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White wine processing by UHPH without SO_2. Elimination of microbial populations and effect in oxidative enzymes, colloidal stability and sensory quality

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Linalool: Pubchem CID 6549
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2,6-Dimethyl-1,7-octadiene-3,6-diol: Pubchem CID 548927
8-Hidroxylinalool: Pubchem CID 5280678

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ABSTRACT

The use of UHPH sterilization in the absence of SO_2 has been used to eliminate wild microorganisms and inactivate oxidative enzymes. A white must of the Muscat of Alexandria grape variety was continuously processed by UHPH at 300 MPa (inlet temperature: 23–25 °C). The initial microbial load of the settled must was 4-log CFU/mL for both yeast and moulds, and slightly lower for bacteria. After UHPH processing, no microorganisms were detected in 1 mL. UHPH musts remain without fermentative activity for more than 60 days. Concentrations of the thermal markers indicated the absence of thermal damage in the UHPH-treated musts, since 5-hydroxymethylfurfural was not detected. In addition, the must treated by UHPH keeps terpene concentrations similar to those of the untreated controls. A strong inactivation of the oxidative enzymes was observed, with no browning at room temperature for more than 3 days. The antioxidant value of the UHPH-treated must was 156% higher than the control.

1. Introduction

The reduction of SO_2 levels is a hot topic in the wine industry, although suitable alternatives capable of controlling oxidation and microbial developments at the same time have not yet been found (Giacosa et al., 2019). Some physical techniques, such as flash thermal treatments, allow the control of microorganisms and oxidative enzymes, but with sensory repercussions. Emerging non-thermal technologies comprise several physical technologies that can reduce or eliminate microorganisms, and some of them also have effects on oxidative enzymes. These gentle technologies do not produce thermal damage and therefore minimise sensory impact (Morata et al., 2017). High pressure technologies have been used at industrial level since 1990 and can now be easily applied to grape must. Initially, high

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Table 1
Oenological parameters of musts processed by UHPH or untreated, and of their respective wines after fermentation. Values are mean ± SD, n = 3. Values with the same letter in the same row are not significantly different ($p < 0.05$). Analyses performed by FTIR.

	Ethanol (% v/v)	Glu/Fruc (g/L)	Total Acidity (g/L Tartaric acid)	Malic Acid (g/L)	Lactic acid (g/L)	α-amino nitrogen (mg/L)	Volatile acidity (g/L Acetic acid)	TSS ° Brix	pH	Ammonia (mg/L)
Musts										
Control	–	188.5 ± 0.2b	2.7 ± 0.0b	3.27 ± 0.1a	–	58.3 ± 4.7b	0.11 ± 0.02a	17.5 ± 0.1b	3.53 ± 0.01b	0.00 ± 0.0a
UHPH	–	192.8 ± 2.2a	2.8 ± 0.1a	3.23 ± 0.2a	–	77.0 ± 3.1a	0.08 ± 0.02a	18.4 ± 0.3a	3.56 ± 0.01a	8.97 ± 12.0a
Wines										
Control	11.1 ± 0.1b	3.2 ± 0.3a	4.3 ± 0.1b	0.4 ± 0.3b	1.5 ± 0.2a	–	0.4 ± 0.0a	–	–	–
UHPH	11.6 ± 0.1a	1.8 ± 0.2b	4.9 ± 0.1a	1.9 ± 0.1a	0.1 ± 0.1b	–	0.3 ± 0.0b	–	–	–

hydrostatic pressure (HHP) was used in batch processes showing interesting abilities not only to remove yeasts and moulds, but also to increase the extraction of phenols (Corrales, FernándezGarcía, Butz, & Tauscher, 2009; Morata et al., 2015; Puig, Olmos, Quevedo, Guamis, & Mínguez, 2008). Additionally, HHPs produce the reduction of wild yeasts and bacteria, thus facilitating the use of new biotechnologies such as fermentation with non-*Saccharomyces* yeast species or yeast-bacteria co-inoculations (Bañuelos, Loira, Escott, & Fresno, 2016). However, it is difficult to completely eliminate gram positive bacteria even with pressures above 500 MPa (Morata et al., 2015). Moreover, the effect on oxidative enzymes is quite limited (Jacobo-Velázquez & Hernández-Brenes, 2010).

High Pressure Homogenization (HPH) is an alternative high pressure technology with the advantage of being a continuous process. It is usually called HPH when the processing is done at 200 MPa or less, and Ultra-High Pressure Homogenization (UHPH) when the pressure is higher than 200 MPa (Comuzzo & Calligaris, 2019; Zamora & Guamis, 2015). In UHPH, the must is pumped at 300 MPa and then depressurised immediately through a specific valve built in high resistant materials. The temperature in the valve can be high, easily reaching 100 °C or more, but for a very short time, usually 0.2 s for the whole process and < 0.02 s in the valve (Loira et al., 2018). In this process, the antimicrobial and antienzymatic effects are produced by mechanical forces, mainly impact and shearing phenomena, but also cavitation and friction (Loira et al., 2018). The design of the valve, the pressure pump and other auxiliary components is essential for the performance of the UHPH device. Strong valves working at 300 MPa and reaching 140–150 °C can eliminate sporulated bacteria (EP2409583; Ypsicon, 2018), being an effective alternative to UHT treatments by providing a lower residence time (< 0.2 s) compared to the typical 3–4 s in UHT. The sensory impact is really low when using the UHPH technology. UHPH, similarly to HHP, can be considered sensory protective as the energy of the process is not enough to affect the covalent bonds, so pigments, aroma compounds and flavouring substances are protected (Bermúdez-Aguirre & Barbosa-Cánovas, 2011).

This technology is available at industrial scale reaching 50.000L/h based on modular systems. UHPH pumps able to work at 300 MPa are available to process at 10.000L/h (Ypsicon, 2018). UHPH is under evaluation by the OIV as authorised practice in oenology. Currently, the project of resolution is in step 5 (OENO-MICRO 16-594B Et5).

The aim of this work is to check the effectiveness of UHPH in the control of wild microorganisms in grape musts and to evaluate the oenological and sensory parameters of the wines obtained after fermentation with *Saccharomyces cerevisiae* in comparison with control wines produced from sulphited must or spontaneously fermented must.

2. Materials and methods

2.1. Must preparation

Grapes of *Vitis vinifera* L. Muscat of Alexandria were pressed in a pneumatic press and the running must was settled at 8 °C using Lafazym CL pectolytic enzymes (1.2 g/HL) (Laffort) until 150NTU (Nephelometric turbidity units). It was then preserved under inert CO_2 atmosphere. The clean must was separated in 2 fractions, one kept as control and the other processed by UHPH. This must was processed using a continuous UHPH equipment (60L/h) (Ypsicon Advanced Technologies, Barcelona, Spain) built under patent by Universitat Autònoma de Barcelona (EP2409583B1). The equipment used a tungsten carbide valve. The processing parameters were flow-rate 60 L/h at 307 ± 3 MPa, inlet temperature 23–25 °C, in-valve temperature 78–65 °C for only 0.02 s and outlet temperature 13–15 °C (Supplementary figure S1). The must was cooled down after the valve to reach the outlet temperature. The heat exchanger used water at 3 °C. The in-valve temperature was measured by a sensor just after the valve. The total volume processed by UHPH was 100 L. The initial parameters

of the must are described in Table 1.

2.2. Next Generation sequencing (NGS)

Samples of must were sent for total DNA extraction and Next Generation Sequencing (NGS) (www.wineseq.com; www.biomemakers.com). Samples were processed using the Qiagen PowerSoil® DNA isolation Kit and analysed for the 16 s rRNA V4 region, and the ITS by amplification of the ITS1 region using WineSeq® custom primers (Patent WO2017096385). After a Quality Control by electrophoresis gel, each library (16S and ITS) was pooled in equimolar amount and subsequently sequenced on Illumina MiSeq instrument (Illumina, San Diego, CA, USA) using 2x301 paired-end reads and according to the Biome-Makers implemented protocol.

All the data produced and collected were subsequently analysed through a QIIME-based custom bioinformatics pipeline (Patent WO2017096385, Biome Makers). A first quality control was used to remove adapters and chimeras. Later, reads were trimmed and OTU clusters were performed using 97% identity. Taxonomy assignation and abundance estimation were obtained comparing Operational Taxonomic Unit (OTU) clusters obtained with WineSeq® taxonomy database.

2.3. Microbial counts

Microbiological analyses were carried out on the control musts and on the UHPH-treated musts. For vegetative forms, 1 mL of serial decimal dilutions in saline peptone (0.85% NaCl and 0.1% peptone) were pour-plated in selective media for total aerobic bacteria and lactic acid bacteria, and 100 μL or 1 mL were spread-plated for yeasts. The media were: Glucose chloramphenicol agar (GCA) incubated aerobically for 4 days at 25 °C (yeast counts); synthetic lysine agar (Oxoid, Hampshire, UK) for non-*Saccharomyces* counts, incubated aerobically for 6 days at 25 °C; PCA supplemented with nystatin (50 mg/L) after sterilization and incubated for 6 days at 30 °C (aerobic bacteria counts); MRS agar and MLO agar supplemented with nystatin (50 mg/L) after sterilization and incubated for 6 days at 30 °C in anaerobic conditions in a jar under CO_2 atmosphere (Lactic Acid Bacteria counts). GCA, MRS and MLO media were purchased from Pronadisa (Barcelona, Spain). For aerobic bacterial endospores analyses, flasks with 100 mL of must were pasteurised at 80 °C/30 min to kill all vegetative forms. Later, flasks were mixed and cooled in ice water. 10 mL volume from each flask passed through a membrane filter, 0.45 μm (Millipore) and was incubated onto the surface of the PCA plates for 6 days at 30 °C.

2.4. Culture-dependent analysis

Endospore-forming bacteria isolates were identified by colony PCR with primers for the V3–V4 16S rRNA region (primers 16SV3-V4-CS1 modified; 5′-CCTACGGGNGGCWGCAG-3′ and 16SV3-V4-CS2 modified; 5′-GACTACHVGGGTATCTAATCC-3′) and submitted to Secugen (www.secugen.es) for sequencing and identification.

2.5. Fermentations and microbial counts

Fermentations were performed in 2L flasks with 1.8L of must in triplicate at 18 °C. Fermenters were inoculated with 32 mL starters of a 24-hour culture in YPD broth (Conda, Madrid, Spain) containing 5×10^7CFU/mL (checked by plating). The strain used was *Saccharomyces cerevisiae* 7VA (enotecUPM, Spain).

A parallel assay was conducted to evaluate the spontaneous fermentation with three other replicates of each UHPH-processed and untreated must. These musts were placed in 100-mL vials with 50 mL of must and allowed to ferment with the wild population. The flasks were sealed with Müller valves to release amicrobically the CO_2 from the fermentation. Fermentation was gravimetrically monitored by recording the weight losses due to the release of CO_2, and proceeded isothermally at 18 °C.

2.6. Particle size after UHPH by laser diffraction

Particle size measurements were performed by laser diffraction, using Malvern Mastersizer 2000® (Malvern Instruments Ltd., Malvern, UK). Sample was previously diluted with distilled water until the appropriate laser obscuration values (5–10%). Refractive index of sample and water were set at 1.340 and 1.333, respectively. The particle size distribution was characterised by the D50 and D90 (particle diameter at 50 and 90% in the cumulative distribution) and d3,2 (surface area average diameter) and d4,3 (volume moment mean) parameters. Determinations were performed at day 3, after UHPH treatment. Measurements were performed in triplicate.

2.7. Electronic microscopy of both control and UHPH-processed musts

Samples of UHPH-treated and control musts particles retained in the filter material (0.22 μm; Teknokroma, Barcelona, Spain) were placed in 12 mm circular glass slides and dried at 40 °C during 72 h. The slides were placed on 25 mm diameter specimen holders using double-face carbon adhesive tape and then coated with gold using a Quorum Q150RS sputter coating machine (Quorum Technologies, East Sussex, United Kingdom). The samples were analysed with a Jeol JSM-6335F scanning electron microscope (SEM) (Tokio, Japan) at 15 kV of accelerating voltage and 15 mm working distance.

2.8. Oenological parameters by Infrared spectroscopy

The equipment OenoFoss™ (FOSS, Barcelona, Spain) using Fourier Transform Infrared Spectroscopy (FTIR) was used to identify and quantify major compounds such as residual sugars, organic acids, total and volatile acidity, and pH value. Relative accuracy is > 0.95 (95%) for all parameters except: TTS (66%) and Total acidity (84%) in musts, and malic acid (74%) in wine.

2.9. Terpenes and aromatic composition by GC–MS

Sample preparation and chromatography conditions were made according to Roda et al. (2019). One hundred milliliters of centrifuged (15 min at 6.000 rpm) grape juice was doped with 100 μL of 2-octanol (as internal standard), and passed through a SPE cartridge (Solid Phase Extraction) Bond Elut ENV of 500 mg and 6 mL (Agilent Technologies, Santa Clara, CA, USA). Cartridges were previously conditioned (5 mL of methanol, 5 mL of hydro alcoholic solution (12% v/v) and 5 mL of water). Aromatic compounds were eluted with pentane-dichloromethane (50/50), dried and finally re-dissolved in pentane-dichloromethane (50/50) up to a final volume of 200 μL. A gas chromatograph 7890A (Agilent Technologies) equipped with a mass spectrometer 5975C inert MSD was used. A constant flow of 2.1 mL/min of He was used as carrier gas. The injected volume was 5 μL in splitless mode with 17.33 psi pressure (septum purge flow 15 mL/min and splitless time 1 min.). A DB-WAX IU column (60 m × 0.25 mm × 0.25 μm) was used. The injector temperature was maintained at 180 °C for 1 min. and then heated up to 260 °C at 250 °C/minute. The oven was maintained at 60 °C for 15 min. and then increased up to 220 °C at (3 °C/min.), for 25 min. The mass spectrometer operated at 70 eV. The analysis was performed in Scan mode (*m/z* 10–1000). The aromatic compounds were identified by their retention times and the mass fragments, to compare with those of pure standard compounds. The quantification was carried out using the method of internal standard patterns.

2.10. Markers of thermal degradation by GC–MS

5-hydroxymethylfurfural (HMF) was analysed by GC–MS. The equipment used was an Agilent Technologies 6890 *N*-MSD-5973 N gas chromatography-mass spectrometer (GC–MS). Chromatography was performed according to Loira et al. (2013) with a DB-WAX column (30 m × 0.25 mm × 0.25 μm) (J&W Scientific, Folsom, CA, USA). The method was calibrated using external standards (Merck, Hohenbrunn, Germany). A liquid extraction with dichloromethane was done before the chromatographic separation. 2.5 mL of must was mixed with 250 μL of dichloromethane and 25 μL of a 3,4-dimethylphenol solution (10 mg/L; Merck, Hohenbrunn, Germany) as IS; 0.37 g of NaCl was added too and stirred in vortex for 5 min. After centrifugation at 7500 rpm and 4 °C for 15 min, the dichloromethane phase was recovered and 1 μL was injected into the GC–MS. The split ratio used was 20:1. The helium flow rate was 1 mL min^{-1}. The oven temperature programme was 60 °C for 1 min, followed by a 3 °C min^{-1} ramp until 150 °C, followed by a 10 °C min^{-1} ramp to 260 °C, then held for 2 min. The mass spectrometer operated at 70 eV. The analysis was performed in SIM mode.

2.11. Determination of polyphenol oxidase (PPO) activity

Polyphenol oxidase (PPO) activity was monitored by following the absorbance at 420 nm in a 1 mm cuvette during 1400 h in triplicate samples with a specific air contact surface of 1 cm^2/mL at 20 °C. Absorbance was spectrophotometrically monitored using a UV–visible spectrophotometer 8453 from Agilent Technologies™ (Palo Alto, CA, USA).

2.12. Antioxidant capacity (ABTS method)

The ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] method allows determining the antioxidant activity trough the discolouration of the cationic $ABTS^{\bullet+}$ radical produced by the oxidation of ABTS with potassium persulphate (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999). A solution of potassium persulfate (2.45 mM) was prepared in an ABTS stock solution (7 mM) to form the radical cation. This solution was stored at 4 °C and diluted with phosphate buffered saline (PBS) at pH 7.4 in order to prepare the working reagent with an absorbance around 0.7AU at 734 nm. The working reagent colouration disappears when the radical is reduced by antioxidants. Trolox and wines were added to the working reagent and the decrease in absorbance was measured. The measurement of the reagent was used as blank. Finally, 50 μL of diluted wines was added and vortexed for 30 s and after 4 min of incubation at room temperature, the absorbance was measured again at 734 nm. Samples were analysed at 4 different concentrations by duplicate.

2.13. Analysis of volatile fermentative compounds by gas chromatography with flame ionisation detection (GC-FID)

Volatile compounds were determined using an Agilent Technologies 6850 gas chromatograph (Network GC System) with a FID detector. A DB-624 column (60 m × 250 μm × 1.40 μm) was used. Calibration was done with external standards ($r^2 > 0.999$). All compounds were from Fluka (Sigma–Aldrich, Buchs SG, Switzerland). The injector temperature was 250 °C, and the detector 300 °C. The column temperature was 40 °C (5 min), rising linearly by 10 °C/min until 250 °C; then held for 5 min. Hydrogen was used as carrier gas. The injection split ratio was 1:10, the in-column flow rate 2.2 mL/min, and the detection limit 0.1 mg/L. One hundred microlitres of IS (4-methyl-2-pentanol, 500 mg/L) were added to 1 mL test samples and filtered through 0.45 μm membrane filters (Teknokroma, Barcelona, Spain). Injection volume was 1 μL.

2.14. Fining and colloidal stability

After fermentation, several fining agents were used to improve wine brightness, as is usual in winemaking. The following agents and doses were tested: bentonite (30, 60 and 80 g/HL), PVPP (20 and 40 g/HL) and the mixed treatments bentonite/gelatin (40/4 and 40/8 g/HL) and bentonite/active carbon (18/8 g/HL). The clean-up effect was evaluated by measuring turbidity in nephelometric turbidity units (NTU).

To check protein stability, a heat test was used, which consisted of subjecting the wine to 80 °C for 2 h with measurements of the turbidity before and after the test, once the room temperature (20 °C) had been recovered (Pocock & Waters, 2006).

2.15. Sensory evaluation

A triangular test (ISO 4120:2004) was performed to compare a non-UHPH treated wine versus a UHPH-treated wine. The samples were presented blindly (named with 3-digit random codes), so that each judge had three samples to evaluate sensorially, two of them equal and one different, being the position of this last one randomly assigned to each judge. The objective of this test is to determine if there is any significant difference between two products. To do this, the null hypothesis (Ho) accepts that the two products are the same and the alternative hypothesis (Ha) that the two products are different. The judges were asked to identify the different sample. All other serving details and environmental conditions were the same as for the attribute difference testing described below.

Additionally, an attribute difference testing was developed to assess the quality of the wines. A panel of nine experienced tasters (age range: from 30 to 60 years old, 4 women and 5 men) evaluated the wines. The blind tasting took place in the tasting room of Chemistry and Food Technology Department, UPM, provided with fluorescent lighting and presenting samples in random order. The wines (20 mL/tasting glass) were served at 20 ± 2 °C in three different standard odour-free wine-tasting glasses. Briefly, the panellists used a scale from 0 to 5 to rate the intensity of different attributes (0 = attribute not perceptible, 5 = attribute strongly perceptible). Each panellist also provided an overall impression of the wines produced, taking into account olfactory and taste features, including any defect, following the same procedure described by Loira et al. (2018).

2.16. Statistical analysis

Means and standard deviations were calculated, and differences examined using ANOVA and the least significant difference (LSD) test. All calculations were made using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). Significance was set at $p < 0.05$.

3. Results and discussion

3.1. Microbial load and antimicrobial effect of UHPH

The control must was analysed by Next Generation Sequencing (NGS) to check the totality of the microbiota in the must. The main yeast population consisted of non-*Saccharomyces* (> 95%), among which the following species were highly prevalent: *Starmerella bombicola, Torulaspora delbrueckii, Candida boidinii, Lachancea thermotolerans, Hanseniaspora vineae* and *Hanseniaspora uvarum*. The remaining yeasts belong to *Saccharomyces* and other residual species (Supplementary figure S2a), including fungi of the genera *Penicillium* and *Alternaria*. As for the bacterial populations, mainly acetic and lactic bacteria were found (Supplementary figure S2b). These results agree with the typical must yeast population consisting mainly of non-*Saccharomyces* yeasts. Several of them have been described for their potential applications in wine technology to improve: structure producing glycerol like

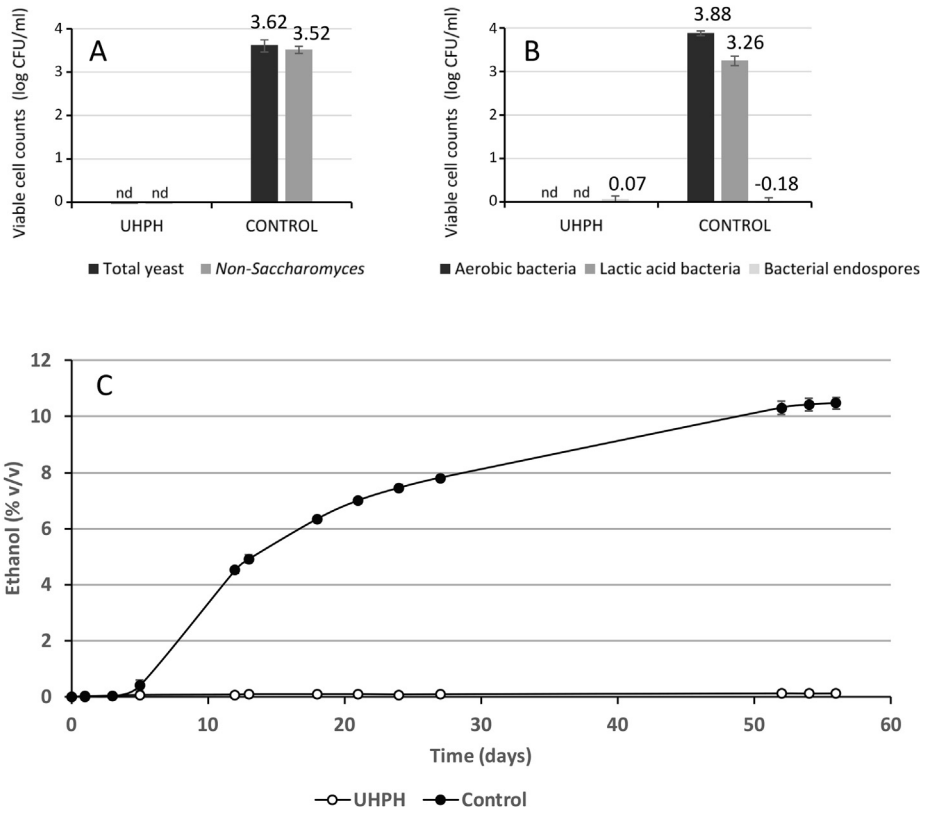

Fig. 1. Yeast (A) and bacteria (B) counts in controls and UHPH-processed musts. Values are means with standard deviations of the triplicates. (C) Fermentative activity monitored in the spontaneous fermentation of UHPH-processed and untreated must. The ethanol content was estimated by the CO_2 losses. Values are means with SDs of triplicate fermentations.

Starmerella (García, Esteve-Zarzoso, Cabellos, & Arroyo, 2018); flavour and aroma by releasing fruity or floral fermentative esters like *Torulaspora* (Ramírez & Velázquez, 2018) or *Hanseniaspora* (Martin, Valera, Medina, Boido, & Carrau, 2018); freshness by increasing acidity like *Lachancea* (Morata et al., 2018).

Total viable yeasts and non-*Saccharomyces* counts in the must were higher than 3-log CFU/mL (Fig. 1a) with a prevalence of non-*Saccharomyces* due to the high population detected in the lysine media and consistent with the NGS results (Supplementary figure S2) and also in the normal range of grape musts (Fleet, 2003).

After UHPH processing, all yeast cells were eliminated and undetected in the lysine and GCA media in 1 mL of must (Fig. 1a). The absence in both media confirms not only the elimination of *Saccharomyces,* but also non-*Saccharomyces* yeasts. The high efficiency of high pressure technologies to process grape musts agrees with previous results on HHP (Morata et al., 2015; Takush & Osborne, 2011) and UHPH (Loira et al., 2018), which reported the elimination of similar yeast populations by HHP discontinuous treatments (400–550 MPa-10 min) and UHPH continuous processing at 300 MPa.

Bacterial counts ranged from less than 4-log for aerobic bacteria in PCA to slightly higher than 3-log for lactic acid bacteria (Fig. 1b). After UHPH processing, they were undetected in 1 mL, in agreement with previous findings in grape must processed at 300 MPa (Loira et al., 2018). The effectivity is much higher than when is used discontinuous high hydrostatic pressure (HHP), in which residual 1-log populations can be found after high intensity treatments at 550 MPa-10 min (Morata et al., 2015). Moreover, softer UHPH treatments at 200 MPa are not enough effective, and require multiple application passes to reach inactivation levels in spoilage bacteria.

Bacterial endospores were cultured and less than 1-log populations were observed in 10 mL after thermal treatment in both control and UHPH-treated musts (Fig. 1b). UHPH can destroy bacterial endospores depending on the in-valve temperature. However, in this trial we tried to preserve the sensory quality of the must and, therefore, it was processed with a maximum in-valve temperature of 78 °C. We have observed in other food products that at least 140 °C together with a pressure of 300 MPa for less than 0.2 s is necessary to destroy bacterial endospores. Bacterial endospores were identified by sequencing of 16S rRNA and belong to endospore-forming genera, such as *Bacillus* and *Paenibacillus*.

A parallel assay was conducted without inoculating the musts in order to verify the absence of long-term fermentative processes produced by viable but not culturable yeasts temporarily damaged by UHPH. The three micro-fermenters with 50 mL of must processed by UHPH showed no fermentation for 2 months (Fig. 1c). In contrast, the untreated control musts started fermenting by the indigenous yeast population after 3 days.

Fig. 1c only shows the values measured in the first 2 months; however, we left them at 18 °C until now (4 months) and the UHPH-processed musts remained unfermented. The absence of long-term fermentation ensures the total elimination of fermentative yeasts and especially damaged non-culturable cells that are sometimes observed when discontinuous HHP is used (Lado & Yousef, 2002).

3.2. Effect of UHPH on colloidal particle size and must structure

Particle size distribution was measured in triplicate in musts processed by UHPH obtaining the following values: maximum diameter of 50% of particles D_{50} = 0.276 ± 0.015 μm; maximum diameter of 90% of particles D_{90} = 2.727 ± 0.373 μm; Sauter mean diameter $d_{3,2}$ = 0.287 ± 0.002 μm; volume moment mean $d_{4,3}$ = 0.982 ± 0.100 μm. It can also be observed that most of the particles are in a size range of 100–400 nm (Supplementary figure S3) as previously observed in UHPH-processed juices (Suárez-Jacobo, Saldo, Rüfer, Guamis, Roig-Sagués, Gervilla, 2012).

A clean appearance can be observed in the control musts after cold settling due to the low turbidity that was set at 150NTU. When a few mL of the UHPH-treated must were filtered through a 0.22 μm membrane filter, electron microscopy showed only a thin slime, and it was difficult to detect clear structures, probably because the strong impact

and shear forces produced very small fragments that crossed the filter (Supplementary figure S4a). However, crystals (probably from tartrates) and plasmolysed globular structures such as yeast cells could be observed in unprocessed control musts (Supplementary figure S4b). When enlarged to over 1200 magnification, the same appearance can be observed in crystals showing their structure undamaged (Supplementary figure S4c-d). Comparing UHPH-treated and control musts, less details on the filtering surface can be observed in control since the thin slime is denser and thicker (Supplementary figure S4d).

3.3. Oenological parameters in grape must. Effect of UHPH processing on must composition

The general analysis of the standard oenological parameters was done by means of FTIR spectroscopy (Table 1). A higher (and significant: $p < 0.05$) content of total suspended solids can be observed in UHPH-treated musts which is related to the intense fragmentation produced by the depressurization in the valve. Furthermore, UHPH-treated musts have higher contents (25% more) of Yeast Assimilable Nitrogen (YAN) or alpha-amino nitrogen than controls, probably as a result of the intense fragmentation of the grape cell wall where compounds that serve as nutrients for yeast are released. This agrees with previous findings in UHPH-treated grape musts (Loira et al., 2018), and enhance the potential of UHPH to produce easily fermentable musts for winemaking. In addition, nitrogen nutrition has effects on the formation of fermentative volatile aromas such as floral and fruity esters (Carrau et al., 2008) that can be enhanced by UHPH.

The variety used in this research, Muscat (*Vitis vinifera* L.), is a terpene producing cultivar and was selected because in the must composition these terpenes affect strongly the sensory profile. Moreover, terpenes are delicate and sensitive to processing treatments and it was intended to evaluate the effect of UHPH processing on varietal aroma. Terpenes and other varietal aromas were analysed by GC–MS (Table 2).

The main terpenes found were α-terpineol, linalool and geraniol according to the typical distribution in terpenic varieties (Mateo & Jiménez, 2000). However, no significant differences ($p > 0.05$) were found between UHPH-processed and control musts for any terpene (Table 2). Polyoxygenated terpenes showed similar contents in both controls and UHPH-processed musts, except for 2,6-dimethyl-1,7-octadiene-3,6-diol and 8-hidroxylinalool with higher and lower contents respectively, but without a clear relationship to treatment (Table 2).

Concerning aldehydes (C6 compounds, alcohols and volatile phenols), most of them remained unaffected and showed no significant differences after UHPH processing, highlighting the gentle effect of this technique on the aromatic quality of the must. Some like benzaldehyde, *cis*-2-hexen-1-ol and isoeugenol showed small significant differences, but without a clear trend (Table 2). No significant differences were found in either alcohols or volatile phenols, except for a lower concentration of isoeugenol in the UHPH-treated samples.

3.4. Thermal damage and control of oxidative enzymes by UHPH

No HMF has been detected in either the controls or the UHPH-treated musts; nor has 5-methyl furfural been detected. This observation agrees with the previous results for UHPH-treated apple juices (Suárez-Jacobo et al., 2012). HMF is produced by thermal degradation of sugars via the Maillard reaction and can be considered a marker of thermal processing in grape and fruit juices. HMF is formed by acid-catalysed dehydration of sugars, mainly fructose (Ortu & Caboni, 2017).

HMF increases the brown tonality of pale juices. HMF is a typical marker in heat-treated juices. Previous works have been reported the absence of HMF in juices processed by discontinuous HHP (Mert, Buzrul, & Alpas, 2013). This is explained by the low capacity of HHP to affect covalent bonds, so HMF cannot be formed by pressurization treatments. UHPH is also a pressurization process, but in-valve the must reaches 78 °C for less than 0.2 s. The absence of HMF indicates the gentle effect of UHPH on fruit juices.

Table 2
Terpenes and other aroma compounds in control and UHPH-processed musts (μg/L). Values are mean ± SD, n = 3. Values with the same letter in the same row are not significantly different ($p < 0.05$). Analyses performed by GC–MS.

		Control	UHPH
Aldehydes	Benzaldehyde	8.9 ± 0.8b	18.8 ± 2.1a
	2-hexenal	8.2 ± 2.0a	7.2 ± 1.0a
C6 compounds	trans-3-hexen-1-ol	11.5 ± 1.5a	8.6 ± 1.2a
	cis-2-hexen-1-ol	19.5 ± 1.6a	6.4 ± 1.4b
	cis-3-hexen-1-ol	45.1 ± 10.9a	43.7 ± 7.1a
	1-hexanol	28.7 ± 6.1a	21.3 ± 3.1a
Alcohols	1-octanol	11.2 ± 1.7a	11.8 ± 1.8a
	1-octen-3-ol	44.6 ± 7.3a	47.6 ± 4.9a
	benzyl alcohol	19.0 ± 1.7a	23.0 ± 3.7a
	Phenylethyl alcohol	82.9 ± 9.1a	74.3 ± 11.4a
Monoterpenes	linalool	79.2 ± 7.7a	77.8 ± 8.4a
	terpinen-4-ol	15.8 ± 2.5a	15.1 ± 0.8a
	Epoxylinalool	18.0 ± 1.8a	20.1 ± 5.1a
	β-citronellol	37.2 ± 7.8a	29.9 ± 2.3a
	geraniol	44.9 ± 9.1a	37.4 ± 2.2a
	α-terpineol	118.0 ± 19.0a	146.5 ± 26.9a
Polyoxygenated terpenes	cis-linalool oxide	12.8 ± 2.3a	13.3 ± 1.5a
	trans-linalool oxide	3.9 ± 0.7a	5.0 ± 0.9a
	cis-pyran linalool oxide	34.4 ± 7.6a	39.8 ± 3.4a
	trans-pyran linalool oxide	36.9 ± 7.8a	30.5 ± 2.0a
	2,6-dimethyl-3,7-octadiene-2,6-diol	645.6 ± 50.0a	723.7 ± 44.5a
	2,6-dimethyl-1,7-octadiene-3,6-diol	96.2 ± 7.6b	125.6 ± 12.9a
	3,7-dimethyl-1,7-octanediol	10.3 ± 0.3a	10.1 ± 2.1a
	8-hidroxylinalool	21.1 ± 1.4a	14.0 ± 1.6b
Volatile phenols	eugenol	1.0 ± 0.3a	1.2 ± 0.3a
	isoeugenol	6.3 ± 1.1a	3.1 ± 0.8b
	methyl salicilate	0.0 ± 0.0	0.0 ± 0.0
	ethyl salicilate	0.0 ± 0.0	0.0 ± 0.0

The evolution of enzymatic oxidation by polyphenol oxidase (PPO) was monitored by measuring absorbance at 420 nm because it is an indirect indicator of the oxidation of ortho-diphenols in o-quinones. The control musts showed an increase in the brown tonality (Fig. 2a), while the absorbance of the UHPH-treated musts remained stable for > 3 days. Darkening reactions produced by enzymatic oxidation reduce wine quality (Hendrickx, Ludikhuyze, Van Den Broeck, & Weemaes, 1998). Exposure to oxygen with a high air contact surface (1 cm^2/mL) in small beakers shows an intense browning from the beginning (1 min) in the control musts. However, the colour of UHPH-processed musts remains pale for several days (Fig. 2b), due to the inactivation of PPO, thus preventing enzymatic oxidation of the must even under these extreme aeration conditions, at room temperature and in the absence of sulphites. Previous works have also shown good PPO inactivation when using UHPH at 300 MPa is used (Loira et al., 2018; Suárez-Jacobo et al., 2012). Conversely, the information published so far is unclear about the inactivation of PPO enzymes by discontinuous HHP in fruit juices, some effectiveness has been observed by associating HHPs with heat treatments (Buckow, Weiss, & Knorr, 2009; Sulaiman, Soo, Yoon, Farid, & Silva, 2015). The strong inactivation of PPO by UHPH at 300 MPa opens clear opportunities for the reduction of sulphites in wines, because of the simultaneous antimicrobial and antioxidasic effects.

By applying a continuous treatment of 300 MPa, a > 50% higher antioxidant activity (Trolox equivalents) is observed in the UHPH-processed must (1165 μmol/L) compared to the unprocessed must (745 μmol/L). This increased antioxidant activity is related to a better preservation of the phenolic fraction, as a consequence of PPO

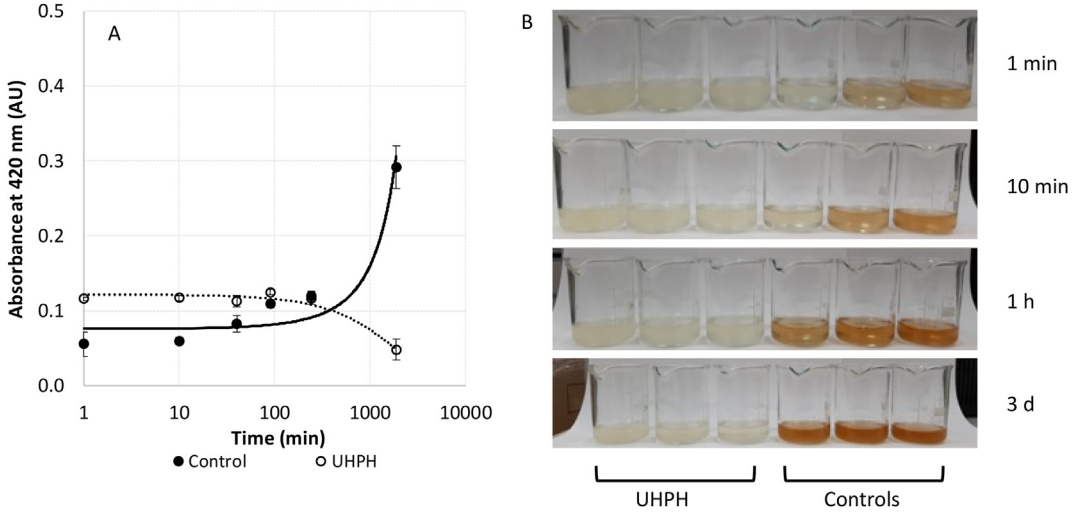

Fig. 2. Absorbance at 420 nm in control and UHPH-processed musts (a) and colour changes by enzymatic oxidative browning in triplicate (b), UHPH-processed (left) and controls (right).

inactivation, and highlights the high efficiency of UHPH treatment at 300 MPa. When UHPH at 200 MPa was used in multi-step mode (1–3 passes) to process mulberry juices, the antioxidant capacity (ORAC value) decreased to 40–30% of the initial value (Yu et al., 2014).

3.5. Oenological parameters and fermentative aroma of the wines

UHPH-treated and control musts fermented during 15 days at 18 °C after inoculation with *S. cerevisiae* in the absence of sulphites. UHPH wines reached a slightly higher alcoholic degree (0.5% vol.) along with the presence of lower residual sugars than the controls (2 g/L) (Table 1). Fermentation can be favoured by the UHPH process through the release of higher levels of YAN (Table 1, Carrau, Medina, Fariña, Boido, & Dellacassa, 2010), which supports the utility of this technique in improving must fermentability. Additionally, the total elimination of yeast and bacteria in the UHPH-treated must helps to produce pure alcoholic fermentation with the inoculated yeast. However, in the controls, since the must microbiota remains during fermentation, a clear development of malolactic fermentation (MLF) can be observed by the simultaneous degradation of malic acid and the formation of lactic acid (Table 1). MLF also decreases total acidity and increases volatile acidity (Table 1), both of which affects the sensory profile of the wine. Undesirable MLF is a typical problem that occurs in warm climates, as it decreases wine acidity and, therefore, affects wine freshness. The use of UHPH helps not only to avoid spontaneous MLF, but also to get a better implantation of non-*Saccharomyces*, which can improve wine freshness by producing both organic acids from sugar metabolism and fresh floral or fruity fermentative aromas (Morata et al., 2020; Morata, et al., 2019).

Fermentative volatiles were quite similar and within the normal range in white wines (Table 3). UHPH wines showed slightly higher contents of isoamyl alcohol and 2-phenylethanol, probably due to the different nitrogen concentration compared to the controls. The higher level of 2-phenylethanol did not influence a higher concentration of 2-phenylethyl acetate ($p < 0.05$). Ester concentrations were higher in control wines mainly due to the ethyl lactate formed as a result of the lactic acid produced by MLF (Table 3). UHPH wines also showed a slightly higher content of acetaldehyde, but in the normal range of white wines (Liu & Pilone, 2000).

3.6. Clarification, colloidal stability and effect of UHPH in protein haze

White wine fining is typically done by using silicates as bentonite, which allows the removal of protein and prevents protein haze in

Table 3
Fermentative volatile compounds (mg/L). Values are mean ± SD, n = 3. Values with the same letter in the same row are not significantly different ($p < 0.05$). Analyses performed by GC-FID.

	Control	UHPH
Acetaldehyde	4.8 ± 1.7b	13.2 ± 0.7a
Methanol	21.0 ± 1.1a	21.7 ± 2.5a
1-propanol	10.3 ± 0.3a	9.8 ± 0.7a
Diacetyl	0.0 ± 0.0a	0.0 ± 0.0a
Ethyl acetate	38.8 ± 0.6a	36.9 ± 1.5a
2-butanol	0.0 ± 0.0a	0.0 ± 0.0a
Isobutanol	12.6 ± 0.2b	14.0 ± 0.4a
1-butanol	0.0 ± 0.0a	1.4 ± 2.4a
Acetoin	5.6 ± 0.4a	5.5 ± 0.1a
2-methyl-1-butanol	86.3 ± 1.7b	116.1 ± 4.7a
3-methyl-1-butanol	22.5 ± 0.9b	28.8 ± 1.6a
isobutyl acetate	0.0 ± 0.0a	0.0 ± 0.0a
ethyl butyrate	0.0 ± 0.0a	0.0 ± 0.0a
Ethyl lactate	23.7 ± 3.8a	3.0 ± 5.2b
2–3 butanediol	655.9 ± 23.8a	679.7 ± 49.2a
isoamyl acetate	4.1 ± 0.1a	3.6 ± 0.3a
Hexanol	0.0 ± 0.0a	0.0 ± 0.0a
2-phenylethanol	38.6 ± 0.9b	62.9 ± 2.0a
2-phenylethyl acetate	6.6 ± 0.2a	6.8 ± 0.0a
Esters	73.2 ± 3.9a	50.2 ± 4.3b
Higher alcohols	170.3 ± 3.9b	233.0 ± 5.2a
Total	930.9 ± 30.7a	1,003.3 ± 49.1a

several white varieties such as Muscat (Lambri, Dordoni, Silva, & De Faveri, 2012; Muhlack et al., 2006). This colloidal fining can be improved by the complementary use of protein products such as gelatine. Phenols responsible for bitterness and browning can be decreased by using PVPP or activated carbon (AC).

UHPH produces a complex and stable colloidal structure by nano-fragmentation of colloidal particles in musts. This effect produces stable nano/submicron suspensions of colloidal pectin and other cellular biopolymers even after fermentation. The stability due to the nano/submicron size strongly decreases the gravity or aggregation forces that produce settling during storage (Dumay et al., 2013).

Even when colloidal turbidity is higher in UHPH wines (14NTU), more than double the turbidity of the controls (6NTU), it can be equally reduced to levels between 3 and 8 NTU after fining with bentonite or bentonite/gelatine (Fig. 3). After these treatments, the wines remained bright for several months. As expected, low effectiveness in removing suspended particles was observed in fining with PVPP and AC.

Before clarification, an intense brown colour could be observed in

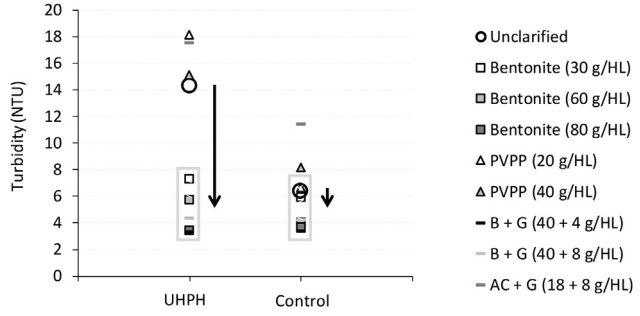

Fig. 3. Turbidity measured after clarification trials with several common fining agents at different doses: bentonite (B), PVPP, gelatine (G) and activated carbon (AC).

the controls compared to UHPH wines in which the PPO were inactivated. However, after fining treatments, browning was reduced in controls. Surprisingly, after a few minutes the wines started to brown again, especially in the wine fraction close to the air contact surface. This is clearly an effect produced by the oxidative enzymes that remain functional even one month after the beginning of fermentation and after the fining treatments (Supplementary figure S5). This effect did not occur in UHPH wines, thus demonstrating the effective protection provided by this technique against oxidation phenomena and supporting the reduction of sulphite doses in the white winemaking process.

In addition, the protein stability test was carried out in all the fining trials, as Muscat wines are very sensitive to protein haze (Pocock & Waters, 2006). Only the trial combining 40 g/L of bentonite with 8 g/HL of gelatine showed protein stability as the variation in turbidity measured before and after the test was less than 2-3NTU. However, it is worth mentioning that all UHPH wines showed less turbidity prior to the protein stability test (Supplementary table S6), so the intense effect on enzymes may also affect the haze-forming proteins and perhaps this technique can help reduce protein instabilities or it could be an alternative cold treatment to denature proteins prior to the application of proteases which is the currently accepted treatment (Marangon et al., 2012). Proteases require a previous thermal denaturalization which directly affects wine aroma quality. In this regard, the use of UHPH can be a solution to this particular problem. Nevertheless, further research is needed to verify this preliminary result.

3.7. Sensory evaluation

The existence of significant differences between the UHPH and control wines was examined by means of a triangular test before the descriptive tasting. 10 out of the 12 judges participating in the tasting were able to differentiate the control wine from the UHPH wine. Therefore, it can be concluded that there are perceptible sensory differences that allow both wines to be clearly identified.

Later, in the attribute difference sensory evaluation of the wines, the main significant differences found between control and UHPH wines corresponded to the attributes perceived in the visual phase. The white control wines obtained higher scores in colour intensity and tonality, which correlates with the analytical data previously described and is indicative of their greater oxidation. However, no notable differences were found in the other parameters evaluated (aroma and taste). Therefore, not only was the sensory quality of the wine not compromised when processing the must with UHPH, but also its visual appearance was improved.

4. Conclusions

UHPH sterilization allows the elimination of indigenous microorganisms in grape juices producing safer fermentations and more stable wines in the long term. UHPH is highly effective in inactivating oxidative enzymes, reducing browning processes and preserving sensory quality. Both effects help to open up new possibilities in the elimination or strong reduction of sulphites in wines, which is a challenge in current oenology. The elimination of indigenous microbiota not only facilitates the implantation of inoculated starters, but also improves the use of new fermentation biotechnologies such as the use of non-*Saccharomyces* yeasts or yeast-bacteria co-inoculations.

CRediT authorship contribution statement

Mª Antonia Bañuelos: Investigation. **Iris Loira:** Investigation, Writing - review & editing. **Buenaventura Guamis:** Conceptualization, Supervision, Writing - review & editing. **Carlos Escott:** Investigation. **Juan Manuel Del Fresno:** Investigation. **Idoia Codina-Torrella:** Investigation. **Joan Miquel Quevedo:** Investigation. **Ramon Gervilla:** Writing - review & editing. **Jesús María Rodríguez Chavarría:** Resources. **Sergi de Lamo:** Resources. **Raúl Ferrer-Gallego:** Investigation. **Rocío Álvarez:** Investigation. **Carmen González:** Writing - review & editing. **José Antonio Suárez-Lepe:** Conceptualization, Supervision. **Antonio Morata:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127417.

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SE SI SK SM TR Designated Extension States: BA ME RS Applicant: Universidad Autónoma de Barcelona 08193 Barcelona (ES).
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