Changes in the microstructure and location of some bioactive compounds in persimmons treated by high hydrostatic pressure

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ABSTRACT

Persimmon fruit are an important source of phenolic compounds, dietary fibre, and carotenoids. However, the location of these elements in the tissue is directly connected with processing techniques used. The aim of this work was to study the effect of high hydrostatic pressure (HHP) on the microstructure of persimmon fruit cv. ‘Rojo Brillante’ during different ripening stages and the relationship of this treatment with changes in the location of some bioactive compounds. Samples from persimmon fruit cv. ‘Rojo Brillante’ from two ripening stages, with and without deastringency treatment (95–98% CO₂), were treated by HHP at 200 and 400 MPa during 1, 3, and 6 min. A microstructural study using cryo-scanning electron microscopy, light microscopy, and confocal laser scanning microscopy (Cryo-SEM, LM, and CLSM) was carried out. Total soluble solid (TSS) content and some textural properties were also analyzed. The microstructural study showed that application of HHP caused cell wall disruption and intracellular component dispersion throughout the tissue, together with some nutritionally interesting compounds (tannins, fibres, and carotenoids). TSS content diminished in astringent samples when HHP was applied. This was attributed to precipitation of soluble tannins, which are responsible for fruit astringency. Therefore, it might be possible to omit a deastringency treatment with CO₂ before HHP treatments. HHP treatments caused an overall decrease in firmness for both ripening stages and an increase in cohesiveness (TPA analysis) in the more advanced stage.

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1. Introduction

The beneficial effect of bioactive compounds and other nutrients in food, both vegetable and animal, depends on appropriate amounts of the compound reaching certain areas of the body in an active chemical form. During food processing, the reaction of enzymes with their corresponding substrates and the extraction of nutrients and bioactive compounds from organelles and other cellular compartments is favoured. This leads to changes that alter sensory (colour, texture, aroma) and nutritional qualities. Accordingly, the bioavailability of nutrients is affected by changes in the microstructure caused by processing (Parada and Aguilera, 2007).

Food processing using high hydrostatic pressure (HHP) involves the application of pressure on the product at a rate of between 50 and 1000 MPa. The main objective of HHP food processing is to obtain safe and wholesome food, while maintaining the sensory qualities. Most studies regarding the potential and limitations of HHP food processing have focused on microbial and enzymatic inactivation. The effect of this technology on nutritional compounds, vitamins, and bioactive compounds in foods has been less studied (Sancho et al., 1999; Patterson, 2000; Cano and De Ancos, 2005; Oey et al., 2008). Improvements in equipment design and research have enabled the development of a wide range of commercial fruit and vegetable produce treated by HHP (orange juice, apple juice, guacamole, fruit jam, mashed tomato, and onion slices) (Torres and Velazquez, 2005; Rastogi et al., 2007). However, there are other fruit and vegetables, such as persimmon, where the implementation of HHP is still at an experimental stage and it has only been tested on purees (De Ancos et al., 2000). Therefore it would be interesting to evaluate the effects of HHP treatments on fresh-cut persimmon.

In recent years, the cultivation of persimmon or kaki (Diospyros kaki L.) cv. ‘Rojo Brillante’ has expanded greatly and the fruit is now considered an important alternative to other crops. The persimmon is a good source of phenolic compounds, mainly consisting of condensed tannins (group B proanthocyanidins). These compounds have the ability to form stable complexes with metals and proteins and are responsible for the astringency of persimmon (Santos-Buelga and Scalbert, 2000; Nakatsubo et al., 2002). The conversion of soluble tannins (in astringent kaki), into insoluble tannins occurs during ripening or treatments such as the application of modified atmospheres with ethanol or CO₂ (Arnal and
Del Río, 2003). Persimmons also contain high levels of phenolic acids, such as ferulic or p-coumaric, and contain twice as much dietary fibre as apples (Gorinstein et al., 2001). Persimmons are also noted for their high level of carotenoid compounds and these contain significant antioxidants (β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene). Some are also active with provitamin A (β-caroteno and β-cryptoxantina) (De Ancos et al., 2000). Persimmons are also high in vitamin C and minerals such as potassium (Wright and Kader, 1996).

There are few studies on the microstructure of ‘Rojo Brillante’ (Salvador et al., 2007, 2008; Pérez-Munuera et al., 2009a, b). ‘Rojo Brillante’ persimmons belong to the group that is astringent when harvested, and astringency is removed with CO2. Firmness is the parameter that best describes the quality of this fruit (Salvador et al., 2007), and this is directly related to cell wall polysaccharides, most notably pectins and hemicelluloses (Yakushiji and Nakatsuka, 2007). The aim of this study was to examine the effect of high hydrostatic pressure (HHP) on the microstructure of ‘Rojo Brillante’ persimmons at two ripening stages and changes in the location of some bioactive compounds. This will help to a better understanding of the commercial viability of fresh-cut persimmons treated by HHP.

2. Materials and methods

2.1. Sample preparation

Persimmon fruit (Diospyros kaki cv. Rojo Brillante) were harvested at two different ripening stages of commercial maturity in Carlet and Alzira (Spain) at the beginning of November and December of 2009, respectively. The maturity index used is a visual observation of the external colour of the fruit (Salvador et al., 2007) and six maturity stages are accordingly defined, ranging from I (yellow-green) to VI (orange-red). Stages III (RS1) and V (RS2) of this scale were used in this work. For each ripening state, half of the batch was treated to remove astringency in closed containers in a temperature freezer (Dairei Europe, Denmark) for 24 h, transferred to the cryostat and allowed to equilibrate with the warmer temperature of the cryostat (−25 °C) for 15 min. The frozen samples were placed in the holder and sections were cut with a disposable blade using an anti-roll plate. Sections were picked up with an artist brush and transferred to coated glass slides, which had been previously put inside the cryo chamber for improved adherence of tissue sections.

Some sections were stained with a 0.2% aqueous solution of toluidine blue. Other sections were stained with 10% vanillin-HCl (1:1, v/v) for the identification of tannins (Valette et al., 1998). Stained samples were examined under a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan). Some non-stained cryostat sections were also studied.

2.2. Microstructural analysis

2.2.1. Cryo-scanning electron microscopy

A JSM-5410 SEM microscope (JEOL, Tokyo, Japan) was used with a Cryo CT-1500C unit (Oxford Instruments, Witney, UK) for the Cryo-SEM observation. Samples (1 mm thick pieces from the persimmon cubes) were placed in the holder, frozen in liquid nitrogen slush (T ≤ −210 °C). The frozen samples were then transferred to the Cryo unit, fractured, etched for 15 min (−90 °C) and gold-coated (2 mbar and 2 mA). Samples were then transferred to the microscope and examined at 10 kV, −130 °C and at a working distance of 15 mm.

2.2.2. Light microscopy

For light microscopy (LM) observation, cryostat sections (10 μm) were obtained from the persimmon cubes (CM1950, Leica Biosystems, Nussloch, Germany).

Persimmon cubes were frozen at −60 °C in an ultra low temperature freezer (Dairei Europe, Denmark) for 24 h, transferred to the cryostat and allowed to equilibrate with the warmer temperature of the cryostat (−25 °C) for 15 min. The frozen samples were placed in the holder and sections were cut with a disposable blade using an anti-roll plate. Sections were picked up with an artist brush and transferred to coated glass slides, which had been previously put inside the cryo chamber for improved adherence of tissue sections.

2.2.2. Microstructural analysis was also performed using confocal laser scanning microscopy (CLSM). This was achieved with a Nikon confocal microscope C1 unit fitted on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan). An Ar laser line (488 nm) was employed as a light source and the autofluorescence of the sample was observed through a 515/530 band-pass emission filter.

2.3. Total soluble solids and textural properties

2.3.1. Total soluble solids

Total soluble solids of the fruit juice were determined using a hand-held refractometer (Atago ATC-1E) and expressed as %. Juice was prepared in triplicate for each treatment and 60 g of persimmon cubes were blended for each juice.

2.3.2. Textural properties

Flesh firmness and cohesiveness were determined at room temperature with a TA.XTplus Texture Analyzer (Stable Micro Systems). Flesh firmness was expressed as the maximum breaking force in newtons (N) using a 4 mm diameter flat-tipped cylindrical probe at 1 mm/s test speed. A texture profile analysis was performed to determine cohesiveness. The samples were axially compressed in two consecutive cycles at 1 mm/s test speed and 75% compression, 3 s apart, with a 50 mm diameter flat plunger. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve (dimensionless). Firmness and cohesiveness values were an average of the measurements from eight cubes.

2.4. Statistical analysis

Results presented in graphs were reported as mean ± standard deviation. Data was subjected to variance analysis (one-way ANOVA), using Least Significant Differences (LSD) multiple comparison test with a 95% confidence interval for differences between means (Statgraphics Plus 5.1, Manugistics, Inc., Rockville, MA, USA). One-way ANOVA and LSD tests were performed between the control and the different HHP treatments for both astringent and non-astringent samples. One-way ANOVA and LSD tests were then performed to show differences between astringent and non-astringent samples with the same HHP treatment.
Fig. 1. Cryo-SEM. Astringent persimmons at the RS1 ripening stage. Control (A and D) and HHP treated samples at 200 MPa, 1 min (B and E) and 400 MPa, 6 min (C and F). IS: intercellular space; FIS: flooded intercellular space; SM: soluble material; TC: tannic cell; D: detached tonoplast/plasmalemma; CV: cavity; IM: insoluble material; CW: cell wall.

3. Results and discussion

3.1. Microstructural study

The parenchyma of astringent ‘Rojo Brillante’ persimmons at RS1 was quite compact, with small air-filled intercellular spaces. However, some of the spaces were filled with soluble material from the cells due to degradation during the maturation process (Fig. 1A). The parenchymatic cells observed in cross section were rounded, 100–130 μm in diameter. The vacuole, full of soluble material, covered totally the interior of the cell, so that the tonoplast could not be observed (Fig. 1D). Soluble solids were observed in the samples as a network; this is the typical eutectic artefact generated during the process of water sublimation while samples are prepared by Cryo-SEM. After HHP treatment at 200 MPa/1 min, a similar but more compact structure was maintained (Fig. 1B). Due to the degradation of the tissue caused by pressure, cells were deformed and a higher number of intercellular spaces were filled with soluble material from the inside of the cells. When pressure was applied, intracellular liquid spread throughout the tissue. The tonoplast or plasmalemma could now be observed as a consequence of the compression during the HHP treatment. Moreover, some cell walls were degraded. Some cells had a compact material inside their vacuoles (Fig. 1E). This structure corresponded to insoluble material, since the typical eutectic artefact could not be observed in these areas, and it has been associated with the precipitation of soluble tannins (Salvador et al., 2007, 2008). This new structural appearance is only observed in what some authors call tannic cells (Gottreich and Blumenfeld, 1991; Yonemori et al., 1997). Some authors (Cheftel, 1992) have already shown that pressure produces a decrease in the total molar volume associated with some chemical reactions. Therefore, pressure could encourage the precipitation and polymerization of tannins. At 400 MPa/6 min, cells collapsed, cell walls were damaged and cells became hardly distinguishable (Fig. 1C). In these samples, some small cavities occurred due to the expansion and compression produced by HHP treatment (Préstamo and Arroyo, 1998). The intercellular spaces were completely filled with liquid and insoluble material was also observed outside the cells in these samples (Fig. 1F).

Non-astringent samples at RS1 showed, after all the treatments (Fig. 2), a similar structure to the corresponding astringent samples (Fig. 1). However, the tissue was more sensitive to the application of pressure because the previous treatment with CO2 slightly affects the cellular structure (Salvador et al., 2007). Non-astringent control samples (Fig. 2A and D) had a higher proportion of intercellular spaces filled with liquid. Moreover, the tannic cells were filled with insoluble material (polymerized tannins) because of the applied deastringency treatment. Probably, the HHP treatment produced polymerization of some of the tannins that remained soluble after the deastringency treatment, not only inside but also outside the cells (Fig. 2E and F).

Tissue from the control samples of persimmon harvested at RS2 showed structural changes related to the maturation process (Fig. 3), when compared with tissue from control samples harvested at RS1 (Figs. 1A and 2). The plasmalemma was highly degraded and separated from the cell wall in many cells (Fig. 3A) and there was an increase in cell–cell separation, above all in non-astringent samples. Moreover, the majority of intercellular spaces were filled with soluble material. Non-astringent samples showed the typical tannic cells from the treatment with CO2 (Fig. 3D). The application of pressure (Fig. 3B, C, E and F) generated changes equivalent to those described for samples at RS1. At 400 MPa/6 min, degradation of the tissue was much greater and most cells were no longer distinguishable (Fig. 3C and F). Tannins were localized before and after HHP treatment using light microscopy. In the control astringent samples at RS1, without HHP treatment, cell walls were stained in purple (Fig. 4A) and soluble tannins stained in red with vanillin-HCl (Fig. 4B). These tannins, which were originally inside tannic cells, were dispersed throughout the tissue while the cryostat sections were being obtained. The effect of pressure on cell wall fracture was noticeable when low pressure was applied (Fig. 4C) for short time periods (200 MPa/1 min). Moreover, because of the pressure applied, the precipitated tannins were found inside tannic cells and in intercellular spaces stained in dark blue with toluidine blue (Fig. 4C). Tannins dispersed from the inside of cells and precipitated in the intercellular spaces were more clearly observed when samples were stained with vanillin-HCl (Fig. 4D). When higher pressure was applied, the tissue was more affected and the dispersion of tannins was greater (Fig. 4E and F). This could be related to a higher accessibility of these compounds in HHP-treated samples. Tissue from control non-astringent samples (Fig. 5A and B) showed polymerized tannins inside tannic cells owing to the effect of CO2 (Gottreich and Blumenfeld, 1991; Oshida et al., 1996). When 200 MPa/1 min was applied (Fig. 5C and D), greater cell distorsion and cell wall damage than in the astringent samples occurred (Fig. 4C). Tannins had been already polymerized inside the cells before the application of HHP. Therefore, they did not spread and
no precipitated tannins were observed outside the cells (Fig. 4D). At 400 MPa/1 min, the tissue was completely degraded and the polymerized tannins from the tannic cells were observed among the cell wall remains (Fig. 5E and F).

The effects of HHP treatment on the structure of persimmons at RS2 were similar to the effects previously described (micrographs not shown). However, the tissue from the control samples was more degraded due to the maturation process in both astringent and non-astringent samples.

In view of the results obtained, it can be deduced that HHP treatment produces changes in the distribution and aggregation of tannins which could affect their extractability when the fruit has not undergone CO2 treatment.

Autofluorescent bodies in the persimmon samples were distinguished using confocal laser scanning microscopy. In a previous study regarding the quantification of carotenoids in yeast cells, An et al. (2000) concluded that autofluorescence at wavelengths over 515 nm using an argon ion laser emitting 488 nm light was primarily due to carotenoids. Therefore, the brightest autofluorescent bodies found in persimmon samples have been identified as carotenoids, although cell walls were also observed as fluorescent structures (Fig. 6A and D). When these non-stained sections were observed using light microscopy, carotenoids were found distributed throughout the tissue (Fig. 6B) and they seemed to be associated with cell walls forming spherical bodies (chromoplasts) surrounded by a membrane (Fig. 6C). These chromoplasts were approximately 15 μm in diameter.

As previously described by cryo-SEM, cell walls were degraded after HHP treatment and this may explain the loss of fluorescent emission, although carotenoids still emit bright green fluorescence (Fig. 6D). In Fig. 6E, it can be observed using light microscopy that pressure favoured carotenoid diffusion from the chromoplasts, whose membrane could have been affected by the treatment. In detail (Fig. 6F), carotenoids seemed to be composed of globules (approximately 0.75 μm in diam.) associated in chains. Pressure favours the dispersion of these globules in the tissue. This could favour the bioavailability of carotenoids since they would now be more dispersed.

3.2. Total soluble solids

The TSS content for control samples in both ripening stages was approximately 17% in astringent samples and 15–16% in non-astringent samples (data not shown). A significant decrease in TSS...
content after CO₂ treatment was observed for both ripening stages. This could be related to the insolubilization of tannins (Salvador et al., 2007).

When HHP was applied to fruit at RS1, a significant decrease in TSS was observed in astringent samples, falling to values similar to those from non-astringent samples. This could be attributed to the effect of high pressure on the precipitation of soluble tannins, as observed in the microstructural study (Fig. 1). Non-astringent samples did not show a variation in the TSS content. Therefore, the application of 200–400 MPa to astringent ‘Rojo Brillante’ persimmon fruit caused a decrease in TSS, mainly tannins, equivalent to the decrease caused by the deastringency treatment with CO₂.

In fruit at RS2, TSS content in the samples treated with HHP was higher than in the corresponding samples at RS1. This can be attributed to broken walls and cell membranes caused by the application of HHP (see cryo-SEM discussion) which encourages the solubilization of certain substances whose concentration in the fruit increases as maturation progresses (sugars, pectic substances from cell walls, etc.).

3.3. Textural properties

3.3.1. Firmness

Firmness is an important quality parameter in persimmons. In this study, persimmons were considered fit for consumption when firmness values were above 2.5 N when measured with 4 mm diameter cylinder (Arnal and Del Río, 2004; Salvador et al., 2004). The samples at RS1 had a firmness of around 10.8 N, while the values at RS2 were between 3 and 4 N (Fig. 7). These values are consistent with those in previous studies (Salvador et al., 2004, 2005, 2007; Besada et al., 2008; Igual et al., 2008). Softening of persimmon fruit during ripening is associated with the solubilisation and hydrolysis of pectic substances in the cell wall due to the activity of enzymes such as pectinmethylesterase (PME) and β-galactosidase (Nakamura et al., 2003; Cardiello et al., 2004).

HHP treatment resulted in diminished firmness at both ripening stages. When 200 MPa was applied for short periods (1 and 3 min) to the astringent persimmon samples at RS1, the result was a significantly lower value for firmness when compared to those
treated with CO₂. According to Basak and Ramaswamy (1998), two effects can contribute to firmness value after HHP treatment. On the one hand, a tissue with completely flooded interstices has higher resistance than that with air-filled spaces. On the other hand, the enzyme pectin methyl esterase (PME), which is bound to the cell wall in the intact cell, is liberated and contacts its substrate, the highly methylated pectin. This causes de-esterification not only during the HHP application but also after the release of the pressure. The de-esterified pectin can form a gel-network with divalent ions such as Ca²⁺ and Mg²⁺, contributing to the firmness value. Persimmon contains approx. 6.5 and 9 mg of calcium and magnesium for every 100 g of fresh fruit, respectively (Park et al., 2006), and this may contribute to strengthening the structure. Since a greater number of intercellular spaces are filled with liquid from the interior of cells in the non-astringent samples, both effects are expected to be more relevant in these samples. However, when the HHP treatment conditions are more severe (200 MPa/6 min or 400 MPa/3 and 6 min), samples may be seriously disrupted leading to a permanent damage to the texture; Basak and Ramaswamy (1998) observed this fact when treating apple with 200 MPa and other vegetables such as carrots and red peppers with 400 MPa.

Firmness values of samples at RS2 were lower than those of the samples at RS1, with a decrease in firmness after the HHP treatments (Fig. 7B). As discussed in the microstructure section, when high pressure was applied to persimmons at an advanced ripening stage, damage in the tissue was much greater than for the earlier ripening stage. This would be the reason why firmness values were below the minimum acceptable levels in this case (Arnal and Del Río, 2004; Salvador et al., 2004). Furthermore, differences in firmness between HHP-treated astringent and non-astringent persimmons were lower than those at RS1. This could be due to the fact that persimmon tissue at RS2 was already degraded before the HHP treatments due to the maturation process.

3.3.2. Cohesiveness

The control samples at RS2 were less cohesive than those of the samples at RS1. In addition, the astringent samples were sig-

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**Fig. 5.** Light microscopy. Non-astringent persimmons at the RS1 ripening stage. Control (A and B) and HHP treated samples at 200 MPa, 1 min (C and D) and 400 MPa, 1 min (E and F). Toluidine blue stain (A, C and E) and vainillin-HCl stain (B, D and F). TC: tannic cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Fig. 6. Carotenoids in astringent persimmon parenchyma at the RS1 ripening stage. Control (A–C) and HHP treated samples at 400 MPa, 3 min (D–F). Confocal laser scanning microscopy (A and D) and light microscopy (B, C, E and F) images. CW: cell wall; CH: chromoplast; CC: carotenoid-lipid globules forming a chain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 7. Firmness (N) of samples of astringent and non-astringent persimmons with different HHP treatments: (A) ripening stage RS1; (B) ripening stage RS2. Means from eight replicates ± standard deviation. Values with the same letter do not have significant differences (P < 0.05). Asterisks mean there are significant differences (P < 0.05) between astringent and non-astringent samples for a specific treatment and ripening stage.

Fig. 8. Cohesiveness of astringent and non-astringent persimmon samples with different HHP treatments: (A) ripening stage RS1; (B) ripening stage RS2. Means from eight replicates ± standard deviation. Values with the same letter do not have significant differences (P < 0.05). Asterisks mean there are significant differences (P < 0.05) between astringent and non-astringent samples for a specific treatment and ripening stage.

Fig. 7.

Fig. 8.

Fig. 6.

4. Conclusions

HHP treatment produced a significant effect on the structure of persimmon, affecting the integrity of cell walls and membranes. Much of the soluble tannins spread outside vacuoles, carotenoid substances were released from the chromoplasts and cell walls

significantly more cohesive than the non-astringent in both ripening stages (Fig. 8).

When applying HHP to the samples at RS1 (Fig. 8A), there was a decrease in cohesiveness that could be attributed to the rupture of cell walls and membranes, as well as to separation between cells. Only non-astringent samples treated at 200 MPa for 3 min maintained values similar to untreated samples.

However, at RS2 (Fig. 8B) the astringent samples treated with HHP maintained, or even increased cohesiveness in comparison with untreated samples; non-astringent samples also experienced a general increase in cohesiveness (which resulted especially high when 400 MPa was applied). Some authors have associated increased cohesiveness in other fruit with the formation of a gel-like network between pectin and divalent ions (Basak and Ramaswamy, 1998), as previously stated in the firmness section.

4. Conclusions

HHP treatment produced a significant effect on the structure of persimmon, affecting the integrity of cell walls and membranes. Much of the soluble tannins spread outside vacuoles, carotenoid substances were released from the chromoplasts and cell walls
were degraded. Due to these effects, the distribution of tannins, carotenoids and some fibre fraction changed after HHP treatments. This could affect the extractability of these compounds. The application of HHP provoked the precipitation of soluble tannins in “Rojo Brillante” persimmons, which could be related to the loss of astringency. Moreover, the deastringency treatment with CO2 and the ripening stage are significant factors in HHP-treated persimmons.

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