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ORIGINAL PAPER

Grape Processing by High Hydrostatic Pressure: Effect on Microbial Populations, Phenol Extraction and Wine Quality

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Abstract *Vitis vinifera* (variety Tempranillo) grapes were subjected to high hydrostatic pressure (HHP) treatments of 200, 400 and 550 MPa for 10 min, and its effect on microbial populations, phenol extraction and wine quality was examined. At ≥400 MPa, the wild yeast population was strongly reduced from 10^4 to <10 cfu/ml. Bacteria showed greater resistance, and a residual load remained even after the treatment at 550 MPa. The extraction of phenolic compounds from the HHP-treated grapes was improved, with higher concentrations of total phenols obtained compared to crushing alone. Anthocyanin extraction was also increased, producing wines with better colour intensity. These wines also had higher methanol and ethanol contents and returned higher aromatic quality and colour scores. The HHP treatment of grapes may assist in the use of yeast starters, increase phenol extraction from grape skins and improve wine quality.

Keywords High hydrostatic pressure · Red grape · Wine · Microorganisms · Phenols

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Introduction

Certain emerging techniques such as the use of high hydrostatic pressures (HHPs), working at low temperature, and reducing the initial wild population of bacteria, yeasts and moulds, can increase wine quality, reduce wine volatile acidity and prevent undesirable alterations during fermentation and ageing. Further, reducing the numbers of wild microorganisms allows for the use of yeast or bacterial starter cultures designed to ensure alcoholic or malolactic fermentation.

HHP treatment for the control of yeasts, moulds and non-sporulating bacteria (Trujillo et al. 2002; Norton and Sun 2008) is becoming increasingly used in the food industry. It can be performed at low temperature and even under refrigeration. Moreover, the pressure applied during HHP processing is insufficient to break covalent bonds; the molecules responsible for aroma, colour and taste are therefore unaffected, and sensorial quality is well preserved (Yen and Lin 1996; Considine et al. 2008), unlike in thermal treatments (Puig et al. 2003; Mok et al. 2006). Conventional industrial scale treatments involve pressures of 400–600 MPa for 3–10 min (Morata 2010; Buzrul 2012). HHP treatment at 600–700 MPa for 2–10 min can also be used to reduce the oxidation of musts via the partial inactivation of polyphenoloxidase activity (Castellari et al. 1997; Weemaes et al. 1998). This effect can be improved by the simultaneous use of moderate heating (Rapeanu et al. 2005). HHP at low temperature is a good way of controlling wine spoilage microorganisms such as *Brettanomyces*/*Dekkera* yeasts (Morata et al. 2012a). HHP also provides a means of reducing the use of SO_2 in winemaking (Buzrul 2012; Santos et al. 2013) and can be used to accelerate ageing in red wine, helping to avoid the traditional dangers of barrel ageing (Tao et al. 2012).

Along with other emerging technologies, such as ultrasound and the use of pulsed electric fields, HHP has been used to increase the extraction of anthocyanins from grape by-

products (e.g. grape pomace) (Corrales et al. 2008a). Pressurization extracts acylated anthocyanins are better than non-acylated anthocyanins, probably due to modifications in the polarity of the must (Corrales et al. 2009). HHP treatment also increases the extraction of other phenolic compounds, such as phenolic acids, over that achieved in conventional winemaking, although flavan-3-ols are less well extracted (Chen et al. 2012). Pressure may increase or reduce the polymerization of proanthocyanins depending on the composition of the must (Chen et al. 2012). HHP plus heating facilitates the formation of vitisin A derivatives in model media when the appropriate precursors are available (Corrales et al. 2008b), but this also causes the degradation of anthocyanins.

The aim of the present work was to study the effect of HHP treatments on the following: (1) wild yeast and bacterial populations, (2) the extraction of phenolic compounds from grape skins and (3) the fermentative and sensorial quality of the wine obtained.

Materials and Methods

Treatment of Grapes by HHP

Vitis vinifera L. grapes (variety Tempranillo) were manually destemmed, and 300 g was vacuum packed in polypropylene bags. Pressure treatments were performed using a pilot-scale model FPG7100:912C HHP device (Stansted Fluid Power Ltd., Harlow, Exxes, UK) designed to provide of up to 900 MPa at temperatures between −20 and 110 °C. The vessel volume was 2 L; water was used as the compressing fluid. Treatments were performed at 200, 400 and 550 MPa for 10 min. The rates of compression and expansion were 7 and 60 MPa/s, respectively. During the treatments, the pressure vessel was thermostatically controlled at 20 °C (Fig. 1). Two thermocouples were installed to monitor temperature, one near the vessel wall (thermostatically set at 20 °C, controlled by heat exchangers) and the other close to the grapes in the centre of the vessel. The first monitors the status of the required temperature during pressurization, while the second records the increase in temperature caused by the adiabatic heat of compression. Pressure, time and temperature were monitored throughout; data were recorded every second. After treatment, the samples were stored at 4 °C until fermentation. All experiments were conducted in quadruplicate.

Post-HHP Fermentations

After pressurization, the vacuum-packed grapes were examined in a laminar flow cabinet. All but ten grapes (see below) from each packet were placed in sterilized 500-ml Erlenmeyer flasks. They were then gently crushed using a sterile glass stick, and the must was inoculated with 0.1 g of commercial yeast CEG (Lallemand, Montreal, Canada) and fermented isothermally at 25 °C in the absence of SO_2. Samples were taken at day 0, 3, 6, 8, 10 and 13 to follow the development of anthocyanins, colour and total phenols. Volatile compounds were analyzed on day 13 only.

Microbial Populations

Microbiological analyses were performed on grapes following the HHP procedure and on wines at the end of fermentation.

After the HHP treatment, ten grapes (previously vacuum-packed) per treatment were crushed using a sterile glass stick and mixed using a vortex mixer. Tenfold dilutions of the must were prepared in saline peptone, and 1 ml was pour-plated on selective media for the enumeration of total aerobic and lactic acid bacteria (LAB). A further 100 μl was spread-plated for yeast enumeration on glucose chloramphenicol agar (incubated aerobically for 4 days at 25 °C). The media used for growing bacteria were plate count agar (PCA) supplemented with 50 mg/l nystatin after sterilization of the agar (incubations were aerobic and for 3 days at 30 °C) and Man-Rogosa-Sharpe (MRS) agar supplemented with 50 mg/l nystatin after sterilization of the media (incubations were anaerobic and for 4 days at 30 °C). All the media were from Pronadisa (Barcelona, Spain).

The same plating methodologies and media were used to determine the bacterial and yeast counts of the wines at the end of fermentation.

Determination of Anthocyanins

Grape anthocyanins and pyranoanthocyanins were determined by HPLC-DAD-ESI/MS according to Morata et al. (2012b). Briefly, solvent A (water/formic acid, 95:5, *v*/*v*) and B (methanol/formic acid, 95:5) gradients were used in an RP C18 column (100×4.6 mm; 2.6 μm) as follows: 0–27 min, 20–50 % B linear (0.8 ml/min); 27–28 min, 50 % B; 28–29 min, 50–20 % B linear; and 29–30 min, re-equilibration. Detection was performed by scanning in the 500–600-nm range. Quantification was performed by comparison against an external standard at 525 nm and expressed as milligram per litre of malvidin-3-glucoside (r^2=0.9999). Anthocyanins were identified by their retention times and by comparing their UV–visible and mass spectra with data in the literature. Mass spectrometry was performed in positive scanning mode (*m*/*z* 100–1,000, fragmenter voltage 150 V from 0 to 23 min). One hundred-microlitre samples of previously filtered (0.45 μm membrane) wines were injected into the HPLC apparatus. The detection limit was 0.1 mg/l.

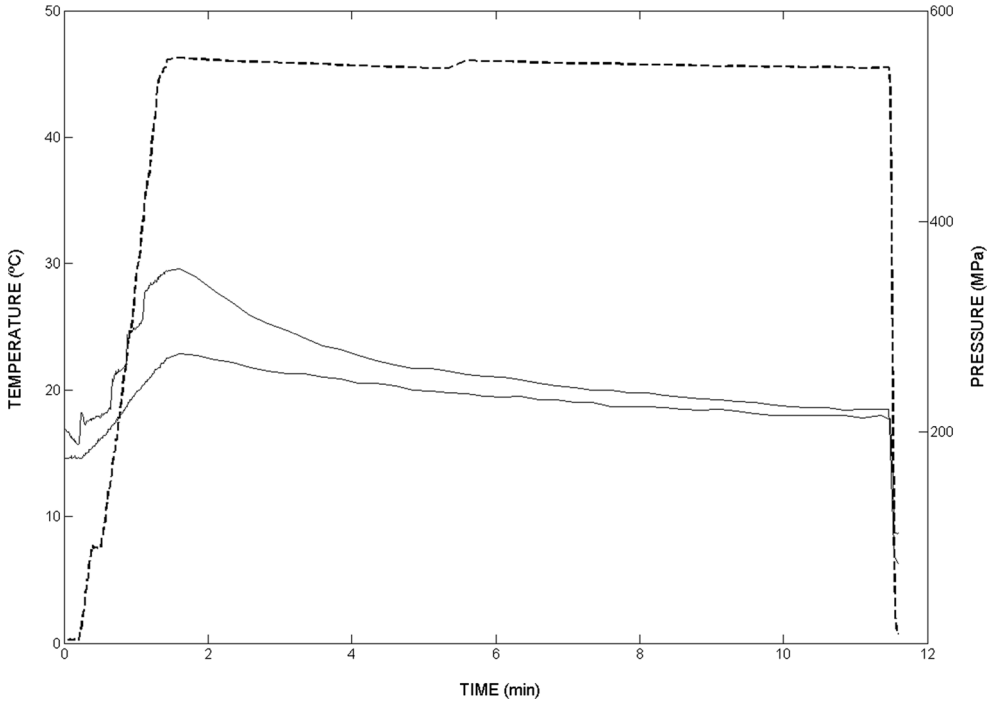

Fig. 1 Pressure and temperature monitoring during HHP treatment at 550 MPa. Temperature was measured by two thermocouples inside the pressure vessel. *Dashed line* pressure, *continuous lines* temperatures

Analysis of Volatile Compounds by GC-FID

Volatile compounds were determined using an Agilent Technologies 6850 gas chromatograph (Network GC System) equipped with an integrated flame ionization detector (GC-FID), as described by Abalos et al. (2011). A DB-624 column (60 m×250 μm×1.40 μm) was used. The following compounds were used as external standards for calibration (r^2>0.999): acetaldehyde, methanol, 1-propanol, 1-butanol, 2-butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethyl acetate, 2-phenylethyl alcohol, diacetyl, acetoin, ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl butyrate, ethyl lactate and hexanol. 4-Methyl-2-pentanol was used as an internal standard. All compounds were purchased from Fluka (Sigma-Aldrich Corp., Buchs SG, Switzerland). The injector temperature was 250 °C, and the detector temperature was 300 °C. The column temperature was 40 °C (5 min), rising linearly by 10 °C/min until 250 °C; this temperature was then held for 5 min. Hydrogen was used as the carrier gas. The injection split ratio was 1:10, the in-column flow rate 2.2 l/min and the detection limit 0.1 mg/l. One hundred microlitres of internal standard (500 mg/l) was added to 1-ml test samples and filtered through syringe membrane filters (pore size 0.45 μm) (Teknokroma, Barcelona, Spain). They were then placed in 2-ml glass vials sealed with a PTFE/silicon septum. One microlitre of this filtrate was injected into the GC apparatus. 3-Ethoxy propanol was identified by GC-MS under the same chromatographic conditions.

Analysis of Acetic Acid and Residual Sugars

Glycerol, acetic acid and residual sugars were measured enzymatically using Y15 Enzymatic Autoanalyzer (Biosystems, Barcelona, Spain).

Ethanol Quantification

Ethanol was analyzed by liquid chromatography with refractive index (LC-RI) detection using a Waters e2695 apparatus (Milford, Massachusetts, USA) equipped with a 2414 Refractive Index Detector. Analyses were performed using a reverse-phase Phenosphere XDB C18 column (4.6×150 mm, 5-μm particle size) (Phenomenex, Torrance, CA, USA). The solvent was Milli-Q water (used in isocratic mode); the flow rate was 0.4 ml/min. The temperature was set at 30 °C in the column and detector. Calibration was performed using an external ethanol/glucose standard (Panreac, Barcelona, Spain). Samples were injected after filtration through 0.45-μm cellulose methyl ester membrane filters (Teknokroma, Barcelona, Spain). The injection volume was 2 μl.

Colour and TPI

Wine absorbance at 420, 520 and 620 nm was determined using a V-530 spectrophotometer (JASCO, Tokyo, Japan) and a 1-mm path-length quartz cell, following the procedure of Glories (1984a, 1984b). Both colour intensity and hue were recorded. The total polyphenol index (TPI) was determined

spectrophotometrically by measuring the absorbance at 280 nm in a 1-cm path-length quartz cell after 1:100 dilution with Milli-Q water.

Sensorial Analysis

Wines were evaluated by an experienced panel of six judges, according to Loira et al. (2013). Briefly, the panellists used a scale from 0 to 10 to rate the intensity of different attributes (0= attribute not perceptible, 10=attribute strongly perceptible). Each panellist also provided an overall impression of the wines produced, taking into account olfactory and taste features, including any defects.

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 $p<0.05$.

Results and Discussion

Effects of HHP on Berry Structure

None of the tested pressures had any effect on berry shape or external appearance (Fig. 2a). Similar findings have been reported for HHP-treated strawberries and cherry tomatoes (Terefe et al. 2009; Iizuka et al. 2013). HHP is applied using a pressurized liquid, normally water, which transmits the pressure isostatically. Thus, equal pressure from all directions compressed the fruit, which returned to its initial shape once the pressure was released. However, the surface of the HHP-treated grapes was brighter than the slightly dusty surfaces of the controls. The Tempranillo variety, and indeed most of the grapes used in oenology, has no pulp or seed pigments; anthocyanins are found only in the skin cells. After treatment, several grapes were crushed by hand to detect any macroscopic effects of HHP on the pulp. Figure 2b shows that the pulp of these grapes had become stained; no pigments were detected in the pulp of control grapes. It would appear that the HHP treatments damaged the grapes' skin cells, causing the migration of anthocyanins (and probably other phenols and aromatic compounds) towards the pulp. Figure 2c shows the seeds of several grapes before the beginning of the fermentation and maceration. The external surface of the seeds from the HHP-treated grapes was clearly stained. Anthocyanins therefore migrate right across the pulp.

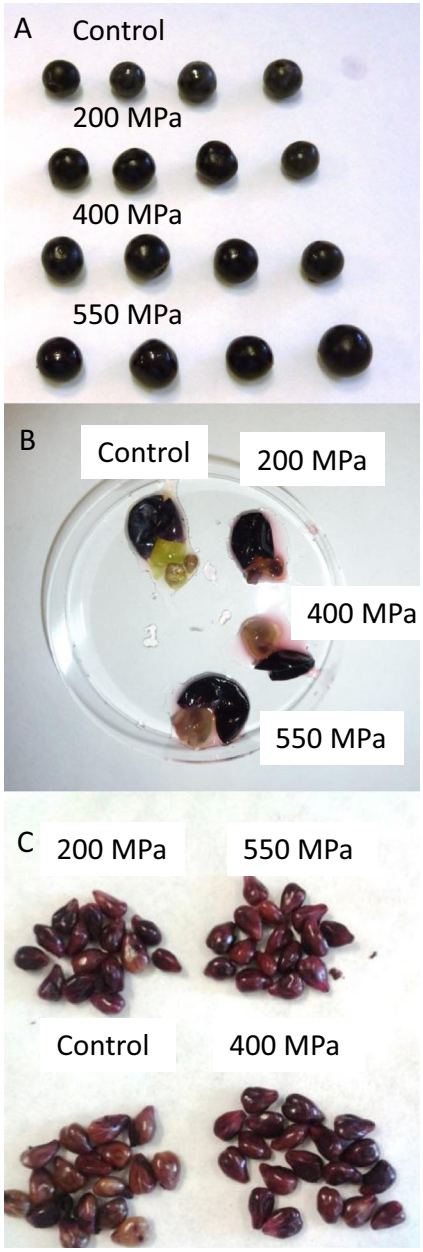

Fig. 2 Effect of HHP treatments on the following: **a** external structure and shape of the grapes, **b** diffusion of anthocyanins towards pulp and **c** anthocyanin adsorption on seeds

Grape Microbial Populations After HHP Treatments and in Wines at the End of Fermentation

Yeast counts for control grapes returned an initial wild population of 4 log colony-forming units (cfu)/ml. HHP treatment at 200 MPa reduced this initial population tenfold. No yeasts were detected at all when the grapes were pressurized at 400 MPa or higher (Fig. 3a). The bacterial populations counted on PCA and MRS showed a similar trend with pressurization (probably because the same types grew in both media). The initial bacterial population was close to 2 log cfu/ml; after

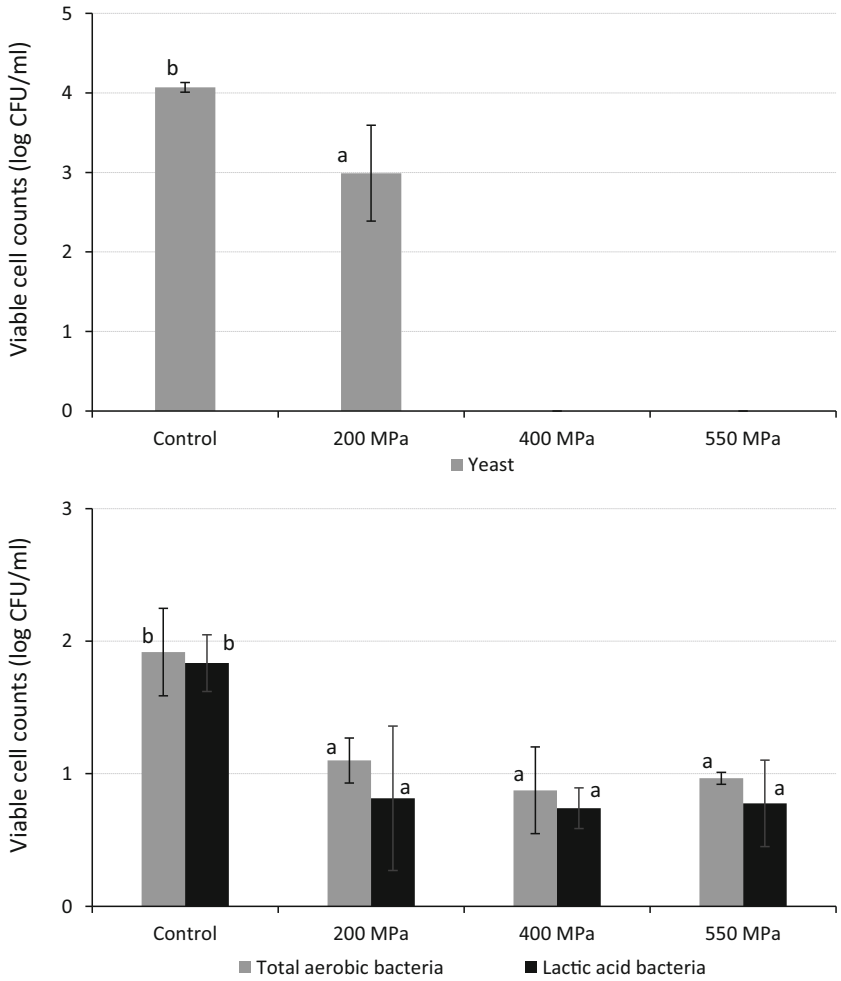

Fig. 3 Microbial counts in crushed control and HHP-treated grapes at the beginning of fermentation. **a** Yeast. **b** Bacteria. Values are means±standard deviations of four replicates. *Different letters* in the same series indicate significant differences between means ($p<0.05$)

pressurization at 200, 400 and 550 MPa, the counts fell to a mean of near 1 log cfu/ml (the samples treated at 200 MPa showed a higher standard deviation than those treated at 400–550 MPa) (Fig. 3b).

These results agree with those reported by Shimada et al. (1993), who indicated that *Saccharomyces cerevisiae* cell wall disruption starts at pressures above 400 MPa. In fruit juices, reductions of 3 log cfu/ml have been observed in *S. cerevisiae* after treatment at 250 MPa for 18 min (Basak et al. 2002); after treatment at 600 MPa for 7 min, yeast populations fell to undetectable levels from initial figures of 10^7 cfu/ml (Marx et al. 2011).

The present bacterial counts were reduced to 1 log cfu/ml. LAB are the bacteria most commonly associated with grapes, with up to ten species belonging to the *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* genera (Lonvaud-Funel 1999). LAB are Gram-positive bacteria, and it is well known that these are more resistant to HHP than Gram-negative bacteria (Hayakawa et al. 1994) (they can withstand 500 to 600 MPa compared to 400 MPa for 10 min at 25 °C) (Trujillo et al. 2002). The resistance of LAB to HHP is due to the configuration of the cell wall, which has a thick layer of peptidoglycan plus rigid teichoic acids (Lado and Yousef 2002).

After inoculation, fermentations were allowed to proceed for 13 days at 25 °C. All fermentations finished with <2 g/l of residual sugar. The volatile acidity remained below 0.6 g/l in all cases. After fermentation, the yeast populations in both the HHP-treated and control wines were in the range of 7–8 log cfu/ml, with no significant differences between them. Bacterial counts at the end of fermentation for the control treatment and the HHP treatments on PCA were 1 log cfu/ml and approximately 0.5 log cfu/ml on MRS. The elimination of wild yeast populations at pressures of >200 MPa, plus the reduction of bacterial numbers at all pressures, provides a way for reducing the amount of SO_2 used in wine production and facilitates the use of commercial starters. This might be particularly important for red wines which usually have a high initial wild population of bacteria and yeasts given that the grape skins remain present over fermentation. Moreover, the high polyphenolic compound content of red wines increases their antioxidant capacity, reducing the need for external

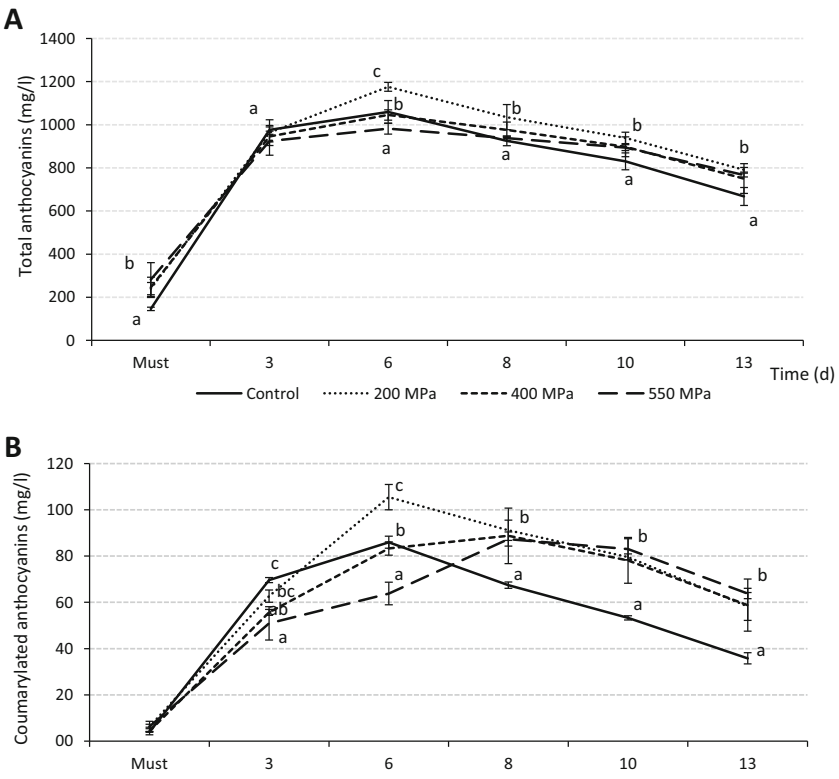

Fig. 4 Anthocyanin extraction over fermentation. **a** Total anthocyanins. **b** Coumarylated anthocyanins. Values are means± standard deviations of four replicates. *Different letters* in the same series indicate significant differences between means ($p<0.05$)

antioxidants such as SO_2. Additionally, HHP might be employed to facilitate the use of selected strains of *Saccharomyces* that produce low ethanol yields (Loira et al. 2012) or the use of mixed/sequential fermentations with aerobic non-*Saccharomyces* yeasts (Contreras et al. 2013).

Anthocyanins

Before fermentation, the mean total anthocyanin content was 146.6 mg/l for the control grapes and 240–282 mg/l for the HHP-treated grapes. HHP affected the structure of the skin cell walls and significantly increased the extraction of anthocyanins in the must after crushing the grapes and before maceration (Fig. 4a). No differences were seen in the amount of anthocyanins extracted from the grapes treated at 200, 400 or 550 MPa; i.e. the mechanical effect of HHP with respect to anthocyanin extraction was already at its maximum at the lowest pressure (200 MPa). During fermentation, anthocyanin extraction increased, reaching a maximum on day 6 in all fermentations; the values then fell since, as the ethanol concentration increased and the polarity of the medium therefore decreased, the solubility of these molecules diminished. At the

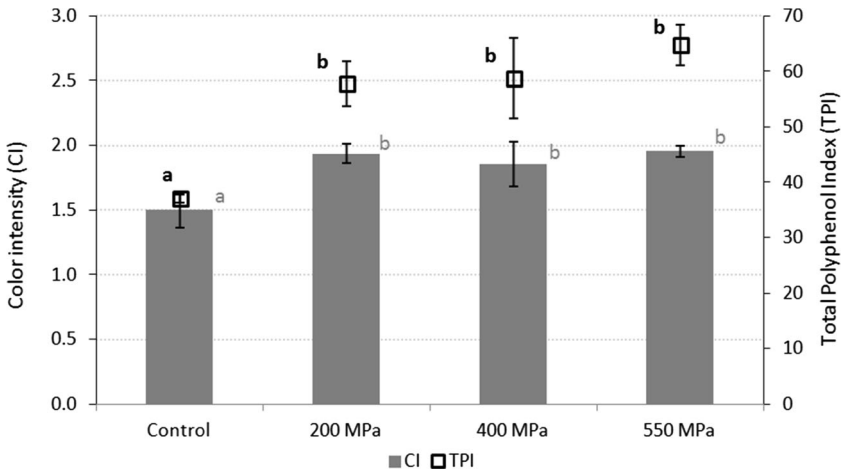

Fig. 5 Colour intensity and total polyphenol index at the end of fermentation (day 13). Values are means±standard deviations of four replicates. *Different letters* in the same series indicate significant differences between means ($p<0.05$)

end of fermentation, all the wines made from the HHP-treated grapes showed significantly greater concentrations ($p<0.05$) of anthocyanins than the controls.

Before fermentation, no differences were seen between the control and HHP treatments with respect to the concentration of *p*-coumarylated anthocyanins ($p>0.05$); indeed, <9 mg/l was recorded for all samples. Nevertheless, its extraction proceeded over fermentation, reaching higher concentrations each day in all fermentations but especially in those involving pressurized grapes. In fact, from day 8 until the end of the fermentation, the latter showed significantly higher concentrations of *p*-coumarylated anthocyanins than did the controls (Fig. 4b). Moreover, by the end of the fermentation, their average content was 68 % higher than that of controls ($p<0.05$). Figure 4b shows a larger proportional extraction for *p*-coumarylated anthocyanins compared to total anthocyanins in wine samples made from pressurized grapes. This finding agrees with that reported for acylated anthocyanins when grape by-products are pressurized (Corrales et al. 2008a). The extraction of acylated anthocyanins, which have a less polar structure than non-acylated anthocyanins, can be explained by a reduction in the pH of the grape tissues by

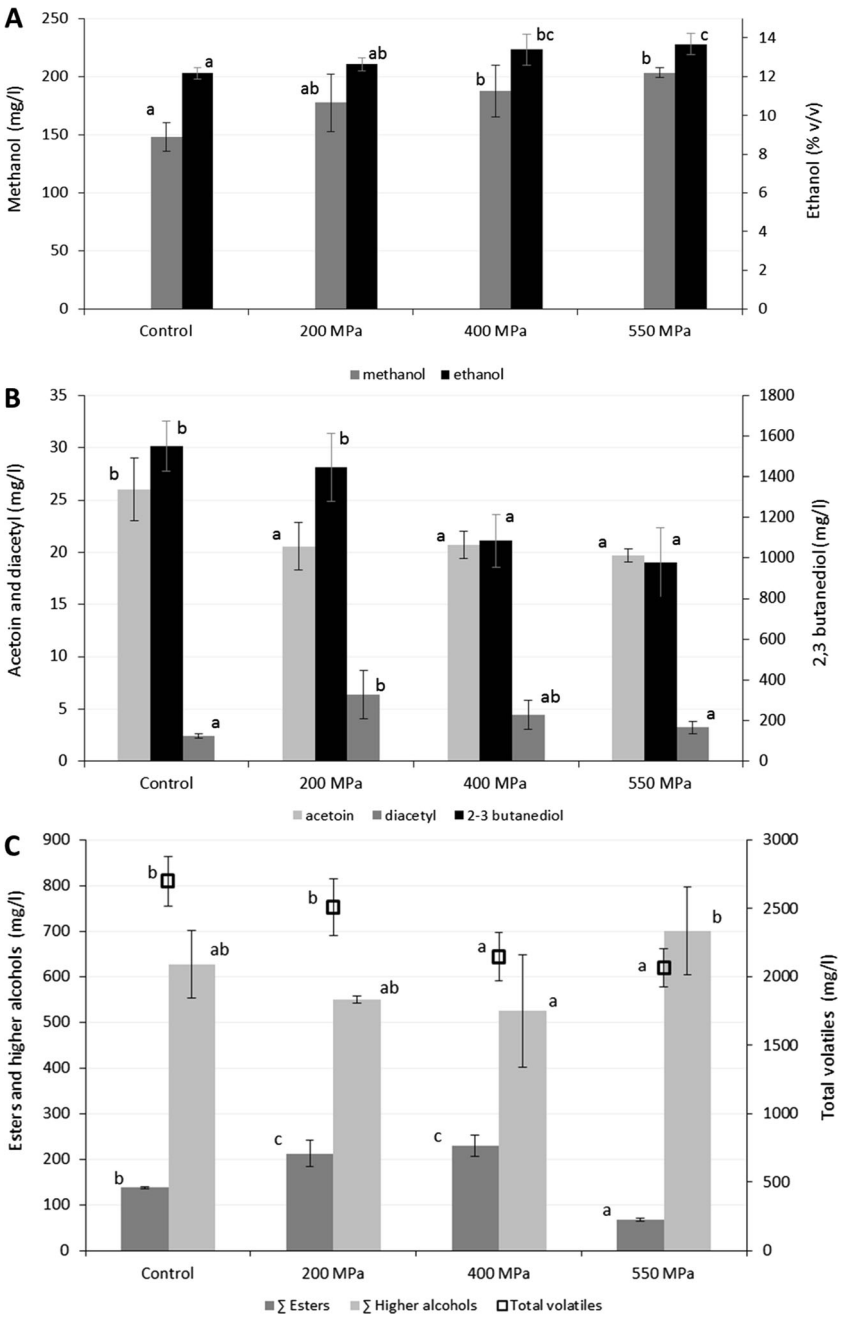

Fig. 6 Volatile compounds: **a** Methanol and ethanol. **b** Acetoin, 2,3-butanodiol and diacetyl. c. Esters, higher alcohols and total volatiles. Values are means± standard deviations of four replicates. *Different letters* in the same series indicate significant differences between means ($p<0.05$)

HHP as it exerts its effect on the polarity of the must (Mathys et al. 2008; Corrales et al. 2008a). *p*-Coumarylated anthocyanins are of interest in the production of wine colour since their maximum absorbance in the visible spectrum is at a higher wavelength than that of other non-acylated and acetylated anthocyanins (535 nm), providing desirable red-bluish colours (Monagas et al. 2003).

Colour and TPI

The larger amount of total anthocyanins extracted from the pressurized grapes at the end of fermentation produced an increase in colour intensity (CI) of 26 % over the control wines (Fig. 5), with no significant differences among the HHP treatments ($p<0.05$). The mean TPI value was 37.0 for the control wines and ranged from 53.2 to 68.9 in wines made from HHP-treated grapes. Once again, no differences were found among the HHP treatments ($p<0.05$). The maximum CI was reached on day 6 of fermentation, but the TPI increased daily in all the samples, reaching its maximum value at the end of fermentation, as is usual in red wine maceration.

The greater extraction of phenolic compounds occasioned by HHP increases the anti-oxidative properties of wine and could therefore reduce the use of SO_2. Together with the reduced populations of wild yeasts and bacteria achieved, this could be a good way to produce wines using less of this antimicrobial and antioxidative additive.

Volatile Compounds

Unlike that observed for the phenolic compounds, no relationship was seen between volatile compounds produced during fermentation and the effect of pressure. As expected, some influence of pressure was seen on methanol given its production from the hydrolysis of pectins in grape cell walls. Figure 6a reflects the greater methanol content of the wines made from HHP-treated grapes, although differences with control wines were only significant for pressures of 400 MPa and higher. These results agree with those of previous studies on cloudy apple juice (Baron et al. 2006) and onions (Gonzalez et al. 2010). An increasing alcoholic content was seen with pressure, with significant differences ($p<0.05$) appearing between controls and the 400 and 550-MPa treatments. These differences are probably due to the greater extraction of sugars from non-accessible cell structures towards the must, a consequence of the pressure exerted. No residual sugars were detected in either control or low-pressure (200 MPa) treatment.

Smaller amounts of acetoin were formed in the wines produced from all pressurized grapes. Smaller contents of

Fig. 7 Spider net graph of taste panel results. Values are means for eight independent tasters. *Different letters* in the same series indicate significant differences between means ($p<0.05$)

2,3-butanediol were also detected, but only at pressures of 400 and 550 MPa (Fig. 6b). Volatile compounds in wines from the HPP-treated grapes would mainly have been produced as metabolic compounds produced by the dry yeast used as a starter. Similarly, the higher concentrations of 2,3-butanediol in the control and 200-MPa treatments might be due to the wild microbial population. No clear trend was seen with respect to diacetyl content, which showed slightly higher concentrations in wines made from grapes pressurized at 200 MPa, while those made from grapes treated at higher pressures had lower diacetyl contents than the control wines. Moreover, total volatile compounds were lower in wines from grapes treated at pressures of 400–550 MPa (Fig. 6c).

Sensory Analysis

Figure 7 shows that the panel recorded a better global perception of the wines produced from the HHP-processed grapes, largely a consequence of their better aromatic quality and more fruity profiles. The panellists also detected a stronger CI of the wines made from the HHP-treated grapes. The greater extraction of phenols in these wines was not perceived by the tasters as any significant difference in astringency, bitterness or tannicity.

Conclusions

HHP treatment of grapes at 200 MPa for 10 min at room temperature is enough to increase phenol extraction during must fermentation. Pressures of 400–550 MPa are needed, however, to reduce the wild microbial populations, but even under these conditions, residual populations of bacteria persist. HHP processing could be a way of producing low SO_2 wines, the microbiological control afforded by this additive being substituted by pressure.

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