 \pm 0.2bc
UM29 UHPH	12.1 \pm 0.6a	4.3 \pm 0.1bc	3.7 \pm 0.0c	0.5 \pm 0.0d	1.3 \pm 0.1bcd	1.3 \pm 0.1bc	13.7 \pm 3.5d	1.3 \pm 0.1bc	1.1 \pm 0.1cd

^a Total acidity expressed in tartaric acid. ^b Volatile acidity in acetic acid. ^c TPI (total polyphenol index).

acetaldehyde were much higher in controls than in UHPH fermentation (2–4 \times) except for Sc.

3.1.5. Other volatile compounds by HS-SPME-GC-MS. The use of a PCA biplot with the aroma compounds analyzed by HS-SPME-GC-MS allows a clear separation of the wines produced from must processed by UHPH (Fig. 3, red cluster) from the unprocessed controls (Fig. 3, black cluster). In general, higher concentrations of several esters are formed in unprocessed controls (Table S1[†]). UHPH can be a useful tool to produce cleaner wines, in which the variety can be less hidden by the fermentation biotechnology. Additionally, β -damascenone, decanoic acid and 2-ethyl-1-hexanol clearly differentiate the composition of UHPH wines when fermented by *H. vineae* (Fig. 3, red cluster, Table S1[†]). Moreover, 2-ethyl-1-hexanol shows similar concentrations in both UHPH and controls when fermented by *S. cerevisiae*; however, higher concentrations were found in UHPH wines when they were fermented by whatever non-*Saccharomyces* (Table S1[†]).

3.1.6. Thiols by GC-MS. The content of 3MH for Lt L31 fermentation samples has values higher than 400 ng L^{-1} in all the fermentation processes and on average close to 600 ng L^{-1} in control fermentation and higher than 800 ng L^{-1} in UHPH fermentation by Lt (Fig. 4). In general, for all UHPH juices the contents of 3MH were on average higher than in controls except for Hv, although with significant differences just for Td.

Other thiols such as 4MMP and 4MMPH were not detected in most of the samples and concerning the 3MHA the concentrations were quite variable and did not follow a clear pattern.

3.1.7. Color. As per the color analyses results, the values of chroma, which refers to saturation, are consistently higher for wines produced with untreated must; nonetheless, only the fermentation samples produced with Sc, Td, and Hv are significantly different. The average values obtained for these parameters are between 10.4% higher, for the least different (Lt), and 36.7% higher for Sc (see Table 4).

With regards to the hue (Table 4), this parameter had no significant differences between pairs of must, and the media values were slightly higher for the UHPH treated must fermented with Sc and Hv. The results observed for this parameter are consistent with the observations got for the a^* axis and b^* axis shown in Fig. 5, where Sc is completely isolated from the rest of the wines having the highest b^* values (more yellow) and the lowest a^* values (less green).

As observed in Fig. 5, the values for wines produced with must without UHPH treatment were larger on the b^* axis, above the dotted line and above 11 units, which is translated into wines having more yellow appearance associated with higher levels of oxidation. On the other hand, all wines from UHPH-treated must and the wines from untreated must fermented by non-*Saccharomyces* yeasts had a^* values larger than -0.6 in comparison to Sc. In this case, all wines except for Sc had a larger contribution of green.

3.2. Discussion

3.2.1. Micro- and macroscopic effects of UHPH. The beginning of browning in the must poured into the beaker just



Table 3 Fermentative volatiles in control and UHPH must fermented by *Saccharomyces cerevisiae* (Sc7VA), *Lachancea thermotolerans* (L31), *Torulaspota delbrueckii* (Td), *Hanseniaspora vineae* (Hv), and *Metschnikowia pulcherrima* (M29). Compounds analyzed by GC-FID, and values are means \pm sd (fermentation in triplicate) in mg L⁻¹. Different letters mean significant differences ($p < 0.05$)

	Sc7VA		USc7VA		L31		UL31		Td		UTd		Hv		UHv		M29		UM29	
	Control	UHPH	Control	UHPH	Control	UHPH	Control	UHPH	Control	UHPH	Control	UHPH	Control	UHPH	Control	UHPH	Control	UHPH	Control	UHPH
Acetaldehyde	101.90 \pm 2.43c	102.88 \pm 7.23c	113.14 \pm 8.85cd	29.59 \pm 2.27ab	226.25 \pm 23.02f	46.88 \pm 4.59b	97.84 \pm 9.43c	26.50 \pm 5.98a	182.49 \pm 18.25e	124.47 \pm 3.52d										
Methanol	43.49 \pm 6.21d	30.66 \pm 3.62bc	29.29 \pm 3.29abc	23.35 \pm 0.66a	61.06 \pm 6.73e	25.22 \pm 4.14ab	56.79 \pm 3.33e	31.16 \pm 1.87bc	44.83 \pm 1.82d	35.49 \pm 5.16c										
1-Propanol	24.76 \pm 1.75a	33.01 \pm 1.08b	50.43 \pm 1.82d	50.66 \pm 5.34d	30.30 \pm 2.71b	62.16 \pm 2.94e	32.06 \pm 1.38b	42.08 \pm 2.10c	29.36 \pm 1.40b	45.45 \pm 3.49c										
Diacetyl	0.00 \pm 0.00a	0.00 \pm 0.00a	0.00 \pm 0.00a	1.41 \pm 0.10b	1.71 \pm 0.16b	1.64 \pm 0.14b	0.00 \pm 0.00a	0.00 \pm 0.00a	2.67 \pm 0.51c	0.00 \pm 0.00a										
Ethyl acetate	18.02 \pm 1.15a	19.06 \pm 2.04a	37.19 \pm 1.90bc	32.50 \pm 0.69bc	40.86 \pm 0.22cd	30.86 \pm 1.87b	49.67 \pm 9.55d	85.39 \pm 13.49e	35.60 \pm 1.99bc	48.41 \pm 5.86d										
2-Butanol	0.00 \pm 0.00a	0.00 \pm 0.00a	0.00 \pm 0.00a	0.00 \pm 0.00a	2.89 \pm 0.35b	0.00 \pm 0.00a	0.00 \pm 0.00a	0.87 \pm 1.50a	3.36 \pm 0.71b	0.00 \pm 0.00a										
Iso-butanol	26.42 \pm 1.23bc	22.53 \pm 2.25ab	51.22 \pm 1.38f	28.14 \pm 1.94c	49.45 \pm 4.24f	34.16 \pm 1.90d	40.11 \pm 1.87e	20.07 \pm 0.81a	65.41 \pm 6.93g	47.22 \pm 2.75f										
1-Butanol	3.90 \pm 0.05b	0.00 \pm 0.00a	4.68 \pm 0.17bcd	5.58 \pm 1.40d	0.00 \pm 0.00a	5.13 \pm 0.22cd	0.00 \pm 0.00a	5.55 \pm 0.80d	5.53 \pm 0.69d	4.19 \pm 0.18bc										
Acetoin	11.18 \pm 1.31b	11.26 \pm 0.82b	10.61 \pm 0.42ab	10.81 \pm 0.99ab	8.91 \pm 1.19a	11.21 \pm 0.70b	11.25 \pm 0.61b	11.74 \pm 0.31b	12.27 \pm 1.43b	12.00 \pm 2.51b										
2-Methyl-1-butanol	58.62 \pm 2.89de	37.20 \pm 4.13a	82.81 \pm 5.49g	55.15 \pm 2.34cd	64.24 \pm 6.84ef	62.10 \pm 1.65def	66.89 \pm 4.69f	44.01 \pm 0.54ab	68.88 \pm 7.62f	48.23 \pm 0.96bc										
3-Methyl-1-butanol	141.50 \pm 6.16c	78.25 \pm 6.27a	187.56 \pm 4.47e	109.60 \pm 1.99b	163.37 \pm 8.32d	133.54 \pm 6.30c	144.13 \pm 9.97c	81.48 \pm 1.72a	176.88 \pm 17.89de	91.39 \pm 4.96a										
Isobutyl acetate	0.00 \pm 0.00a	0.00 \pm 0.00a	0.00 \pm 0.00a	3.10 \pm 0.26b	10.55 \pm 0.26d	0.00 \pm 0.00a	3.54 \pm 1.14b	3.27 \pm 0.41b	4.83 \pm 0.86c	3.63 \pm 0.40b										
Ethyl butyrate	3.44 \pm 0.53b	4.68 \pm 0.31c	2.95 \pm 0.19b	0.00 \pm 0.00a	4.60 \pm 0.82c	4.31 \pm 0.67c	0.00 \pm 0.00a	0.00 \pm 0.00a	4.83 \pm 0.40c	0.00 \pm 0.00a										
Ethyl lactate	15.17 \pm 1.20c	14.52 \pm 0.84c	60.60 \pm 2.78e	26.37 \pm 2.92d	10.00 \pm 0.68b	14.83 \pm 0.98c	5.53 \pm 0.86a	15.69 \pm 4.91c	12.28 \pm 2.91bc	9.32 \pm 1.58ab										
2-3 butanediol	484.44 \pm 19.15bc	565.98 \pm 52.62cde	362.72 \pm 25.92a	444.27 \pm 34.85 ab	370.25 \pm 29.25a	673.93 \pm 29.92f	512.73 \pm 65.93bcd	593.12 \pm 25.27def	435.27 \pm 60.09 ab	643.34 \pm 86.96ef										
Isoamyl acetate	2.42 \pm 0.67b	0.00 \pm 0.00a	2.14 \pm 0.30b	0.00 \pm 0.00a	2.31 \pm 0.34b	0.00 \pm 0.00a	2.37 \pm 0.12b	1.90 \pm 0.06b	4.72 \pm 1.06c	2.05 \pm 0.29b										
Hexanol	0.00 \pm 0.00a	0.00 \pm 0.00a	3.84 \pm 0.12c	3.62 \pm 0.12b	3.87 \pm 0.15c	3.88 \pm 0.06c	0.00 \pm 0.00a	3.64 \pm 0.15b	0.00 \pm 0.00a	0.00 \pm 0.00a										
2-Phenylethanol	47.18 \pm 1.31d	23.81 \pm 1.93a	73.39 \pm 9.42e	30.37 \pm 2.88 ab	69.35 \pm 3.08e	42.77 \pm 6.10cd	38.60 \pm 4.46bcd	23.44 \pm 1.02a	67.82 \pm 8.16e	34.52 \pm 4.30bc										
2-Phenylethyl acetate	5.38 \pm 0.05a	5.36 \pm 0.12a	5.51 \pm 0.10a	5.77 \pm 0.03a	7.70 \pm 0.46b	7.34 \pm 0.64b	5.43 \pm 0.20a	5.56 \pm 0.34a	7.67 \pm 0.52b	7.23 \pm 0.25b										
Higher alcohols	302.37 \pm 12.97bc	194.79 \pm 13.06a	450.09 \pm 17.99g	279.50 \pm 13.31b	379.61 \pm 18.46e	339.86 \pm 17.72d	321.81 \pm 19.68bc	217.51 \pm 4.31a	417.25 \pm 39.37f	271.01 \pm 9.22b										
Carbonyl compounds	11.18 \pm 1.31ab	11.26 \pm 0.82ab	10.61 \pm 0.42a	12.22 \pm 1.05ab	10.62 \pm 1.04a	12.86 \pm 0.83bc	11.25 \pm 0.61ab	11.74 \pm 0.31ab	14.94 \pm 1.86c	12.00 \pm 2.51ab										
Esters	44.44 \pm 0.33a	43.63 \pm 2.65a	108.38 \pm 0.65e	67.74 \pm 3.33cd	76.02 \pm 1.63d	57.34 \pm 1.16b	66.54 \pm 9.59bc	111.80 \pm 11.66e	69.94 \pm 5.52cd	70.63 \pm 4.82cd										
Floral and fruity esters	26.42 \pm 0.90b	24.57 \pm 1.06b	71.19 \pm 2.53d	35.25 \pm 3.17c	35.16 \pm 1.86c	26.48 \pm 1.24b	16.87 \pm 1.42a	26.42 \pm 5.41b	34.33 \pm 3.82c	22.22 \pm 1.82b										

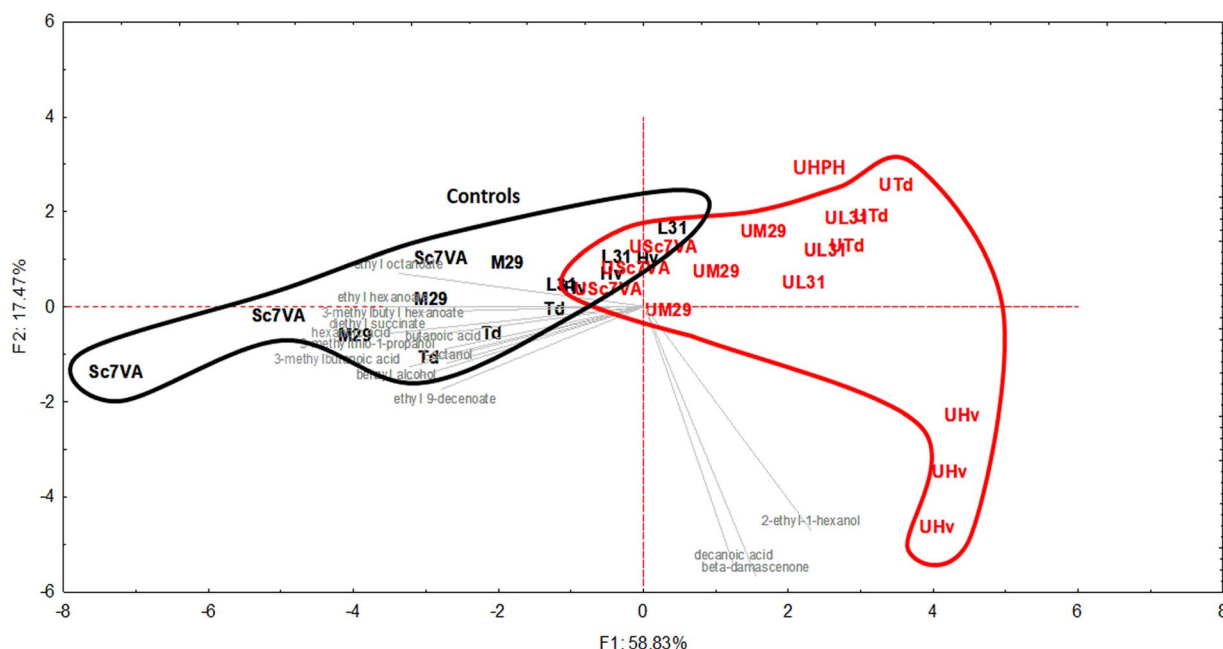


Fig. 3 PCA biplot of values and means in controls and UHPH (U) must fermented by *Saccharomyces cerevisiae* (Sc7VA), *Lachancea thermotolerans* (L31), *Torulaspora delbrueckii* (Td), *Hanseniaspora vineae* (Hv), and *Metschnikowia pulcherrima* (M29). Compounds analyzed by HS-SPME-GC-MS.

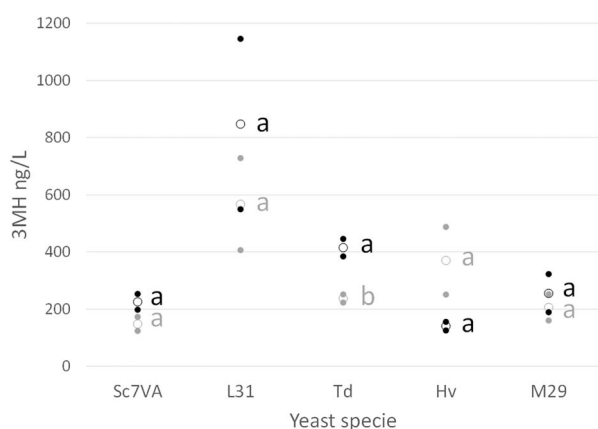


Fig. 4 Content of 3-mercaptohexanol (3MH) (ng L^{-1}) in the fermentation of several yeast strains: *Saccharomyces cerevisiae* 7VA (Sc7VA), *Lachancea thermotolerans* L31 (L31), *Torulaspora delbrueckii* (Td), *Hanseniaspora vineae* (Hv), and *Metschnikowia pulcherrima* (M29). Black dots show fermentation of UHPH processed grape juice (black circles are the average value) and grey dots show fermentation of control unprocessed juice (grey circles are the average value). Fermentation was performed in duplicate. Different letters mean significant differences ($p < 0.05$).

after the settling is a typical effect of oxidation by the grape polyphenol oxidase enzymes (PPOs) working on juice *o*-diphenols; the process is very fast and clearly observable under exposition to air in the absence of reducing agents such as SO_2 . This slightly higher hue can be observed in the control beaker (Fig. 1A) but not in the UHPH processed must (Fig. 1B), thanks to the effective inactivation of PPOs by UHPH as it has been

Table 4 Color parameters chroma and hue for all wines produced from untreated and treated Verdejo must. Values are means with standard deviations for $n = 3$. Values for each pair of yeast species that share the same letter do not differ significantly ($p < 0.05$)

Wine	Chroma	Hue ($^\circ$)
Sc7VA	$14.4 \pm 2.7a$	$91.1 \pm 4.3a$
USc7VA	$9.7 \pm 1.2b$	$95.5 \pm 6.9a$
L31	$11.4 \pm 0.2a$	$97.8 \pm 3.3a$
UL31	$10.2 \pm 1.1a$	$98.0 \pm 4.0a$
Td	$12.6 \pm 1.4a$	$97.4 \pm 2.1a$
UTd	$9.9 \pm 0.6b$	$97.6 \pm 5.8a$
Hv	$11.4 \pm 1.0a$	$93.4 \pm 4.0a$
UHv	$9.3 \pm 1.0a$	$98.0 \pm 2.6a$
M29	$12.5 \pm 0.4a$	$95.5 \pm 0.7a$
UM29	$9.3 \pm 2.6b$	$94.4 \pm 0.3b$

previously reported.^{5,7,11,30} Concerning the microscopic aspect, the thin regular structure in the sediment of the UHPH juice is due to the intense and regular nanofragmentation produced by the mechanical effects on colloidal particles and microorganisms in the UHPH valve.^{5,11} This microscopic observation agrees with the size range of 235–744 nm previously measured by AFM in grape juice.⁷

3.2.2. Yeast counts. The resistance of yeasts to UHPH is not very high and inactivation levels of 6 log have been observed for *Saccharomyces* and non-*Saccharomyces* in grape juice.¹¹ The absence of yeasts in the must after the 6th day of fermentation guarantees a better expression and prevalence of the inoculated species during the fermentation. We have observed the absence of wild yeasts in several trials during full fermentation,^{5,7} and it



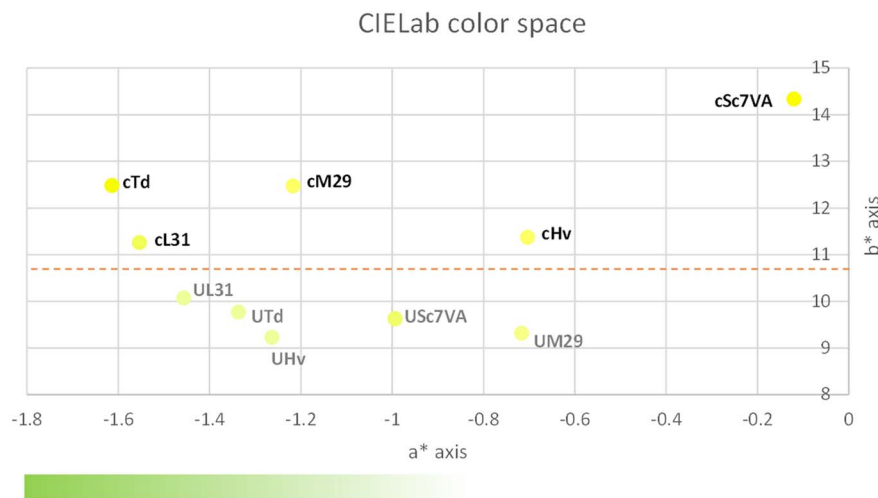


Fig. 5 CIE Lab parameters for control (c) and UHPH (U) must fermented by *Saccharomyces cerevisiae* (Sc7VA), *Lachancea thermotolerans* (L31), *Torulaspora delbrueckii* (Td), *Hanseniaspora vineae* (Hv), and *Metschnikowia pulcherrima* (M29). Color of wines expressed as the contribution of the a^* axis and b^* axis from the CIE Lab color space coordinates. Negative values of the a^* axis correspond to green, and positive values of the b^* axis correspond to yellow.

is possible to maintain unfermented musts for months or even years when sterilized by UHPH.

We have observed a slightly lower fermentation speed in UHPH processed must inoculated with the different species than in controls [data not shown]. This can be due to the lower loads of wild yeasts or the lower availability of thiamine or some nitrogen compounds that can be nano-encapsulated and less available, but more research is necessary to support these tentative explanations.

3.2.3. Lactic acid and enological parameters. Recently the use of acidifying yeasts to reduce pH is a hot topic in the wine industry to improve the freshness and long-term stability of wines.¹⁷ *Lt* is the most important species from this point of view, due to its ability to reduce significantly wine pH by producing lactic acid from sugars.^{23–25} It also provides advantages for the consequent increase of molecular SO_2 obtained at lower pH, also allowing total SO_2 reduction with the same stability influencing the production of off-flavors³¹ and having a bioprotective effect.³² Although the implantation of *Lt* has been good in controls and UHPH processed must, many times at the industrial level acidification fails because of a low implantation of the yeast. Wild yeast elimination by UHPH is a guarantee for good implantation for *Lt* starters promoting good acidification even in juices with high loads of competitive wild yeasts.

3.2.4. Volatile compounds by GC-FID and SPME-GC-MS. The lower contents of higher alcohols in UHPH wines (30% less, Table 3) have a positive impact on wine aroma because they give a winy and weighty sensory perception,^{33,34} which reduces the freshness and gives the impression of a flat and simple wine. The reduction of higher alcohols in the fermentation of UHPH wines has been already reported in white wines.¹¹ Additionally, a low content of fermentative esters is also positive in a variety such as Verdejo with a clear thiol profile,³⁵ in which excessive fermentative esters can mask this sensory quality. Previously, high production of esters has been observed in must processed

by UHPH;¹¹ however that wine was processed without settling and had more precursors after the nanofragmentation of the colloidal particles by UHPH. The degree of settling may be one way to modulate ester production during fermentation of UHPH-processed juices as indeed is nitrogen nutrition during fermentation.^{36,37} No significant differences have been found in the concentration of 2-phenylethyl acetate, an ester with a clear floral impact on wine (Table 3).

Concerning carbonyl compounds, the excessive amount of acetoin and diacetyl can be correlated with excessive dairy notes in wines, but in this case, both compounds were at a moderate amount and without significant differences between controls and UHPH wines (3%, Table 3). Finally, the low content of acetaldehyde in UHPH wines is positive, because of the impact of such a compound on the development of flat and oxidized aromatic characters.³⁸ Indeed, in high amounts, this compound can confer to the wine nutty aromas, which turn to green/grassy or apple-like off-flavors at even higher concentrations; in contrast, if the levels are low, it can be connected with fruity notes.³⁸ The lower acetaldehyde content in UHPH wines may be reasonably explained due to a higher presence of wild non-*Saccharomyces* yeasts in controls. However, it is also reported that acetaldehyde formation during alcoholic fermentation can be negatively affected by a deficiency of thiamine, because this vitamin is a cofactor of pyruvate decarboxylase.³⁹ When heated in water at 140 °C under pressure, thiamine may be degraded⁴⁰ and, due to the in-valve temperature reached during the experiment, UHPH processing might have reduced its natural content in the grape juice. The low concentrations of acetaldehyde in UHPH wines can be complementary to the suppressive effect of thiols in the oxidative profile produced by this molecule in wines.⁴¹

The same trend highlighted by GC-FID analysis for fermentative volatile compounds was also found for the other VOCs analyzed by SPME-GC-MS (although not always in a statistically



significant way); indeed many of such compounds also have a fermentative origin. The slight decrease of different fatty acids in UHPH wines may also have a positive role on wine freshness, because of the consequent reduction of their typical pungent notes. Finally, limited to the samples fermented with *H. vineae*, the lower concentration of β -damascenone found in the samples processed by UHPH is an interesting feature, because this compound can modify the sensory perception of some esters. Indeed, some researchers observed that the addition of low levels of β -damascenone ($0.85 \mu\text{g L}^{-1}$) to a model solution containing esters increased the fruity notes of the mixture, while when the concentration was higher ($3.5 \mu\text{g L}^{-1}$), it determined the development of intense notes of raisins and dried plums.⁴²

3.2.5. Thiols by GC-MS. Thiols are the main aroma compounds in the sensory profile of the Verdejo variety with herbal like box tree nuances. The highly intense effect of Lt L31 in the formation of 3 MH is very significant with average values of $567\text{--}847 \text{ ng L}^{-1}$ compared with the other species $140\text{--}415 \text{ ng L}^{-1}$. This fact agrees with the positive effect of Lt in the release of thiols.⁴³ It is also noticeable that most of the fermentation samples of the UHPH must had higher contents of 3 MH than the control must even when just the differences were significant in Td probably because of the high dispersion among replicates (Fig. 4). The non-detection or variable behavior in other thiols such as 4MMP, 4MMPOH, A3MH and benzene methanethiol indicates a low influence in the general profile of these wines.

3.2.6. Color. Regarding the color of the wines, two parameters reflect the differences observed between wines from untreated must and those from UHPH-treated must. These two parameters are chroma and the b^* axis. In this way, the only outstanding value was observed for untreated Sc wine with the largest difference in chroma from its treated counterpart, and the highest yellow fraction from the b^* axis. The higher the value on the b^* axis, the higher the expression of the yellow fraction in wines. The yellow color of wines is associated with oxidation. This phenomenon was observed in wines from untreated must, and this is also related to the inhibition of PPO enzymatic activity in treated must as it has been shown that UHPH has the ability of inactivating these enzymes.^{5,11,13} The color of wines from treated must had a less yellow fraction as observed in the *Vitis vinifera* L. variety "Hondarribi zuri" inoculated with 7VA.¹¹ It is also interesting to observe that all wines produced from treated must and the wines produced from untreated must, but fermented with non-*Saccharomyces* yeast, retain the green color potentially coming from the Verdejo variety. From this observation, the species *Lachancea thermotolerans* and *Torulaspora delbrueckii* preserve the green color to a larger extent. Biological acidification of wines may play an important role in avoiding oxidation in the first case as it has been described that Lt reduces the evolution of color in Airén wines with lower chroma and hue values.⁴⁴

4. Conclusions

UHPH is a promising emerging non-thermal technology environmentally friendly with very low consumption of energy and

water resources able to control microbial loads and oxidation in grape juices. The effect, when associated with the use of non-*Saccharomyces* yeasts, is an effective implantation of the yeast inocula and a positive metabolomic impact facilitating the preservation of varietal aroma from grapes and the improvement of the freshness if acidifying species such as *L. thermotolerans* are used. Some of the aroma effects of the association of UHPH and specific non-*Saccharomyces* species might occur only in Verdejo or maybe just in other thiolic varieties. Additionally, lower pH values together with the control of the full wild microbiome of grapes, and the inactivation of oxidative enzymes, facilitate the production of wines with very low levels of SO_2 . A new enology is possible with cleaner labels and a more stable sensory quality even with grapes from regions affected by global warming.

Author contributions

C. Escott (investigation and methodology), C. Vaquero (investigation and methodology), J. M. del Fresno (investigation, methodology, and writing – original draft), A. Topo (investigation), P. Comuzzo (methodology, supervision, data curation, writing – original draft, and writing – review & editing), C. Gonzalez (methodology, supervision, and writing – review & editing), and A. Morata (conceptualization, funding acquisition, methodology, project administration, supervision, writing – original draft, and writing – review & editing).

Conflicts of interest

There are no conflicts to declare.

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