Use of high hydrostatic pressure (HHP) to obtain an ingredient rich inbioactive compounds from cv. Tempranillo red pomace

M. Esperanza Valdés[1,*], Matilde D'Arrigo[1], Jonathan Delgado[1], Javier Rocha Daniel Moreno[1], M. Jesús Mateos[1] y Rosario Ramírez[1]

[1]Instituto Tecnológico Agroalimentario de Extremadura (CICYTEX_INTAEX). Avda Adolfo Suárez s/n. 06071 Badajoz

Abstract. With the aim to obtain an ingredient rich in bioactive compounds to be used in meat products, a red grape pomace (RGP) cv. Tempranillo, was subjected to the application of different hydrostatic high pressure (HHP) treatments: i) 600 MPa/1s; ii) 600 MPa/300s and two others treatments of 2 cycles of HHP, iii) 2 cycles of 600MPa/1s and iv) 1 first cycle of 400 MPa/1s and second cycle 600 MPa/1s. The microbiological populationand polyphenoloxidase activity (PPO) of treated RGP was studied immediately after the treatments and at 270 days after the treatments at temperatures of 4 and 20 ºC respect a control sample untreated. The treatments significantly reduced the microbial population; the effect of HHP3 and HHP4 (two cycles) did not differ from those of HHP1 and HHP2 (1 cycle). Phenolic compounds (anthocyanins, flavanols, flavonols, phenolic acids and stilbenes) were extracted from GRP, identified and quantified by HPLC. The values of all these phenolic families were maintained immediately after HHP with exception of a decrease of anthocyanins in HHP3. However, the HHP treatments did not affect the polyphenoloxidase enzyme, since the phenolic compounds were notably reduced during storage although phenolic compounds were better well-preserved at refrigeration than at room temperature.

Resumen. Con el objetivo de obtener un ingrediente con propiedades antioxidantes para ser empleado en productos cárnicos, un orujo de uva tinta (RGP) cv. Tempranillo, se sometió a diferentes tratamientos de alta presión hidrostática (HHP): i) 600MPa/1s; ii) 600MPa/300s, iii) 2 ciclos de 600MPa/1s y iv) 1 primer ciclo de 400MPa/1s y segundo ciclo 600MPa/1. El efecto sobre la población microbiana, la actividad polifenoloxidasa (PPO) se investigó tras los tratamientos y tras 270 días a 20 ºC y a 4 ºC frente a una muestra control sin tratar (C). Los tratamientos redujeron significativamente la población microbiana; el efecto de HHP3 y HHP4 (dos ciclos) no difirió de los del HHP1 y HHP2 (1 ciclo). Se procedió a la extracción y posterior identificación y cuantificación mediante HPLC de antocianinas, flavanoles, flavonoles, ácidos fenólicos y estilbenos del RGP. Tras la aplicación de HHP se observó un descenso significativo de antocianos en HHP3 y una tendencia al alza de los polifenoles totales en HHP3 y HHP4. Ninguno de los tratamientos logró inactivación de PPO, y los valores de todas las familias fenólicas se redujeron de forma notable y significativa durante el almacenamiento. Los descensos fueron significativamente menores en las muestras conservadas a 4 ºC que a temperatura ambiente (20 ºC).

1 Introduction

Currently, by-products from wine making industries have a high economic and environmental impact, near of 25% of them is mainly grape pomace [1]. The seeds and skins remaining after the fermented must-wine pressing operations constitute the red grape pomace (RGP), which is rich in bioactive substances as polyphenols and soluble fiber with antioxidant, antimicrobial and anti- carcinogenic effects [2]. Moreover, the content and phenolic profile of the RGP depends on different factors as variety, year, viticulture techniques and the extraction kind of the phenolic fraction [3,4].

Into grape pomace, skins are rich in anthocyanins, a group of polyphenols well-known for their beneficial properties [5]. Resveratrol, flavonoids, procyanidins, and phenolic acids from grape seeds give them antioxidant and microbiological activity. The pomaces barks have a great potential to eliminate free radicals, closely linked to the structure of stilbenes, flavonols, and anthocyanins [6]. The use of grape pomace in food products could reduce discoloration and lipid oxidation during life storage due to their anthocyanins and other phenolic compounds.

These properties are currently leading to research into the use of GRP as antioxidants in the preparation of other foodstuffs. Specifically, at the Agrifood Technology Institute of Extremadura (INTAEX) belonging to the Centro de Investigaciones Científicas y Tecnológicas de la Junta de Extremadura (CICYTEX), our recent works are being carried out to use them as substitutes for nitrites in

*Corresponding author: esperanza.valdes@juntaex.es.

meat preparations natural, easyto prepare and to preserve over time.

The hydrostatic high pressure (HHP) processing is a non-thermal emergent technology for food facilities, giving new type of preservation. HHP subjects' products to pressures between 400 and 600 MPa instantly, uniform and independent of size and geometry. This is considered a green technology because is energy fully efficient, replacing traditional preservation technologies with others that avoid the negative consequences of heat treatments [7,8]. In addition, HHP maintains the levels ofcompounds with antioxidant activity, increasing the extraction of compounds without the overuse of solvents [9].

HHP technology is gaining more relevance in the food industry not only for the above reasons but also it allows a reduction/inactivation of spoilage and pathogenic microorganisms in food equivalent to pasteurization and is presented as one of the technologies of interest for the valorization of by-products of the wine industry [8]. Recent researchs conducted by our research group [10] suggested the possibility of applying two cycles to increase the inactivation of the resistance forms of microorganisms, the spores. The first cycle would activate spore forms, for later, the forms vegetative cells would inactivate them in a second consecutive cycle. This multiple-cycle application in HHP has been scarcely evaluated for food processing, especially for vegetable products.

It is known that polyphenoloxidase (PPO) is present in some fruits as red flesh and peel plum purée and grape pomace [11]. This enzyme is responsible for enzymatic browning reactions and reduce bioactive compounds. So, its inactivation is essential for the enzymatic stabilization of these products. However, because of incomplete inactivation of certain microorganisms and enzymes such as polyphenoloxidase (PPO), it is sometimes necessary to apply physical or chemical pretreatments (blanching, incorporation of additives, etc.) prior to HHP to achieve complete inactivation of these enzymes [12].

Under these premises, the principal aims of this work are a) to characterize the composition of GRP cv. Tempranillo; b) to study the effect of one and two cycles of HHP on the microbial effectiveness of the process, the PPO activity, the phenolic profile and total phenolic content and, c) to investigate the effect of storage temperature on these parameters.

2 Materials and methods

2.1 Red Grape Pomace cv. Tempranillo. Sampling and preparation

RGP cv. Tempranillo were provided by Santa Martade los Barros Coop. (Badajoz, Spain) in September 2020. RGP was vacuum-packaged in 1 kg-plastic bags and stored at -80 ºC. Then, frozen RGP was ground in Thermomix TM5 (Thermomix-Vorwerk, Madrid, Spain), at maximum speed for around 3 minutes, and mash-like product was obtained. Subsequently, the milled pomace was homogenized and packed in 50 g vacuum bags and stored at -80 ºC until the application of the HHP treatment.

2.2 Experimental Design

The RGPs vacuum packaged pomace were processed in semi-industrial equipment (6000/55, Hiperbaric, S.A., Burgos, Spain) with 55 L capacity container and the initial temperature of the water was 16 °C. The equipment is located in CICYTEX-INTAEX center.

Four different treatments were applied.
i) Non treated (Control)
ii) 600 MPa/1s
iii) 600 MPa/300s
iv) 2 cycles of 600 MPa/1s
v) 1 first cycle of 400 MPa/1s and second cycle 600 MPa/1s.

When the two cycles were applied, the first and the second cycle were separated for 3 hours and 30 minutes.

In order to evaluate the stability of RGP after HHP, three samples (bags) from each HHP-treatment and control were stored for 270 days at refrigerated storage (4 ºC) or at room (20 ºC) temperatures both in darkness.

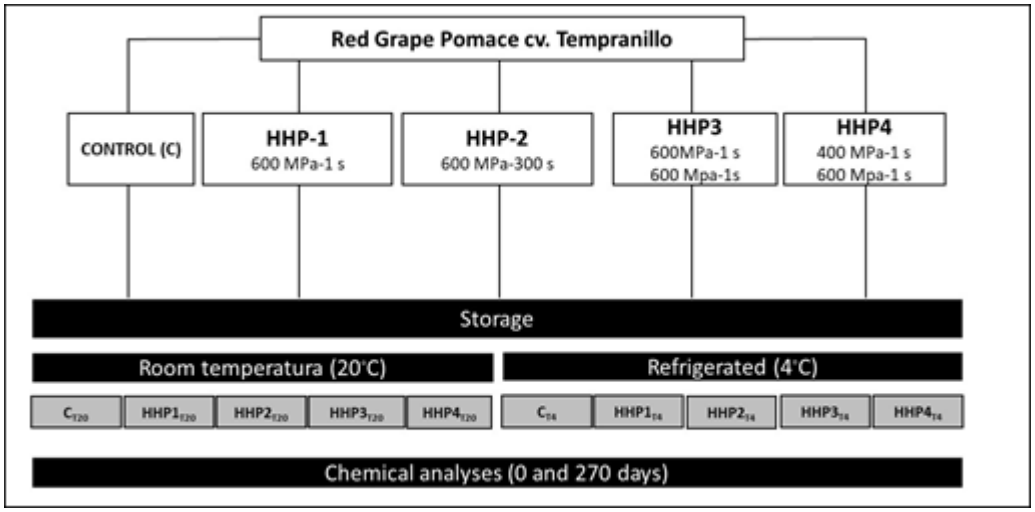

Figure 1. Experimental Design.

2.3 Microbiological determinations

For microbiological analysis, 10 g of RGP was aseptically weighted in sterile plastic bag and homogenized with 90 mL of sterile solution peptone water (Merck, Darmstadt Germany) in masticator blender (Stomacher 400 Circulator), 1/10 dilution. Serial 10-fold dilutions were prepared by mixing 1 mL of the previous dilution with 9 mL of sterile peptone water. Total viable counts were enumerated in Plate Count Agar (PCA; Merck, Darmstadt Germany) and incubated at 30 °C for 72 hours; moulds and yeasts were enumerated using CG Agar Base (Merck, Darmstadt, Germany) with CG Selective Supplement (Merck, Darmstadt, Germany), and incubated at 25 °C for 4-5 days and Enterobacteriaceae (VRBG Agar, 37ºC, 24-48 hours). After incubation, plates with 30-300 colonies were counted. All microbial counts were expressed as log of colony-forming units (CFU) per g of sample weight (log CFU g^{-1}).

2.4. Polyphenoloxidase (PPO) enzyme activity.

Extraction and enzyme activity analysis was carried out as described by Terefe et al. [13]. Absorbance was measured at 420 nm and 25 °C for 3 minutes in Thermo Scientific Evolution 201 UV Vis spectrophotometer (Fisher Scientific SL, Madrid, Spain), in kinetic mode. The results were expressed as percentage of activity with respect to the control samples.

2.5 Extraction, identification and quantification of phenolic compounds from red grape pomace

Polyphenolic substances of 25 g of RGPs were extracted with 50 mL of methanol/water/formic acid (50:48.5:1.5, v/v/v). This mixture was homogenized (180 W Moulinex grinder, Alençon, France), sonicated during 10 min. at 50 Hz (Grant XUB5, Cambridge, England) and centrifugated at 5000 rpm during 10 min (Allegra 25R, Beckman Coulter, Delaware, USA). The supernatant was separated, and the resulting pellet was extracted up to three times. Supernatants obtained were combined in a flask and the volume was brought to 100 mL with the extraction mixture and stored at -20 °C until analysis. Three extractions were performed for each sample. For the analysis of anthocyanin substances (ANT), the extract was injected directly into the HPLC. The isolation of non-anthocyanin compounds was carried out based by passing the extracts through PCX SPE cartridges, (Chromafil PET 20/25, Machery-Nagel, Düren, Germany). The non-anthocyanin phenolic substances were eluted with 3 x 5 mL of methanol and the eluate containing flavanols (FLAVA), flavonols (FLAVO), phenolics acids (AC) and stilbenes (ST) was separately dried in a rotary evaporator (40 °C) and redissolved in 1.5 mL of 20 % (v/v) methanol aqueous solution.

Identification and quantification of phenolic compounds were performed by HPLC analysis. An Agilent 1200 LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with UV-Vis diode-array detector (DAD), fluorescence spectrophotometer detector (FLD), and the Chemstation software package for LC 3D systems (Agilent Technologies, Palo Alto, CA, USA), to control the instrument and for data acquisition and analysis, were used. Separation was performed in a Licrospher® 100 RP-18 reversed-phase column (250 × 4.0 mm; 5 µm packing; Agilent Technologies, Palo Alto, CA, USA) with pre-column Licro-spher® 100 RP-18 (4 × 4 mm; 5 µm packing; Agilent Technologies, Palo Alto, CA, USA). 20 µL of each sample in triplicate were injected and chromatographic conditions were based on those described by Castillo-Muñoz et al. [14], with the following eluents used: (A) acetonitrile/water/formic acid, (3:88.5:8.5, v/v/v), (B) acetonitrile/water/formic acid (50:41.5:8.5, v/v/v), and (C) methanol/water/formic acid (90:1.5:8.5, v/v/v) and the column was maintained at 40 °C.

Phenolic compounds were identified by their elution order and by comparison with the retention times of commercially available standards. The diode array detector was employed on four wavelengths: 220 nm for identification of the flavanols (FLAVA), 320 nm for stilbenes (resveratrol) (ST) and phenolics acids (PA); 360 nm for flavonols (FLAVO) and 520 nm for ANT compounds. Excitation at 280 and emission at 320 nm were measured by FLD for identification of the following compounds: (+)-catechin and pro- cyanidin B2. For quantification and calibration of each compound, calibration curves of their respective standards ($R2$ > 0.999) were used. Quantification of non-commercial compounds was made using the straight calibration compound belonging to the same family which was next in the order of elution.

The total amount of AN was given in mg of malvidine-3-glucoside·kg^{-1} fresh berry weight (FW). FLAVA were quantified as mg of (+)-catechin·kg^{-1} FW, while FLAVO as mg of quercetine-3-glucoside·kg^{-1} FW and phenolic acid as caffeic acid·kg^{-1} FW. Total phenolic compounds were calculated as ∑ (AN, FLAVO, FLAVA, AP and ST).

2.6 Statistical analysis

A two-way ANOVA was used to investigate the influences of treatment, storage temperature and their interaction on each parameter. One way-ANOVA was applied to test the effect treatment on a specific storage temperature. When ANOVA indicated significant differences among the treatments for the same storage temperature, the Tukey test ($p < 0.05$) was applied to compare mean values. Finally, to evaluate the effect of storage for a given parameter and treatment, statistical comparisons between mean values were established with Student's test. The data were analyzed by using XLstat- Pro (2011 Version, Addinsoft, Paris, France).

3 Results and Discussion

3.1 Effect of treatments at initial time

3.1.1 Microbial load population and polyphenoloxidase (PPO) activity

Table 1 displays the significant effect of treatments against the growth of mesophilic, moulds, yeast and Enterobacteriaceae ($p < 0.05$) immediately after the HHP treatments (0 days). Any treatments reduce to one logarithmic (CFU g^{-1}) in molds and yeast and Enterobacteriaceae. Our data are in agree whit Morata et al. [15] who reported that the counts fell to a mean of near 1 log $CFUg^{-1}$ (samples treated at 200, 400, 500 Mpa). García-Parra and Ramírez [8] indicated that Gram negative microorganisms are the most sensitive to HPP, followed by yeasts and molds, whose inactivation is reached by applying pressures of 300-400 MPa at room temperature, then Gram positives and finally spores can survive pressures up to 1000 MPa.

The lack of statistical differences ($p > 0.05$) on PPO activity (Table 1) for RGP treated with HHP confirms that some products derived from waste from the wine industry with high PPO content are not affected by this treatment. Therefore, a pre-treatment is necessary, to control enzymatic browning by removal or transformation of the substrates (oxygen and phenols) or by reducing the pH below the range of activity by addition of organic [11]. González- Cebrino et al. [11] shown a positive effect when a "Songold" Plum purée was tried by thermal blanching and 600 MPa in the maintenance of the polyphenols than the other purees. This allows us to confirm the need for bleaching before treatment to preserve not only the polyphenol content but also its profile.

3.1.2 Phenolic composition

At initial time (0 days), respect to Control, the application of HHP treatments had not a significant impact on the values of FLAVA, FLAVO, PA ST and Total Phenolic compounds (TPC). Only significant decreases were observed in ANT of -HHP3 samples.

Table 1. Effect of the HHP treatments on Microbial counts (log colony forming units, CFU g-1) and Polyphenoloxidase activity (percentage of activity with respect to the control samples*)* on red grape pomace (RGP) cv. Tempranillo.

	Control	HHP-1	HHP-2	HHP-3	HHP-4	*p*
Mesophilics	2.95	2.67	2.64	2.63	2.45	ns
Molds and yeasts	2.03A	<1.00B	<1.00B	<1.00B	<1.00B	*
Enterobacteriae	2.12A	<1.00B	<1.00B	1.16AB	1.20AB	*
PPO	103.3	125.0	145.8	151.7	139.2	ns

HHP1: 600 MPa/1s; HHP2: 600 MPa/300s; HHP3: 600 MPa/1s (2 cycles); HHP4 cycles (b): 400 MPa/1s (first cycle) / 600 MPa/1s (second cycle).
Means values with different superscript numbers in the same row and different letters in the same column indicate significant difference by the Tukey test ($p < 0.05$).

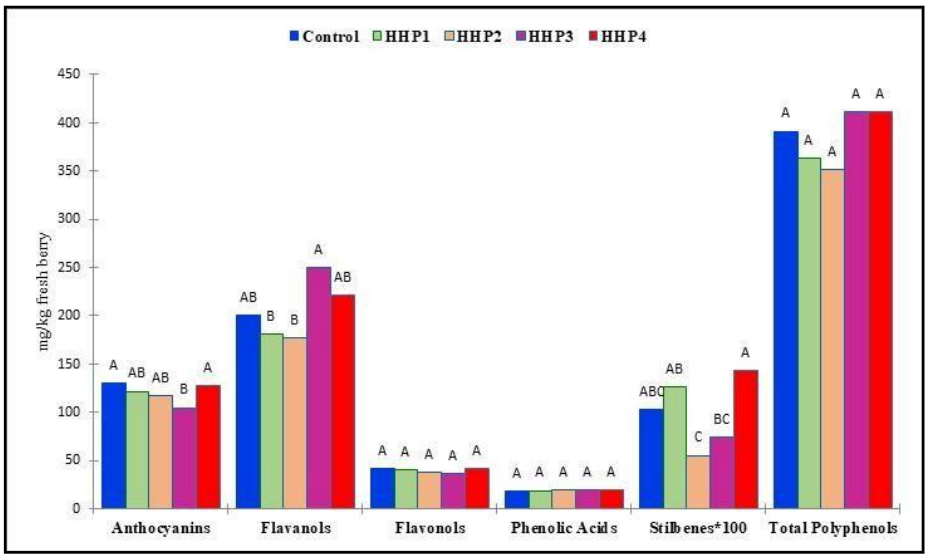

Figure 2. Effect of HHP treatments on polyphenolic composition families at initial time.
HHP1: 600 MPa/1s; HHP2: 600 MPa/300s; HHP3: 600 MPa/1s (2 cycles); HHP4 cycles (b): 400 MPa/1s (first cycle)/600 MPa/1s (second cycle). Means values with different superscript numbers in the same row and different letters in the same column indicate significant difference by the Tukey test ($p < 0.05$).

Specifically, HHP1 and HHP2 had a similar effect: a tendency towards a decrease was observed in the values of ANT, FLAVA FLAVO and ST in the samples from both treatments with higher decreases registered in HHP2 than in HPP1. On other hand, the application of two cycles had different effects, depending on the combination used. Thus, while HPP3 caused significant decreases in ANT, HHP4 did not significantly alter the values of any of the families analyzed. Finally, the highest values of ANT, FLAVO, PA, ST and TPC were registered in RGP-HHP4 samples. In front, the lowest values of FLAVA, ST and TPP were in RGP-HHP3. The results are not in complete agreement with previous work on other vegetable matrices. Ferrari et al. [16] showed that HHP high pressure treatment at room temperature improves the quality of pomegranate juice, increasing the intensity of red color of the fresh juice and preserving the content of natural anthocyanin. On other hand, when the effects of HHP on phenolic compounds and parameters related to their stability in Mesoamerican fruits were studied, increases in total phenolic content were observed by Gómez Maqueo et al. [17]. The main mechanism which led to a higher phenolic content after processing with HHP, was an increase in extractability of bound phenolic compounds due to the modification of cell walls which promoted the release of cell-bound phenolic compounds, that where otherwise inaccessible.

3.2 Effects of HHP treatments and storage temperature at 270 days

3.2.1 Statistical effect of HHP treatments and storage temperature on microbiological counts, PPO and phenolic composition of RGP

Table 2 shows the results of the two-way ANOVA applied to the results obtained from the microbiological, enzymatic, and phenolic compound analyses performed on the RGP cv. Tempranillo. As the table shows, HHP treatments had a highly significant effect on mesophilic, mold and yeast ($p < 0.001$) (48.8% and 97.3% of the total variation respectively) and on Enterobacteriaceae populations ($p < 0.05$, 28.5% of the total variation) also. However, HHP have not a significant effect on PPO activity. Respect to phenolic compounds, treatment had not significance in ST and a statistical significance of 5% in the rest of families analyzed.

However, the storage temperature had a highly significant effect on the most of parameters analyzed. With exception of PA, it was the main cause of variation in the values of the most of phenolic families in the samples, accounting for 89.7% of the total variation in the case of the FLAVO. Therefore, according to the results of the two-way ANOVA carried out, FLAVA were the most sensitive compounds to the HHP treatments, while FLAVO and ST, and in this last phenolic group, a significant interaction treatment*storage temperature was observed.

Table 2. Effect of the HHP treatments and storage temperature on microbial counts (log colony forming units, CFU g^{-1}) PPO activity and polyphenolic profile of the GRP.

Parameter		Treatment	Storage Temperature	Treatment × Storage Temperature
Mesophilics	Significance	***	***	***
	% Variation	48.8	43.7	4.8
Molds and yeasts	Significance	***	ns	ns
	% Variation	97.3	0.2	0.4
Enterobacteriacea	Significance	*	ns	*
	% Variation	28.5	7.1	28.5
PPO activity	Significance	ns	ns	ns
	% Variation	6.1	15.0	7.6
Anthocyanin	Significance	*	***	n.s.
	% Variation	5.0	82.9	3.8
Flavanols	Significance	*	***	*
	% Variation	15.0	35.6	26.9
Flavonols	Significance	*	***	*
	% Variation	2.7	89.7	3.2
Phenolic Acids	Significance	*	ns	ns
	% Variation			
Stilbenes	Significance	ns	***	*
	% Variation	2.0	87.1	4.8
Total polyphenols	Significance	**	***	**
	% Variation	9.2	69.3	13.0

HHP1: 600 MPa/1s; HHP2: 600 MPa/300s; HHP3: 600 MPa/1s (2 cycles); HHP4 cycles (b): 400 MPa/1s (first cycle) / 600 MPa/1s (second cycle).

3.2.2 Microbial load population and Polyphenoloxidase (PPO) activity

Storage conditions at different temperatures influenced the development of viable mesophilic count. Thus, a trend to higher population was found in not treated and stored at room temperature sample. This is not the case on Enterobacteriaceae, moulds and yeasts. So, the refrigeration temperature and previous treatment of HHP keeps no-growth of these microorganisms. As Fig. 2 reflects, when the samples were stored at 4ºC (T4samples), a trend to higher values of PPO in the control than in HHP samples was observed, while similar values of this enzymatic activity were registered on all samples stored at 20 ºC (T20 samples). On other hand, for a given treatment PPO activity was higher in the samples stored at 20 ºC. However, no significant differences were registered in any case. The direct consequence of these result was the decrease in the content of phenolic substances in GRP as it will be seen in the following section. A trend to decrease PPO was reported by Gonzalez-Cebrino et al. [11] on treated plum puree during refrigerated storage.

3.2.3 Phenolic profile

Figure 4 reflects the values of phenolic families found in the RGP control and HHP treated after 270 days of storage at 4 ºC and 20 ºC (T4 and T20 samples respectively). Since regardless the phenolic group considered, the values displayed are lower that found at initial time (Fig. 2), decreases were registered in all phenolic families. Besides, regardless treatment, the highest decreases respect to the initial values were found in ANT (around 90%) both in T20 and T4 samples. However, in T20 the lowest decreases corresponded toPA while in T4 to ST. Stilbenes such as resveratrol, is an important polyphenol for its antioxidant activity.

In the light of the above-mentioned results, the remain activity of the polyphenoloxidase (PPO) caused enzymatic reactions that requires oxygen to catalyse the hydroxylation of monophenols to diphenols, and further on, the oxidation of the resulting diphenols into o-quinon. These quinones formed rapidly react non-enzymatically, with other quinones, aminoacids and, proteins andphenols (including anthocyanins) causing discoloration of anthocyanins. In this sense, Ferrari et al. [16], reported the residual activity of some enzymes, such as the polyphenoloxidase (PPO), at the end of high-pressure processing of pomegranate juice, independently on the processing conditions. This activity caused the degradation of the nutraceutical compounds of pomegranate juice.

On the other hand, the results obtained reflect that the storage temperature was a critical factor in the values of all phenolic families extracted from RGP. With exception of FLAVO and PA–Control samples, the T4 samples (in both control and HHP treated), had higher FLAVO, PA, ST and in consequence of TPC values than the respective T20 ones. It is known that stability of ANTdecreases as the temperature increases. The rise in their breakdown is

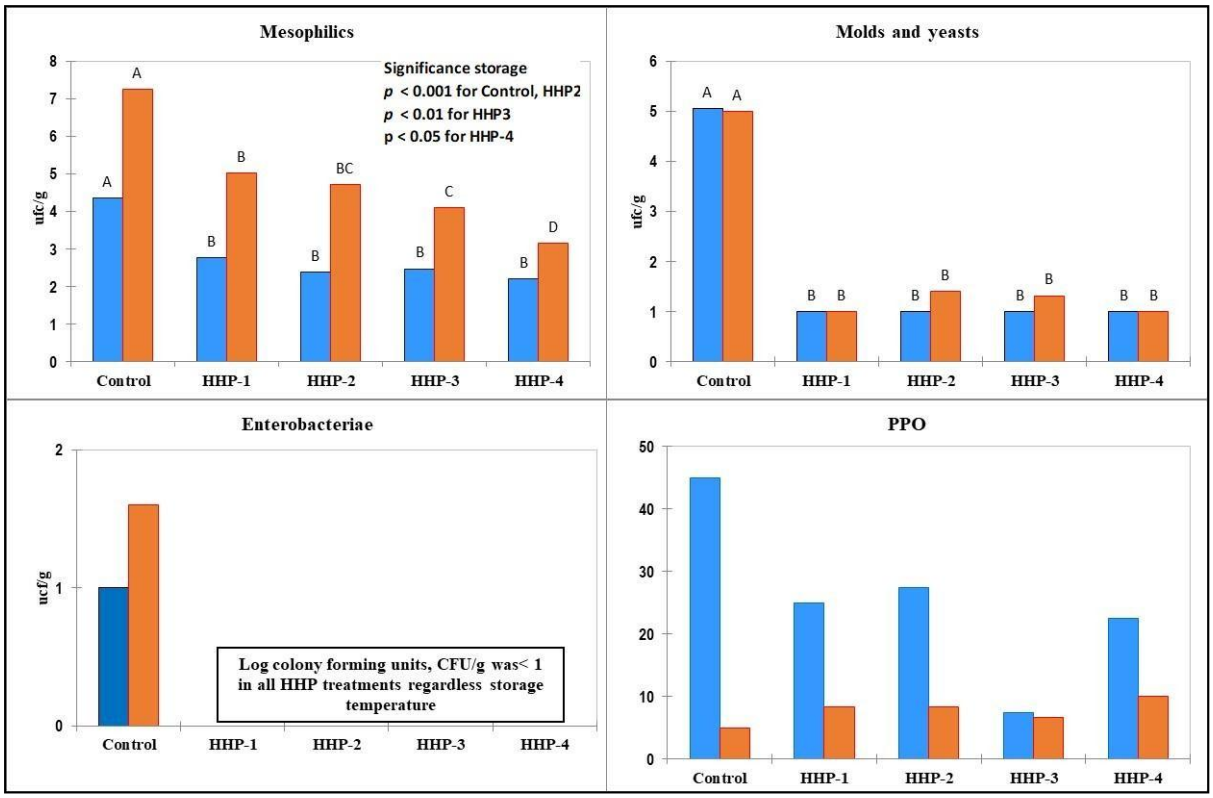

Figure 3. Effect of HHP treatments on microbiological parameters and polyphenoloxidase activity.
HHP1: 600 MPa/1s; HHP2: 600 MPa/300s; HHP3: 600 MPa/1s (2 cycles); HHP4 cycles (b): 400 MPa/1s (first cycle)/600 MPa/1s (second cycle).For a given storage temperature, means values with different letters indicates significant difference by the Tukey test ($p < 0.05$).

associated with an increase shift inequilibrium towards the trans-chalcone form. At high temperatures, the glycosylating sugar is lost in the C3 position of the molecule and the opening of the ring with the consequent production of colorless chalcones [18].

Finally, when the HHP effect is investigated, the results show that treatment had no significant effect on the values of ANT, FLAVA, PA, ST either TPC on T4 samples. However, control had higher values of FLAVA and TPC than all HHP treated samples on the T20 samples. When Corrales et al. [19] in 2008 investigated the anthocyanin condensation reactions under HHP, they reported that temperature/pressure treatments accelerate the synthesis of complex anthocyanin pyruvic acid adducts, such as vitisin A-type derivatives. This pyruvic acid adducts, non-analyzed in this work are also precursors of highly polymerized anthocyanins with different color ranges which may be of interest from an industrial point of view [19]. Christofi et al. [20] demonstrated that HHP influenced the phenolic composition of red wines after 4 months of storage. The lower content of total phenols and monomeric anthocyanins observed in the pressurized samples after 6 months of storage was probably due to an increase of condensation and oxidation reactions.

4 Conclusion

Immediately after the HHP treatments (0 days) the application of different HHP treatments to a grape pomace (RGP) cv. Tempranillo, had a significant effect of treatments against the growth of mesophilic, moulds, yeast and Enterobacteriaceae, the activity of PPO remained, and the values of phenolic groups did not were affected. Besides it is particularly important to highlight the effect of HHP3 and HHP4 (two cycles) did not differ from those of HHP1 and HHP2 (1 cycle). After a storage of 270 days, the results indicate that the storage temperature and the remain PPO activity were the major causes of the variations of phenolic compound values. Therefore, and since HHP did not have a significant effect over PPO further experiments and technologies are required to verify

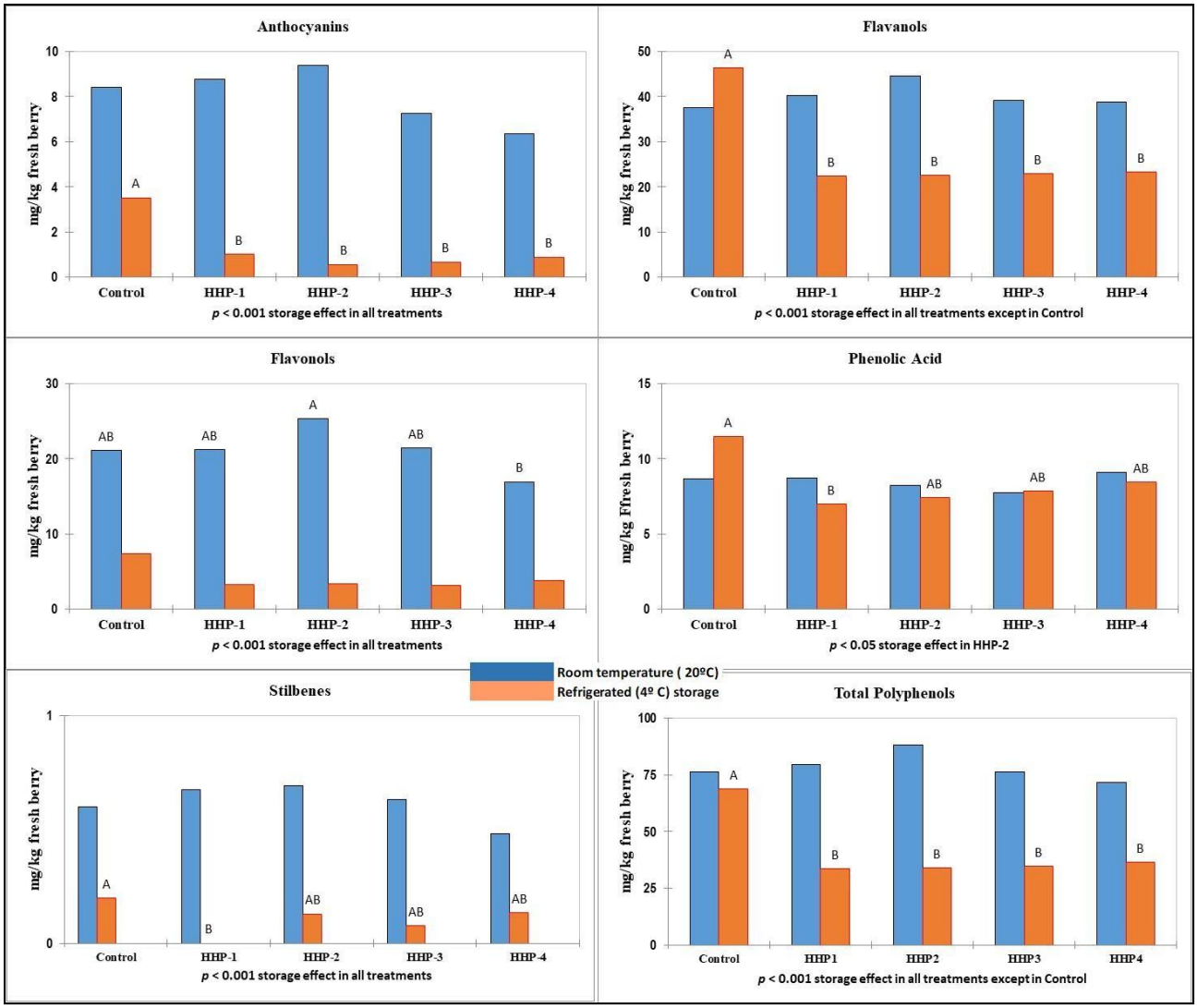

Figure 4. Effect of HHP treatments and storage temperature on polyphenolic composition families.
HHP1: 600 MPa/1s; HHP2: 600 MPa/300s; HHP3: 600 MPa/1s (2 cycles); HHP4 cycles (b): 400 MPa/1s (first cycle)/600 MPa/1s (second cycle).For a given storage temperature, means values with different letters indicates significant difference by the Tukey test ($p < 0.05$).

and to the ensure the stability of phenolic compounds during storage as well as to define the shelf-life of the product processed under optimized treatment conditions.

The results have been financed by the project "Valorisation of the by-products of the enological industry through alternative technologies to conventional ones to improve the preservation of meat products" (IB20073) financed from the Junta de Extremadura and FEDER funds. Authors grants to research group AGA 001-GR21186 from Junta de Extremadura.

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