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Biochemical and microstructural assessment of minimally processed peaches subjected to high-pressure processing: Implications on the freshness condition

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ABSTRACT

Since the condition of freshness is closely linked to the definition of minimally processed fruits, it is generally assumed that they should be constituted by living tissues. High-pressure processing (HPP) has been proven to be successful in maintaining the freshness of foods, although the integrity of some tissues could be altered. The aim of this work was to assess different biochemical and microstructural changes experimented by the HPP-treated tissues. The following determinations were conducted in control and treated diced peaches: viability assay, microstructure analysis, relative expression of RNAm, and enzyme activities related to browning and anaerobic metabolism. Results showed that although HPP-treated tissues were not viable, changes in the expression of the RNAs of the enzymes evidenced that some metabolic processes were still active. In turn, the microstructure remained rather unaltered, while the enzymes tested were significantly inhibited. Then, although some characteristics of living tissues were modified in the HPP-treated fruits, they could be considered as fresh in appearance. *Industrial relevance:* It is well known that conventional thermal treatments applied to preserve fruit products usually cause important changes in flavor, color, texture, and consequently the loss of fresh appearance. However, in the case of novel technologies such as high-pressure processing (HPP), this causality is not straightforward. Interestingly, the claim of "freshness" in foods has become a controversial topic, since some regulations indicate that for a food to be regarded as fresh, it should not have been subjected to any preservation processing. However, a list of exceptions has been considered for processes such as irradiation at low doses, or application of edible coatings. This list of exception is expected to increase, accordingly to the raise in successful experiences with the application of novel technologies such as HPP. This work provides information on the effect of HPP on different biochemical and structural aspects of treated peaches, with the aim to contribute toward the commercial application of HPP to minimally processed fruits.

1. Introduction

According to the definition, minimally processed fruits should have been subjected to processes that do not substantially modify the characteristics of the raw material, with the aim to be transformed into a product suiting the convenience of consumers and/or retailers. Since the products under this category implicitly require a condition of freshness, some authors assume that these products should be entirely constituted by living tissues (Artés & Artés-Hernández, 2003; Wiley, 1994). When fruits are preserved by conventional thermal treatments, the difference between fresh and processed product is clear, since these treatments interrupt all metabolic processes, with the consequent loss of the fresh appearance, causing significant changes in the flavor, color, and texture. However, when novel "nonthermal" technologies such as high-pressure processing (HPP) are applied to plant products, this causality is not straightforward (Sánchez-Moreno et al., 2005). Therefore, it becomes apparent that some traditional definitions should be reviewed and accordingly adapted, by setting apart, for example, the concept of "freshness" from the condition of "living tissue."

Although canned peaches, which constitute one of the most popular canned desserts, can be considered as an alternative for the consumption of fruits (Saura, Laencina, Pérez-López, Lizama, & Carbonell-Barrachina, 2003), from a nutritional point of view, one of the major problems associated with this kind of products is the need of adding sugar and the loss of heat-labile nutrients because of the use of

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preservative thermal treatments (Miller, Pang, & Broomhead, 1995; Rickman, Barrett, & Bruhn, 2007). Therefore, consumer preferences have been evolved until leaning nowadays toward the selection of fresh or, at least, minimally processed products without additives.

The application of HPP for the preservation of minimally processed fruits constitutes an innovative area of research. Therefore, the literature about specific topics such as the effect of HPP on structural and biochemical characteristics of pressurized fruit tissues is rather scarce. However, some of the subjects already studied are the effect of HPP on the microstructure, texture, and some bioactive compounds (Vázquez-Gutiérrez, Hernández-Carrión, Quiles, Hernando, & Pérez-Munuera, 2012) and the development of biochemical reactions taking place in certain plant tissues (Van der Plancken et al., 2012).

Considering the well-established success of HPP in preserving the nutritional value and the sensory characteristics of foods (i.e. their freshness), one of the aims of this work was to analyze the biochemical response of the tissues subjected to HPP, through the determination of the viability of cells conforming the tissues, the relative expression of enzymes linked to enzymatic browning (phenylalanine ammonia-lyase—PAL and polyphenol oxidase—PPO) and anaerobic metabolism (alcohol dehydrogenase-ADH and pyruvate decarboxilase-PDC), and the direct measurement of the activity of related enzymes (PPO and ADH). The conditions of the HPP applied in the present study were defined, according to our preview experience (Denoya et al., 2016), as those leading to the minimal alteration of peach texture while causing the maximal inactivation of microorganisms and enzymes.

Another aspect evaluated in the present work was the microstructure of tissues, by assessing the changes brought about by the HPP treatments. These changes can be caused by both enzymatic and non-enzymatic transformations of the cell wall polymers, as well as by the compression of the cell structure after the degassing of the tissue. In this regard, several publications (Denoya, Vaudagna, & Polenta, 2015; Perera, Gamage, Wakeling, Gamlath, & Versteeg, 2010; Vercammen et al., 2012) pointed out the absence of significant changes in the texture of pressurized fruit or vegetable products. Therefore, it is expected that the assessment of the microstructural aspects underlying the application of HPP can provide with a better explanation for the visual changes experimented by fruits, such as the appearance of translucency, and can also help to unveil issues related to the cell viability.

2. Material and methods

2.1. Plant material

Peaches (*Prunus persica* (L.) Batch) cv. Flavorcrest were harvested from an experimental orchard in San Pedro Buenos Aires, Argentina (Latitude 33°41 S, Longitude 59°41 W). Fruits were carefully selected according to their uniform size and maturity by ground color. Soluble solids were 12° Brix on average. Firmness of fruit was between 20 N and 30 N. The pH was between 3.4 and 3.5. The fruit were stored at 0 °C in a cold chamber until processing.

2.2. Sample preparation

Prior to processing, the peaches were washed in running tap water. Cylinders (15 mm length, 15 mm diameter) from the parenchyma tissue were obtained using a cork borer and a stainless steel knife. In order to obtain homogeneity in the samples, the cylinders were taken from the middle zone of the mesocarp, parallel to the major axis of the fruit. Subsequently, the cylinders were dipped into tap water containing 20 ppm of HClO for 2 min. After being drained, the cylinders were dipped into an aqueous solution containing 1% ascorbic acid (ACS, Biopack, Argentina) and 1% citric acid (USP, Anedra, Austria) for 2 min to prevent surface browning and to wash the remaining HClO. The cylinders were drained again and vacuum-packed in Cryovac BB2800 bags (O_2 transmission rate: 6–14 $cm^3/m^2/24$ h at 23 °C, 1 atm) filled with eight units each, using a double-chamber vacuum-packing machine (Rapivac, Model Maximax 800, Argentina). The samples were divided into two different groups: 1) HPP-treated samples (P); 2) control samples (C), with no additional treatments.

HPP-treated samples were packed into an additional outer bag to prevent leakages. HPP was performed in a high hydrostatic pressure system Stansted Fluid Power Ltd. High Pressure IsoLab System model FPG9400:922 (Stansted, UK) with a vessel of 2 L capacity and a maximum working pressure of 900 MPa. A mixture of propylene glycol 30% (v/v) and distilled water was used as the pressure-transmitting medium. The process parameters of the HPP treatment were selected considering the optimal conditions determined in a preliminary study (Denoya et al., 2016) and were 600 MPa for 5 min. Compression rate was 5 MPa s^{-1}. The pressurization was carried out at an initial temperature of 22 °C, which was only modified by adiabatic heating (up to 38 °C).

2.3. Viability assay

Viability assay was determined according to the method described by Martínez, Nieto, Castro, Salvatori, & Alzamora (2007) with some modifications. Cross-sections of peach tissue were immersed for 5 min in a Fluorescein Diacetate (FDA, MP Biomedicals, France) solution prepared from a 5 mg mL^{-1} FDA solution in acetone that was diluted with phosphate buffer 0.05 M pH 6 to a final concentration of 0.1% w/v. Sections were observed with an epifluorescent microscope Axiolab HB050X (Carl Zeiss, Germany) using a blue filter (excitation: 450–500 nm and emission: 520 nm).

2.4. Change in a* color parameter

The color parameter a* of peach cylinders was measured with a Minolta CR-400 chromameter (Konica Minolta Sensing, Inc. Osaka, Japan), using the CIE scale, where a* represents chromaticity on a green (−) to red (+) axis. The parameter was measured on the surface of the cylinders immediately after opened the packages and after 180 min. The change of a* in time was calculated as a ratio (Δa*/min) and was measured as a browning rate parameter. The instrument was set up for illuminant D_{65} and 2° observer angle.

2.5. Relative expression of enzymes at RNAm level

2.5.1. RNA extraction

Total RNA was isolated from 4 g of tissue using the method described by Meisel et al. (2005). RNA pellets obtained were then dissolved in 40 μL of GIBCO® ultra-pure water (Invitrogen, Massachusetts, USA) and incubated at 56 °C for 5 min.

In order to avoid DNA contamination, samples were then treated with Deoxiribonuclase I (Invitrogen, Massachusetts, USA) according to the manufacturer instructions.

2.5.2. Primers design

Primer Express 3.0 (Applied Biosystems, California, USA) was used to design the qPCR primers, based on EF1 (elongation factor 1), TEF2 (translational elongation factor 2), ADH, PAL, and PDC gene sequences from species of *Prunus* genus, preferable *persica*, which were all retrieved in GenBank (Table 1). These sequences were aligned with Clustal W2 software (available at the European Bioinformatics Institute website: http://www.ebi.ac.uk/Tools/clustalw2/). In order to check in silico specificity, BLAST software *(NCBI—www.ncbi.nlm.nih.gov)* was employed. The nucleotide sequences of the primers obtained for each gene are listed in Table 2.

2.5.3. Quantitative real-time RT-PCR

Relative gene expression was determined by quantitative one-step real-time RT–PCR in a PCR Real-Time StepOne Plus System (Applied

Table 1
Nucleic acid sequences used for the primers design of the studied enzymes.

Gen	GeneBank access no.	Source	Sequence (bp)
EF1	FJ267653.1	*Prunus persica*	1771
TEF2	JQ732180.1	*Prunus persica*	3060
PAL	HM543574.1	*Prunus persica*	2663
PPO	JQ388483.1	*Prunus persica*	2356
PPO	AY865623.2	*Prunus salicina*	2155
ADH	HM240512.2.	*Prunus dulcis* × *Prunus persica*	1360
PDC	HM362681.1	*Prunus dulcis* × *Prunus persica*	2275

References: bp—base pairs; EF1—elongation factor 1; TEF2—translational elongation factor 2; PAL—phenylalanine ammonia-lyase; PPO—polyphenoloxidase; ADH—alcohol dehydrogenase; PDC—piruvate decarboxilase.

Table 2
Sequences of the oligonucleotide primers used for quantitative real-time RT-PCR.

Gene	Forward primer	Reverse primer	Product
EF1	5′- ccaaggcaaggtacgatgaaa-3′	5′-tgtacccaaccttcttcagatagga-3′	63
TEF2	5′-tttccagtgggcttcaaagg-3′	5′-caaagcaaataccccctcatgttt-3′	62
PAL	5′-caatggaaatcggcaataagg-3′	5′-gccccacgcaaaacga-3′	60
PPO	5′-tggtttccggtgccaaaa-3′	5′-ggtccccggccctgtac-3′	60
ADH	5′-cggggaagccactactatcaa-3′	5′-cgtaaatgcgagggaaca-3′	150
PDC	5′-ggcgtcaccgacgtcttc-3′	5′-tggtcgaggagggtcaagtt-3′	56

In the last column, the longitude of the product (in base pairs) amplified by the corresponding primers is indicated. References: EF1- Elongation factor 1; TEF2- Translation elongation factor 2; PAL- Phenylalanine ammonia-lyase; PPO- Polyphenoloxidase; ADH- Alcohol dehydrogenase; PDC- Piruvate decarboxilase.

Biosystems, California, USA), using the intercalation dye SYBR Green through the kit QuantiFast® SYBR® Green RT-PCR (Qiagen, USA) according to the manufacturer recommendations and the following reverse-transcription and amplification protocol: 50 °C for 10 min (reverse transcription), followed by 95 °C for 5 min (HotStar Taq Plus DNA polymerase activation). Then, samples were subjected to 40 cycles of 95 °C for 10 s (denaturalization) and 60 °C for 30 s (annealing-extension). No-template controls were included in every PCR run. A final denaturation step (melting curve) was carried out to assess amplicon melting temperature and to check for non-specific amplification products.

Relative gene expression was calculated using the "Comparative efficiency $^{-2\Delta\Delta}$CT (Threshold cycle) method" (Livak & Schmittgen, 2001) with EF1 as the reference gene. To test whether EF1 and TEF2 behave as housekeeping genes in the analyzed samples, their expression both in control and HPP samples were compared (data not shown). EF1 was the most stable against the treatment, so it was chosen as the reference gene. Lara et al. (2011) used also this gene for comparing the expression of RNA in peaches subjected to different postharvest treatments. Results for HPP-treated samples (P) were expressed in relation to the values obtained for control samples (C). Each RNA sample was run in triplicate. To experimentally determine PCR efficiency, dilutions of RNA were prepared and amplified by real-time RT-PCR using the conditions described above. Plots were made from the log of the total RNA input versus the CT values and the PCR efficiency was calculated from the slope of the plot using the equation $E = 10^{[-1/slope]}$ (Pfaffl, 2001). All genes had slopes ranging from −3.2 to −3.5, with the PCR efficiencies within the acceptable range of 90%–105%.

2.6. Enzyme activities

2.6.1. Enzyme extraction

The enzyme extractions were carried out according to the method described by Denoya et al. (2015).

2.6.2. PPO activity assay

The PPO activity assay was carried out according to the method described by Jiménez & García-Carmona (1997) with some modifications. A volume of 1 mL of 50 mM phosphate buffer solution pH 6.5 with 4.95 mM 4-*tert*-butylcathecol (Aldrich Chemistry, France) as a substrate was mixed with 100 μL of the enzyme extract. Then, the absorbance at 400 nm was continuously recorded at 30 °C for 5 min by using a spectrophotometer (Gilford/CIBA-Corning Diagnostics, USA). The activity was expressed as units per gram of fruit. One unit represents the amount of enzyme necessary to change A_{400nm} in 0.01 min^{-1} at 30 °C.

2.6.3. ADH activity assay

The ADH activity assay was carried out according to the method described by Walker (1992) with slight modifications. ADH activity was followed by using a spectrophotometric method based on the measurement of the initial rate of increase in absorbance at 340 nm due to NADH (β-Nicotinamide Adenine Dinucleotide, reduced) accumulation during ethanol oxidation. A volume of 1 mL of 200 mM TRIS (2-Amino-2-hydroxymethyl-propane-1,3-diol; USB Corporation, USA) pH 8.8 and 100 mM ethanol (98.5%, Dorwil, Argentina) as substrate was mixed with 100 μL of the enzyme extract. The reaction was initiated by the addition of 1 mM of NAD+ (β-Nicotinamide Adenine Dinucleotide, Sigma, USA). The absorbance at 340 nm was continuously recorded at 30 °C for 3 min by using a spectrophotometer (Gilford/CIBA-Corning Diagnostics, USA). The activity was expressed as units per gram of fruit; one unit represents the amount of NADH produced in μM min^{-1} at 30 °C.

2.7. Light microscopy (LM) and transmission electron microscopy (TEM)

For the LM, samples were fixed with a 3% glutaraldehyde solution, post-fixed in 2% OsO_4 solution (2 h), and dehydrated using a graded ethanol series (50, 70, 96, 100% v/v) and embedded in Spurr resin. The samples were cut using an ultramicrotome (Ultracut-E Reichert Jung, Leica AG, Vienna, Austria). Semi-fine sections (1.5-μm-thick) were stained with 1% toluidine blue and examined in a light microscope (Nikon Eclipse E200, Tokyo, Japan). For TEM, the samples followed the same protocol of fixation, dehydration, and infiltration as for LM, but in this case, 0.05-μm-thick sections were collected. Ultrathin sections were stained with 4% lead citrate and 2% uranyl acetate solutions and observed with a JEOL 1200EX Transmission Electron Microscope (Tokyo, Japan).

2.8. Experimental design and statistical analysis

This work was analyzed as a completely randomized design. Two different treatments (P and C) were compared and the experimental unit was each bag with eight cylinders. In case of enzyme expression and the change in a* parameter, four replicates (i.a. pooled cylinders from four bags) were analyzed for each treatment. In case of enzyme activity, three replicates (i.a. pooled cylinders from three bags) were analyzed for each treatment.

In case of enzyme activities and the change in a* parameter, the mean values for both treatments (P and C) were subjected to a t student test ($p < 0.05$) using SAS software (Version 9.2 2002–2003 SAS Institute Inc. Cary, NC, USA) because these determinations involved only the comparison between two treatments.

Relative gene expressions were analyzed by InfoStat software (Di Rienzo et al., 2010). Results from HPP samples were expressed in relation to the values obtained for control samples.

3. Results

3.1. Viability assay

Micrographs in Fig. 1 show the cell viability as evidenced by the Fluorescein Diacetate (FDA) method. Fresh peach tissue (untreated) can be compared with samples obtained at the different processing

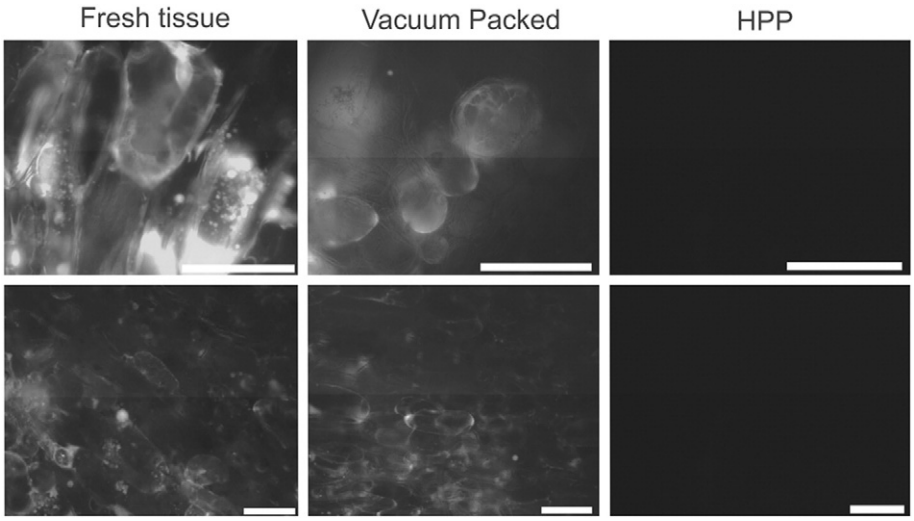


Fig. 1. Micrographs of fresh, vacuum-packed, and high-pressure-processed (HPP) peach tissue, using Fluorescein Diacetate to identify viable cells. Viable cells are distinguished by a bright fluorescence. Scale bars, 100 μm.

stages of the minimally processed peaches, which were finally subjected to HPP (600 MPa for 5 min).

The intense fluorescence in samples from fresh fruit is indicative of the viability of cells prior to processing. In turn, fluorescence was less intense, with distinguished dark zones in fresh-cut and vacuum-packed peaches before HPP. Probably, this effect was due to the loss of membrane integrity of some cells, as a consequence of processing such as cutting and/or vacuum packaging. In the case of HPP-treated peaches, the complete absence of fluorescence evidences the lack of cell viability as a consequence of the treatment.

3.2. Relative expression of enzymes at the RNAm level: Comparison with the enzymatic activity

Figs. 2 a, b, and c show the expression at the transcriptional level (RNAm synthesis by real-time RT-PCR) of genes associated with the most relevant alterations in this type of products: enzymatic browning (PPO and PAL), and the development of a fermentative metabolism (PDC and ADH).

Although the partial repression caused by the HPP on the expression of ADH, PAL, and PDC becomes evident, with significant differences ($p < 0.05$) between P and C samples, this effect was not observed in the case of PPO (related with browning), with RNAm levels of this enzyme being similar between P and C samples. However, when PPO activity was directly measured, HPP-treated samples showed a significant ($p < 0.05$) reduction in comparison to the control (Fig. 2-a). These results suggest that HPP was able to partially inactivate the PPO enzyme present in the tissues at the moment of the exposure, without altering the mechanism of expression of this protein at the RNAm level.

Fig. 2-b shows the effect of HPP on the parameter $\Delta a^* \text{ min}^{-1}$, which is indicative of the rate of development of browning in fruit tissue, and on the RNAm expression of the enzymes PPO (closely linked to this alteration), and PAL (indirectly related to browning, because of its involvement in the synthesis of the phenolic compounds that would act as substrates for this reaction).

These figures clearly show that the HPP had a significant effect on both the repression of PAL expression and on the inactivation of the enzyme PPO already synthesized, although not on the expression of PPO. This effect was physically evidenced by the reduction in the browning rate, as determined by the decrease in the $\Delta a^* \text{ min}^{-1}$ parameter.

Fig. 2-c shows the relative expressions at the RNAm level of the genes encoding for PDC and ADH, two enzymes closely linked to the anaerobic metabolism of fruit tissues. This figure also shows the activity of ADH, which catalyzes the last step of that metabolic pathway. Results show that the inactivation of ADH by the HPP is the outcome of the combination of its genetic repression with the inhibition of the activity of the enzyme present in the tissue. Therefore, as found in previous studies carried out by our group (Denoya et al., 2015), HPP is able to inhibit the development of the fermentative metabolism.

3.3. Micro- and ultrastructural study

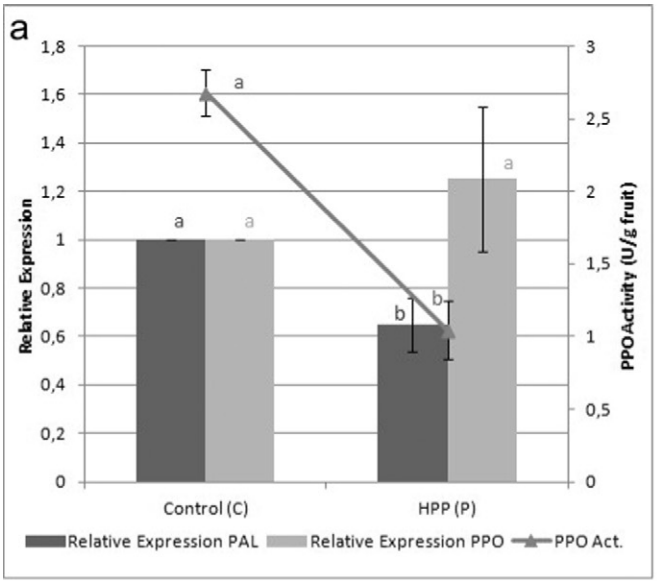
Figs. 3, 4, and 5 allow the microstructural comparison of the effect of the treatments on the morphological aspects of peach tissues. Therefore, tissues micrographs are shown without any treatment (Fig. 3-a–f), after minimally processing and vacuum-packaging (Fig. 4-a–f) and after minimally processing, vacuum-packaging, and exposure to 600 MPa for 5 min (Fig. 5-a–f).

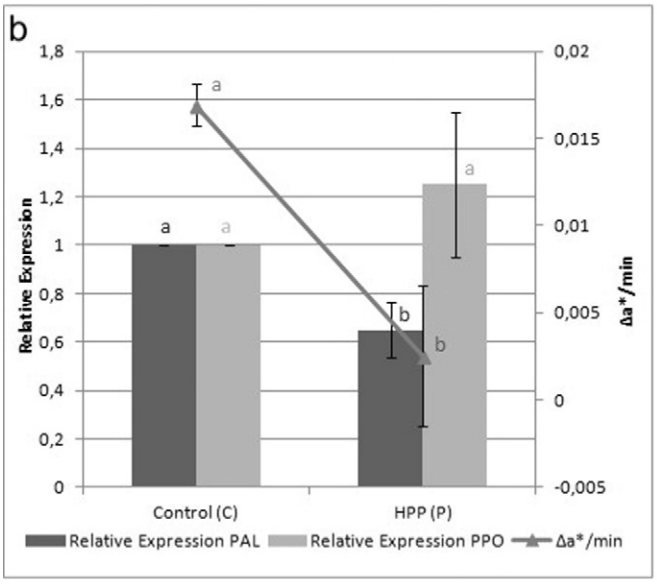
Cells of untreated peach tissue (Fig. 3-a–c) presented a rounded shape and turgid aspect, with well-defined intercellular spaces, and no evidence of transfer of cytoplasmic content from the intracellular medium. TEM micrographs reveal the homogeneous aspect of cell walls (Fig. 3-d–f), with the cell membrane in close association.

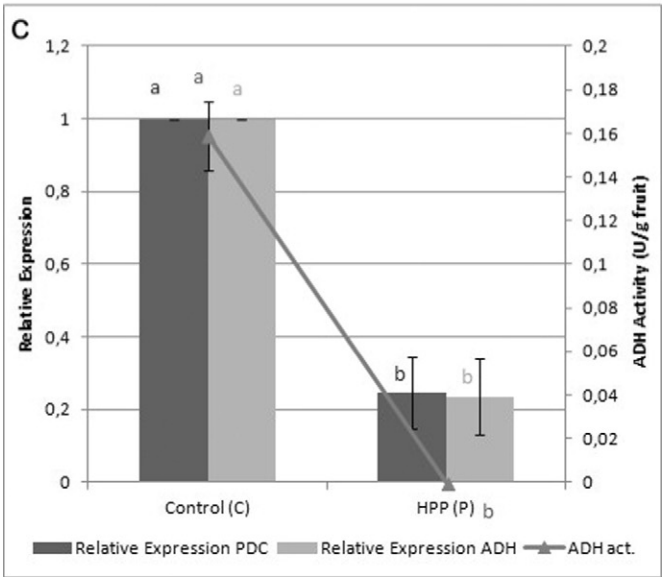
In turn, vacuum-packed minimally processed peaches maintained similar characteristics although the shape of the cells became rather altered, acquiring an elongated aspect, with the intercellular spaces being more reduced than in the fresh tissue (Fig. 4-a–b). In some cases, membranes showed certain level of disruption and were partially separated from the cell walls (Fig. 4-c). TEM micrographs evidenced that cell walls maintained a homogenous structure, although some roughness could be distinguished at the inner edges (Fig. 4-e and f).

In the case of HPP-treated tissue, cells in the semi-fine cuts acquired a totally irregular shape (Fig. 5-a), with the intercellular spaces drastically reduced in size, with rests of cytoplasmic material inside or even totally absent (Fig. 5-a–b). In turn, cell walls (Fig. 5-b–c) became wider than in fresh tissue, with unfolded zones (Fig. 5-c) or even a complete separation in layers. This suggests that HPP could have caused the disruption of cell membranes, their separation from the cell wall, and a hydration effect as a consequence of the swelling.

TEM micrographs of HPP-treated tissue (Fig. 5-d–f) allow the identification of zones located in the center of the cell walls, with lighter







appearance than the borders. This could have resulted from the solubilization and separation of the unions between the cellulose fibrils, as a consequence of the hydration of the wall matrix. Therefore, walls present a swollen aspect in some zones, with a variable thickness along the cellular contour.

4. Discussion

4.1. Viability assay

The study of the cell viability through the evaluation of the membrane integrity helps unveil whether the tissue is able to maintain the metabolic and physiologic characteristics of the fresh fruit. The test with FDA has been used in fruits to study the integrity of parenchyma tissue of species such as grapes (Krasnow, Matthews, & Shackel, 2008). This method detects the existence of an active cell metabolism through the visualization of fluorescence that will only occur if the FDA is first transported through the membranes, then suffer the cleavage of its diacetate group by intracellular esterases, and finally can be retained by the intact membranes of the living cells.

According to this principle, the total absence of fluorescence in the HPP-treated tissue is an evidence of either the complete loss of the membrane integrity and/or the full inactivation of intracellular esterases. In contrast, in untreated tissues, the fluorescence was detected in most of the cells.

The lack of viability of cells from HPP-treated samples was also observed by Gonzalez, Jernstedt, Slaughter, & Barrett (2010), in onion tissue treated with high pressure in the range from 50 to 600 MPa for 5 min. These authors found that in samples untreated or subjected to 50 MPa, most of the cells remained viable. However, a significant decrease in the amount of viable cells was observed at 200 MPa, while at pressure levels of 300 MPa or higher, the absence of viable cells was complete.

In another research, Dörnenburg & Knorr (1997) found that barely one third of potatoes cells exposed to 150 MPa for 10 min remained viable, while the exposure at 200 MPa for 10 min caused the complete loss of this condition. At this level of pressure, cell damage evidenced by the loss of compartmentalization, and the release of lytic enzymes and acid compounds from the vacuoles to the cytoplasm, led to the cell death. In turn, the application of pressure levels of 175 MPa or higher led to an increase in the amount of proteins and phenols in the medium, which constitutes a clear sign of irreversible permeation of the tonoplast surrounding the vacuole.

In relation to the mechanism that causes the loss of the cell viability, Préstamo & Arroyo (1999) suggest that HPP would cause alterations in the ionic channels, with the resulting changes in the cell permeability. These changes were also evidenced at the microscopic level by the redistribution of the water as well as by the gelatinization of cell biopolymers (Hills, Costa, Marigheto, & Wright, 2005).

4.2. Relative expression of enzymes at the RNAm level: Comparison with the enzymatic activity

The assessment of the relative expression of enzymes at the RNAm level showed that HPP was able to inhibit the expression of most of

Fig. 2. Relative expression of enzymes related with browning (phenylalanine ammonia-lyase—PAL and polyphenoloxidase—PPO) and relation with the effect on the PPO activity (*a*) or relation with the effect on Δa*min-1 (*b*) and (*c*), of enzymes related to a fermentative metabolism (piruvate decarboxilase-PDC and ADH) and relation with the effect on the ADH activity, in minimally processed peaches treated with 600 MPa-5 min (P-bars) in comparison with the same parameter in control samples (C-bars). The left y-axis represents the fold difference in transcripts level present in HPP-treated samples relative to the amount present in control samples. Error bars correspond to standard deviations. Values with different letters are significantly different ($p < 0.05$).

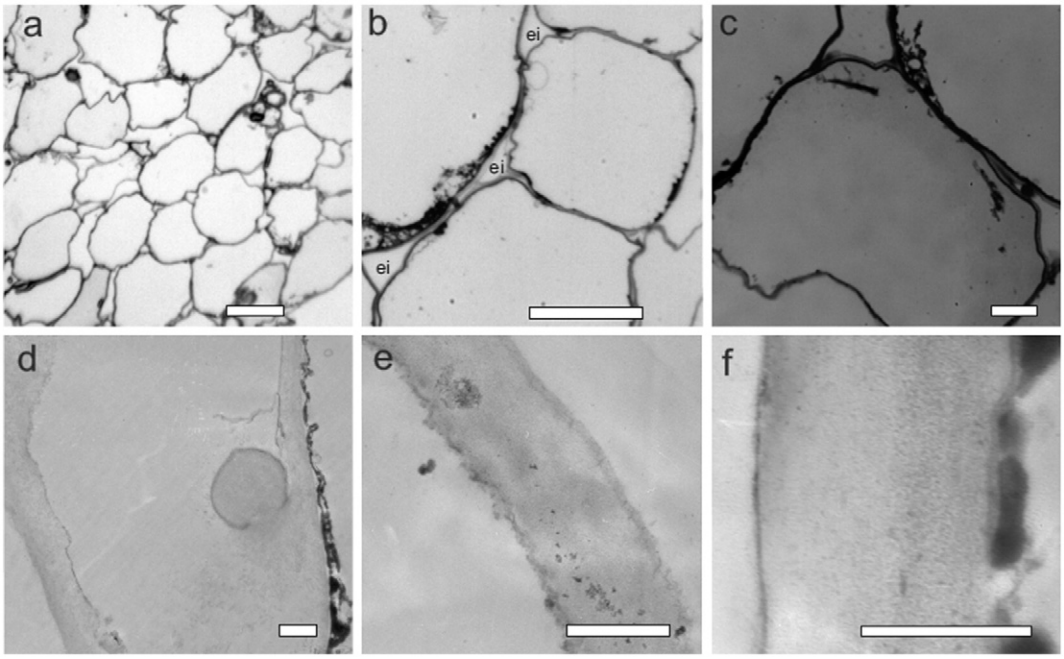


Fig. 3. Micrographs of parenchymatic tissue of fresh peaches. (a–c, semi-fine sections observed at optical microscope; d–f, ultrathin sections observed at transmission electronic microscope). References: ei-intercellular spaces. Bars: a—100 μm; b—50 μm; c—10 μm; d, e—1 μm; f—0.5 μm.

the enzymes studied (PAL, PDC and ADH), although this repression was not total.

PAL is an important enzyme that catalyzes the conversion of the L-phenylalanine to *trans*-cinnamic acid, which constitutes the first step in the metabolic pathway toward the synthesis of phenylpropanoids (Li-Qin, Jie, Shu-Hua, & Lai-Hui, 2009). The analysis of the effect of HPP on the RNA expression of both PAL and PPO makes evident that the decrease in the browning rate is the outcome of the limited synthesis of the substrates of the reaction as a consequence of the repression of the transcription of PAL, together with the diminished activity of the PPO enzyme, even though the levels of transcripts of the last were similar to the control. Mentré & Hoa (2001) suggest that the decrease in the enzymatic activity as exerted by HPP treatments would be linked to the inhibition of the genetic translation. These authors determined that pressures above 70 MPa would be enough to affect the functionality of the ribosomal complex in unicellular organisms.

It has been suggested that the exposure of plant tissues to stress conditions such as HPP, or mechanical processes such as cutting and

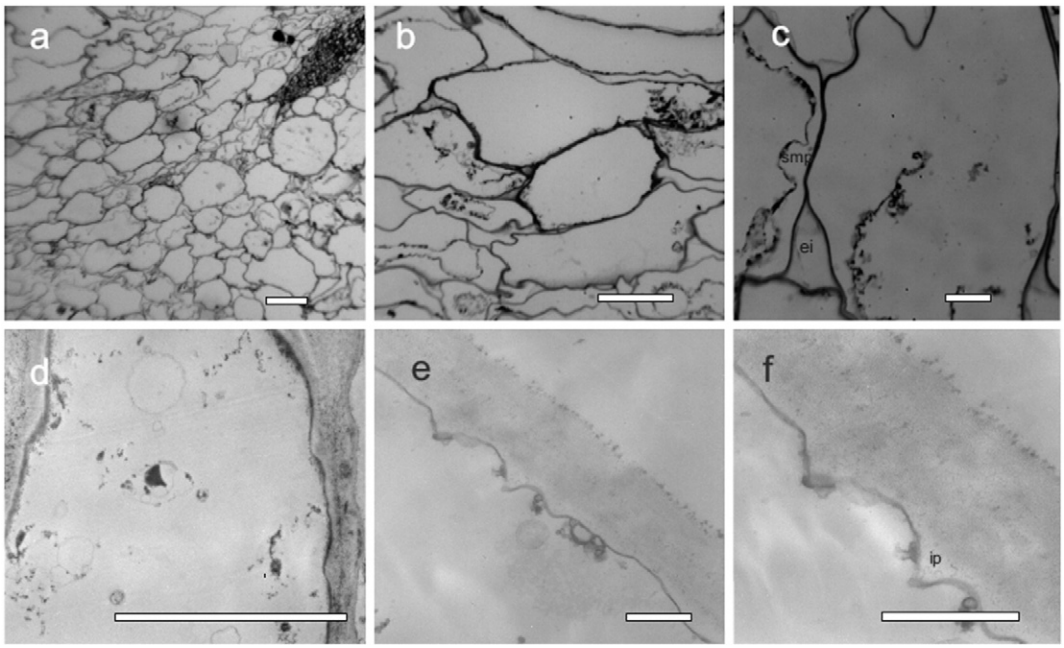


Fig. 4. Micrographs of parenchymatic tissue of vacuum-packed minimally processed peaches. (a–c, semi-fine sections observed at optical microscope; d–f, ultrathin sections observed at transmission electronic microscope). References: ei—intercellular spaces; ip—irregular cell wall morphology; smp—separation of the cell membrane. Bars: a—100 μm; b—50 μm; c–d—10 μm; e–f—1 μm. Bars: a—100 μm; b—50 μm; c, d—10 μm; e, f—1 μm.

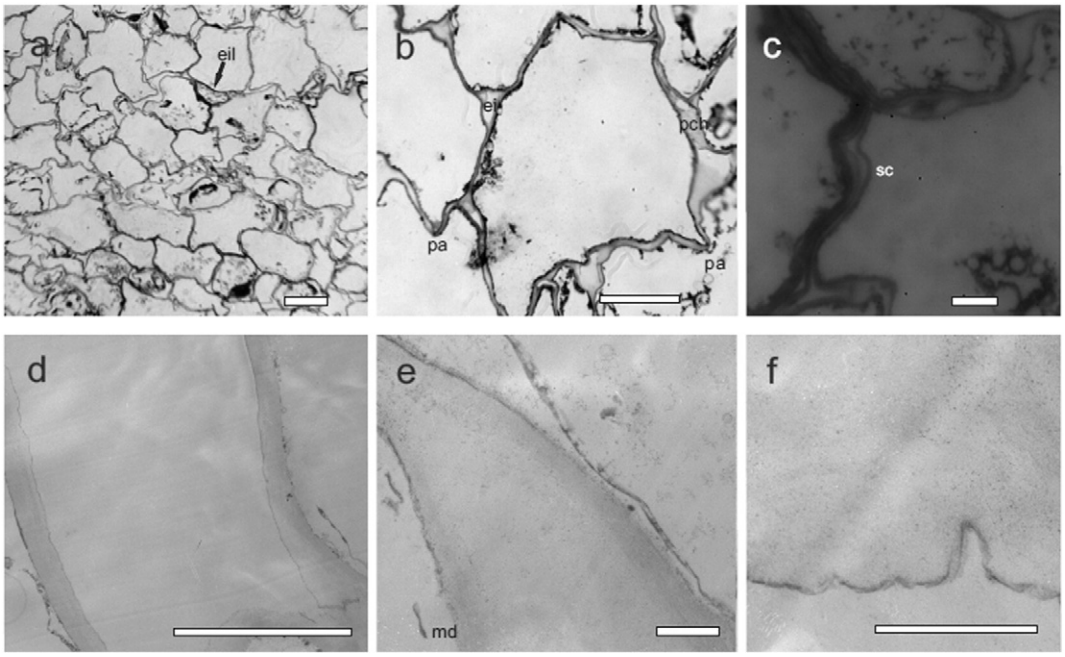


Fig. 5. Micrographs of parenchymatic tissue of vacuum-packed minimally processed peaches treated with 600 MPa for 5 min (a–c, semi-fine sections observed at optical microscope; d–f, ultrathin sections observed at transmission electronic microscope. References: eil—intercellular spaces filled with cytoplasmic content; md—damaged cell membrane; pa—irregular cell wall morphology; pch—swollen cell wall; sc—cell wall disorganization. Bars: a—100 μm; b—50 μm; c, d—10 μm; e, f—1 μm.

peeling, can induce the activation of genes related to the defense mechanisms, among them the biosynthesis of enzymes like PPO (Jacobo-Velázquez & Hernández-Brenes, 2010). This enzyme is closely linked to the enzymatic oxidation of polyphenols, which is in turn responsible for the color change in HPP-treated products such as fruit smoothies (Keenan, Brunton, Gormley, & Butler, 2011). However, the present work evidenced that in minimally processed peaches, the inhibitory effect of pressure on both the PPO activity and the synthesis of phenols was enough to control this oxidative mechanism. This effect was reflected on the product by the decrease in the rate of browning, as measured by the $\Delta a^{*}min^{-1}$ parameter.

In relation to the fermentative metabolism, the qRT-PCR determination showed that HPP significantly ($p < 0.05$) inhibited the expression of the RNAm linked to the most important enzymes involved in this alteration: PDC and ADH. In the case of ADH, this treatment was also able to completely inhibit the enzyme activity. These observations are in agreement with the results obtained in a previous work (Denoya et al., 2015), where HPP prevented the accumulation of ethanol in vacuum-packed peaches.

The present work can effectively contribute to unveil some of the effects of high pressure on the genetic expression in fruits, considering that studies on the effect of HPP on the gene expression of plants in general are rather scarce. Among the investigations found in the literature, Liu, Zhang, Duan, & Wu (2008) reported that the exposure of rice plants and seeds to HPP inhibited the expression of esterases and decreased the activity of the enzymes α- and β-amilase. These effects suggest that HPP can trigger physiological adaptive responses in seeds and plants through changes in the expression of the genes involved in biochemical phenomena such as the transcriptional regulation, the metabolism, and the stress response. This issue constitutes a prominent area of research.

4.3. Micro- and ultrastructural study

TEM micrographs evidenced that the cells of the peaches subjected to HPP suffered only minor morphological changes, with still well-defined cell borders and very limited damage. Although some evidence of disorganization can be observed in the cell walls, with a hydration process apparently starting to develop, the treatment caused in general no marked disruption. In agreement with this, Woolf et al. (2013) reported that HPP did not substantially modify the microstructure of avocado, with no evidence of considerable damage.

Morphological changes similar to those observed in our work were previously described in HPP-treated onions by Vazquez-Gutierrez, Hernandez-Carrion, Quiles, & Hernando (2014), who detected the solubilization of the cell wall and the concomitant separation of the cell membrane. In turn, Tangwongchai, Ledward, & Ames (2000) observed in tomato tissues the folding and corrugation of the cell wall after exposing the fruit to pressures of 300–600 MPa for 20 min.

The increase in the thickness/swelling of the cell walls was also reported in species like carrot subjected to treatments of 300 and 400 MPa (Trejo Araya et al., 2007). These authors remarked that, despite the conformational changes evidenced in the cell walls, only few areas were broken, with the structure of the carrot tissue remaining rather unaltered. Hartmann and Delgado (2004) explain that the alteration visualized in the cell walls is a consequence of the effect of HPP, which causes an extensive strain on the membranes and transmits a considerable force onto the cell walls. In this regards, it has been reported that changes in the polysaccharides of the cell wall of HPP-treated fruit tissues could be involved in this alteration (Hilz, Lille, Poutanen, Schols, & Voragen, 2006).

Also interesting is the reduction of the intercellular spaces, which were filled with cytoplasmic material leaked out from the cells as a consequence of the application of the treatment. This phenomenon was also observed in yellow peaches treated at 600 MPa for 5–30 min (Zhang et al., 2012) and in persimmon treated at 200 MPa for 5 min (Hernández-Carrión, Vázquez-Gutiérrez, Hernando, & Quiles, 2014). These studies suggest that the structural changes caused by the HPP may contribute, at a cellular level, with the movement of the cytoplasmic content, probably conducted by the apoplastic, symplastic, and transmembrane transport routes. From the technological point of view, this process would considerably increase the extractability and availability of some bioactive compounds, contributing to their diffusion from the cells to the extracellular medium. This would be an additional advantage of the application of HPP for the development of functional plant foods with augmented concentration of bioactive compounds.

It is important to mention that in fruits with the structural characteristics of peaches, HPP can increase the cell permeability with the consequent movement of water out of the cells, resulting in a translucent or watery aspect. This can be considered as a negative side-effect because of its impact on the general appearance of the product (Rastogi, 2009). However, this effect is mainly caused by the vacuum packaging, since it is already evidenced before the application of the HPP (Denoya et al., 2015). The implications of this effect from the sensory point of view should be evaluated and improved accordingly.

The fact that most of the textural parameters remained rather unaffected in minimally processed peaches subjected to HPP, as previously found by our group (Denoya et al., 2015), evidences that the textures of fresh and treated fruit are reasonably similar. In addition, the microstructural analysis carried out in the present research contributes to confirm the previous claim, by showing the invariability of the general structure of the tissue in fruits subjected to this kind of processing. The minor decrease in some of the texture parameters, such as hardness and cohesiveness, as previously informed (Denoya et al., 2015), could reflect the slight changes verified in the microstructure of the treated peaches.

On the other hand, the higher rate of softening observed in that work during the cold storage of control peaches, by comparison to the treated ones, could be caused by the effect of HPP over cell wall-degrading enzymes such as pectinmethylstearase (PME) and polygalacturonase (PG). In this regard, Hernández-Carrión, Hernando, and Quiles (2014) linked the maintenance of hardness in HPP-treated sweet peppers to the inhibition of PG provoked by this treatment. It is important to consider that in fruits, PME catalyzes the demethoxylation of pectins, rendering a demethoxylated polymer. This polymer could, in turn, either interact with divalent ions forming a gel-like structure (firming effect) or suffer a hydrolytic process by the action of PG (softening effect). It can be considered that the behavior of the tissue during storage could reflect the combined effect of the increased activity of PME together with the inhibition of PG (Jolie et al., 2012; Sila et al., 2008). Therefore, to better understand the textural behavior of pressurized peaches, it would be necessary to assess the inhibitory effect of HPP on the activity and expression of PME and PG. In this regards, additional studies are currently underway in our lab.

5. Conclusions

Although the present work demonstrates that a tissue subjected to high-pressure processing is not viable in terms of the classic viability tests like the one with FDA, the assessment of different biochemistry variables such as the relative expression of genes related to different enzymes, evidences that some metabolic processes are still active in the tissues after the application of the high-pressure treatments.

The expression of polyphenoloxidase was not significantly affected by the HPP treatment; however, the decrease in the activity because of the pressure, together with the diminished expression of phenylalanine ammonia-lyase, an enzyme related with the synthesis of phenols, was able to successfully limit the development of browning. On the other hand, the concomitant inhibition in the expression of alcohol dehydrogenase and piruvate decarboxilase, together with the decrease in the activity of ADH, resulted in the prevention of the development of the fermentative metabolism in the vacuum-packed product.

As a whole, the micro- and ultrastructural studies provided a better understanding as well as a strong evidence that HPP-treated peaches still preserve most of the features that characterize a fresh product, with only some minor modifications in the properties typical of a living tissue.

All the evidences found in the present study suggest that a deep review of the traditional definition of fresh product would be necessary, especially those that relate the physiology and biochemistry of tissues with aspects having a significant impact on the consumers perception. Since these aspects may reflect the preferences of consumers, it is expected that they will also strongly influence the decision-making process at the purchasing occasion. In this regards, one of the most important drivers for the consumption of minimally processed fruits and vegetables is precisely the fresh-like condition. This re-definition will encourage the spread out and adoption of innovative preservation technologies such as HPP.

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References

Artés, F., & Artés-Hernández, F. (2003). Etapas decisivas y diseño de instalaciones Para la elaboración de productos procesados en fresco. In M. G. L. y. M. González, & G. de Canarias (Eds.), *Productos hortofrutícolas mínimamente procesados* (pp. 57–78).

Denoya, G. I., Polenta, G. A., Apóstolo, N. M., Budde, C. O., Sancho, A. M., & Vaudagna, S. R. (2016). Optimization of high hydrostatic pressure processing for the preservation of minimally processed peach pieces. *Innovative Food Science & Emerging Technologies*, *33*, 84–93.

Denoya, G. I., Vaudagna, S. R., & Polenta, G. (2015). Effect of high pressure processing and vacuum packaging on the preservation of fresh-cut peaches. *LWT - Food Science and Technology*, *62*(1, Part 2), 801–806.

Di Rienzo, J., Casanoves, F., Balzarini, M., González, L., Tablada, M., & Robledo, C. (2010). *InfoStat, software Estadística. Vol. 2011*, Grupo InfoStat: Universidad Nacional de Córdoba, Facultad de Ciencias Agrarias.

Dörnenburg, H., & Knorr, D. (1997). Evaluation of elicitor-and high-pressure-induced enzymatic browning utilizing potato (*Solanum tuberosum*) suspension cultures as a model system for plant tissues. *Journal of Agricultural and Food Chemistry*, *45*(10), 4173–4177.

Gonzalez, M., Jernstedt, J., Slaughter, D., & Barrett, D. (2010). Influence of cell integrity on textural properties of raw, high pressure, and thermally processed onions. *Journal of Food Science*, *75*(7), E409–E416.

Hartmann, C., & Delgado, A. (2004). Numerical simulation of the mechanics of a yeast cell under high hydrostatic pressure. *Journal of Biomechanics*, *37*(7), 977–987.

Hernández-Carrión, M., Hernando, I., & Quiles, A. (2014). High hydrostatic pressure treatment as an alternative to pasteurization to maintain bioactive compound content and texture in red sweet pepper. *Innovative Food Science & Emerging Technologies*, *26*, 76–85.

Hernández-Carrión, M., Vázquez-Gutiérrez, J., Hernando, I., & Quiles, A. (2014). Impact of high hydrostatic pressure and pasteurization on the structure and the extractability of bioactive compounds of persimmon “Rojo Brillante”. *Journal of Food Science*, *79*(1), C32–C38.

Hills, B., Costa, A., Marigheto, N., & Wright, K. (2005). T 1 — T 2 NMR correlation studies of high-pressure-processed starch and potato tissue. *Applied Magnetic Resonance*, *28*(1–2), 13–27.

Hilz, H., Lille, M., Poutanen, K., Schols, H. A., & Voragen, A. G. J. (2006). Combined enzymatic and high-pressure processing affect cell wall polysaccharides in berries. *Journal of Agricultural and Food Chemistry*, *54*(4), 1322–1328.

Jacobo-Velázquez, D., & Hernández-Brenes, C. (2010). Biochemical changes during the storage of high hydrostatic pressure processed avocado paste. *Journal of Food Science*, *75*(6), S264–S270.

Jiménez, M., & García-Carmona, F. (1997). 4-substituted resorcinols (sulfite alternatives) as slow-binding inhibitors of tyrosinase catecholase activity. *Journal of Agricultural and Food Chemistry*, *45*(6), 2061–2065.

Jolie, R. P., Christiaens, S., De Roeck, A., Fraeye, I., Houben, K., Van Buggenhout, S., ... Hendrickx, M. E. (2012). Pectin conversions under high pressure: Implications for the structure-related quality characteristics of plant-based foods. *Trends in Food Science & Technology*, *24*(2), 103–118.

Keenan, D. F., Brunton, N., Gormley, R., & Butler, F. (2011). Effects of thermal and high hydrostatic pressure processing and storage on the content of polyphenols and some quality attributes of fruit smoothies. *Journal of Agricultural and Food Chemistry*, *59*(2), 601–607.

Krasnow, M., Matthews, M., & Shackel, K. (2008). Evidence for substantial maintenance of membrane integrity and cell viability in normally developing grape (*Vitis vinifera* L.) berries throughout development. *Journal of Experimental Botany*, *59*(4), 849–859.

Lara, M. V., Budde, C. O., Porrini, L., Borsani, J., Murray, R., Andreo, C. S., & Drincovich, M. F. (2011). Peach (*Prunus persica*) fruit response to anoxia: reversible ripening delay and biochemical changes. *Plant and Cell Physiology*, *52*(2), 392–403.

Li-Qin, Z., Jie, Z., Shu-Hua, Z., & Lai-Hui, G. (2009). Inhibition of browning on the surface of peach slices by short-term exposure to nitric oxide and ascorbic acid. *Food Chemistry*, *114*(1), 174–179.

Liu, X., Zhang, M., Duan, J., & Wu, K. (2008). Gene expression analysis of germinating rice seeds responding to high hydrostatic pressure. *Journal of Plant Physiology*, *165*(18), 1855–1864.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 — ΔΔCT method. *Methods*, *25*(4), 402–408.

Martínez, V., Nieto, A., Castro, M., Salvatori, D., & Alzamora, S. (2007). Viscoelastic characteristics of Granny Smith apple during glucose osmotic dehydration. *Journal of Food Engineering, 83*(3), 394–403.

Meisel, L., Fonseca, B., González, S., Baeza-Yates, R., Cambiazo, V., Campos, R., ... Silva, H. (2005). A rapid and efficient method for purifying high quality total RNA from peaches (*Prunus persica*) for functional genomics analyses. *Biological Research, 38*(1), 83–88.

Mentré, P., & Hoa, G. H. B. (2001). Effects of high hydrostatic pressures on living cells: A consequence of the properties of macromolecules and macromolecule-associated water. *International Review of Cytology, 201*, 1–84.

Miller, J. B., Pang, E., & Broomhead, L. (1995). The glycaemic index of foods containing sugars: Comparison of foods with naturally-occurring v. added sugars. *British Journal of Nutrition, 73*(04), 613–623.

Perera, N., Gamage, T. V., Wakeling, L., Gamlath, G. G. S., & Versteeg, C. (2010). Colour and texture of apples high pressure processed in pineapple juice. *Innovative Food Science & Emerging Technologies, 11*(1), 39–46.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research, 29*(9), e45.

Préstamo, G., & Arroyo, G. (1999). Protective effect of ascorbic acid against the browning developed in apple fruit treated with high hydrostatic pressure. *Journal of Agricultural and Food Chemistry, 47*(9), 3541–3545.

Rastogi, N. K. (2009). 16 effect of high pressure on textural and microstructural properties of fruits and vegetables. *Novel food processing: Effects on rheological and functional properties* (pp. 301).

Rickman, J. C., Barrett, D. M., & Bruhn, C. M. (2007). Nutritional comparison of fresh, frozen and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds. *Journal of the Science of Food and Agriculture, 87*(6), 930–944.

Sánchez-Moreno, C., Plaza, L., Elez-Martínez, P., De Ancos, B., Martín-Belloso, O., & Cano, M. P. (2005). Impact of high pressure and pulsed electric fields on bioactive compounds and antioxidant activity of orange juice in comparison with traditional thermal processing. *Journal of Agricultural and Food Chemistry, 53*(11), 4403–4409.

Saura, D., Laencina, J., Pérez-López, A. J., Lizama, V., & Carbonell-Barrachina, A. A. (2003). Aroma of canned peach halves acidified with clarified lemon juice. *Journal of Food Science, 68*(3), 1080–1085.

Sila, D. N., Duvetter, T., De Roeck, A., Verlent, I., Smout, C., Moates, G. K., ... Van Loey, A. (2008). Texture changes of processed fruits and vegetables: Potential use of high-pressure processing. *Trends in Food Science & Technology, 19*(6), 309–319.

Tangwongchai, R., Ledward, D. A., & Ames, J. M. (2000). Effect of high-pressure treatment on the texture of cherry tomato. *Journal of Agricultural and Food Chemistry, 48*(5), 1434–1441.

Trejo Araya, X. I., Hendrickx, M., Verlinden, B. E., Van Buggenhout, S., Smale, N. J., Stewart, C., & John Mawson, A. (2007). Understanding texture changes of high pressure processed fresh carrots: A microstructural and biochemical approach. *Journal of Food Engineering, 80*(3), 873–884.

Van der Plancken, I., Verbeyst, L., De Vleeschouwer, K., Grauwet, T., Heiniö, R. -L., Husband, F. A., ... Viljanen, K. (2012). (Bio) chemical reactions during high pressure/high temperature processing affect safety and quality of plant-based foods. *Trends in Food Science & Technology, 23*(1), 28–38.

Vazquez-Gutierrez, J. L., Hernandez-Carrion, M., Quiles, A., & Hernando, I. (2014). Influence of storage at 4C on the stability of high hydrostatic pressure treated onion. *Czech Journal of Food Sciences, 32*(1), 96–101.

Vázquez-Gutiérrez, J., Hernández-Carrión, M., Quiles, A., Hernando, I., & Pérez-Munuera, I. (2012). Impact of high hydrostatic pressures on the structure, diffusion of soluble compounds and textural properties of persimmon 'Rojo Brillante'. *Food Research International, 47*(2), 218–222.

Vercammen, A., Vanoirbeek, K. G., Lemmens, L., Lurquin, I., Hendrickx, M. E., & Michiels, C. W. (2012). High pressure pasteurization of apple pieces in syrup: Microbiological shelf-life and quality evolution during refrigerated storage. *Innovative Food Science & Emerging Technologies, 16*, 259–266.

Walker, J. (1992). Spectrophotometric determination of enzyme activity: Alcohol dehydrogenase (ADH). *Biochemical Education, 20*(1), 42–43.

Wiley, R. C. (1994). *Minimally processed refrigerated fruits & vegetables: Springer Science & Business Media.*

Woolf, A. B., Wibisono, R., Farr, J., Hallett, I., Richter, L., Oey, I., ... Requejo-Jackman, C. (2013). Effect of high pressure processing on avocado slices. *Innovative Food Science & Emerging Technologies, 18*, 65–73.

Zhang, F., Dong, P., Feng, L., Chen, F., Wu, J., Liao, X., & Hu, X. (2012). Textural changes of yellow peach in pouches processed by high hydrostatic pressure and thermal processing during storage. *Food and Bioprocess Technology, 5*(8), 3170–3180.