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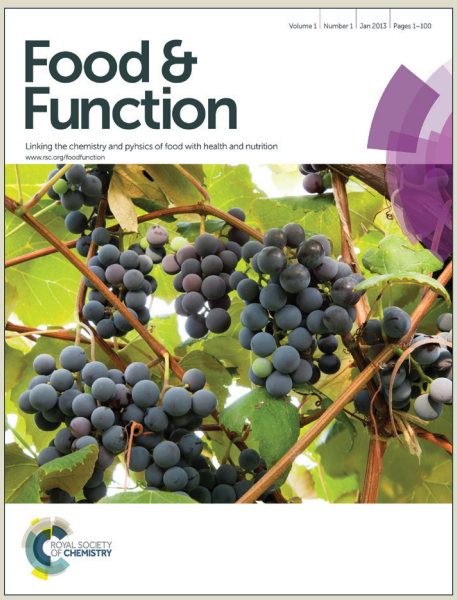

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High hydrostatic pressure treatment provides persimmon good characteristics to formulate milk-based beverages with enhanced functionality

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Abstract

High hydrostatic pressure (HHP) applied during food processing can improve the retention of food quality attributes and nutritional values in comparison with pasteurization. Persimmon is a good source of bioactive compounds but they are a seasonal fruit that cannot be consumed throughout the year. The aim of this work was to compare the HHP and pasteurization treatments to formulate milk-based beverages containing this carotenoid rich ingredient and to evaluate their performance in these beverages. The carotenoid and tannin content of persimmon were determined and the microstructure and rheology of the new milk-based persimmon beverages were examined. The results showed that HHP treatment favoured the release of carotenoids from the fruit matrix and precipitation of the tannins. The milk-based beverages prepared with the high-pressure persimmon presented the best rheological properties because unlike the untreated and pasteurized persimmon milk-based beverages, they did not form a gel-like structure or separate out.

Keywords: carotenoids, high hydrostatic pressure, microstructure, persimmon, rheological properties, tannins.

Introduction

Persimmon (*Diospyros kaki* L. f.) is an important and widespread fruit crop in China. In Europe, Spain is one of the major producers reaching in Valencia more than 130 000 tonnes in 2012 [1]. Persimmons are among the fruits with the highest levels of bioactive antioxidant compounds [2], such as carotenoids and polyphenols. The main polyphenols in persimmons are tannins, which have degenerative disease prevention effects [3], but when they are in their soluble form they can impart astringency to the persimmon fruits [4]. Carotenoids such as lycopene, lutein, and zeaxanthin have considerable antioxidant potential [5-6]. Another important role of carotenes, especially β-carotene, is provitamin A

activity [7-8]. A major variety of persimmon grown in Spain is 'Rojo Brillante', an astringent cultivar which requires postharvest deastringency treatment before the fruit can be marketed such as exposure to carbon dioxide in high concentrations, appropriate ethylene treatment or drying after peeling [9]. Exposure to high levels of carbon dioxide (950 g $kg^{-1}$for 24 h at 20 ºC) has proven to be the most effective way to remove astringency while maintaining fruit firmness [10].

Persimmons are normally sold in fresh form but are a seasonal fruit that cannot be consumed throughout the year. Therefore, it would be useful to develop industrial processes that prolong their shelf life and that make it possible to produce persimmon derivatives for fruit juice mixtures, jams, yoghurts or ice creams from astringent varieties, in order to obtain products with high nutritional value. Until now, thermal processing has been the method most commonly employed because of its ability to inactivate microorganisms and spoilage enzymes (polyphenoloxidase, pectinmethylesterase, etc.). However, heat may induce chemical and physical changes that damage organoleptic properties and may reduce the content or bioavailability of some bioactive compounds, particularly if applied under severe conditions [11-13]. According to Rawson et al. [14], most authors have reported that pasteurization leads to a decrease in the bioactive content (carotenoid content, phenolic content and antioxidant activity) of some fruits and vegetables such as exotic fruits [15], mangos [16-18] and mulberry fruit extract, durian juice, pineapple juice, and cashew apple juice [19-22]. High hydrostatic pressure (HHP) processing would appear to be a suitable alternative to thermal processing as it is considered one of the most economically viable of the non-thermal technologies and makes it possible to obtain products with high nutritional and quality parameters compared to conventional thermal processing [14, 23]. HHP treatment is expected to be less harmful to low molecular weight food compounds such as flavouring agents, pigments and vitamins and preserves the nutritional value of treated food better than heat due to its limited effects on covalent bonds [14, 24]. HHP

treatment at ambient temperature is reported to have minimal effect on the bioactive content of various fruits and vegetables [24].

Both HHP treatment and pasteurization can cause changes in the structure of the plant tissue which could be related to modifications in the bioavailability of some bioactive compounds. So, the microstructural study of food products could clarify the relationship between structure and functionality. In this sense, confocal laser scanning microscopy (CLSM) could be a suitable technique to study the interactions among components using specific dyes and their fluorescent excitation-emission features. CLSM has been employed to study the microstructure of some dairy products such as the interaction between carrageenan and milk proteins [25], the distribution of fat and protein in different dairy products [26], the structure dairy products with different compositions [27], or the effect of whey protein addition on the structural properties of stirred yoghurt systems at different protein and fat content [28].

The aim of this work was to study the effect of HHP and pasteurization processes on some bioactive compounds of the persimmon, as carotenoids and tannins, in order to formulate milk-based beverages with improved nutritional and functional properties. The microstructure and rheological behaviour of these milk-based beverages were also examined.

Materials and methods

Materials and milk based beverages preparation

Persimmon fruits (cv. Rojo Brillante) were harvested in Carlet (Valencia, Spain) at the beginning of November 2012. The maturity index was selected following the method of Salvador et al. [29], which defines six maturity stages based on the external colour ranging from I (yellow green) to VI (orange red); fruits on their commercial maturity stage (stage IV) were used in the present work. Cubes (15 mm) were taken from the equatorial area of the fruits and heat-sealed in 110 x 220 mm plastic bags (Doypack type, Amcor, Spain). Each bag contained approximately 80 g of persimmon. One-third

of the bags were not subjected to any treatment (untreated persimmon). Another third of the bags (HHP-treated persimmon) was placed inside a hydrostatic pressure unit with a 2350 mL capacity (GEC Alsthom ACB 900 HP, type ACIP 665, Nantes, France) using water as the pressure medium; the pressure employed was 200 MPa for 6 min at 25 ºC (energy consumption 244 kJ kg^{-1}), based on previous studies where HHP treatment at 200 MPa was applied for 1, 3, and 6 min and the treatment at 200 MPa for 6 min gave the highest level of carotenoid compound extraction [30]. The last third of the bags was pasteurized in a water bath at 70 ºC for 15 min with an energy consumption of 3600 kJ kg^{-1} (pasteurized persimmon). Each type of persimmon was homogenised during 90 s and freeze-dried during 120 h at -45 ºC and $1.3 \cdot 10^{-3}$ mPa in a freeze-drier (Lioalfa-6®, Telstar, Terrassa, Spain) before their use in the milk based beverages.

Nine different milk-based persimmon beverages were studied varying the type of milk and the treatment of the persimmon. Three different types of milk were used: whole milk (36 g L^{-1} fat content), semi-skimmed milk (15.5 g L^{-1} fat content), and skimmed milk (2.5 g L^{-1} fat content) from Central Lechera Asturiana, Siero, Spain. Three different types of freeze-dried persimmon were employed: untreated, HHP-treated and pasteurized persimmon. The quantity of persimmon used in the formulation of 1 L of milk-based beverage was calculated to have the same carotenoid content to that in 200 g of fresh persimmon (0.743 mg β-carotene/100 g fresh weight). .

The milk-based beverages were prepared by placing the corresponding amount of freeze-dried untreated persimmon (115.9 g kg^{-1}), freeze-dried HHP-treated persimmon (52.8 g kg^{-1}) or freeze-dried pasteurized persimmon (95.1 g kg^{-1}) in a food processor (Thermomix TM31, Wuppertal, Germany) and stirring at increasing agitation speeds (1100 rpm, 3250 rpm and 10200 rpm), for 10 s at each speed, in order to reduce the particle size of the freeze-dried persimmon samples. The milk was added and stirred following the same procedure (1100 rpm, 3250 rpm and 10200 rpm, for 10 s at each speed). All the milk-based beverages were kept at 4–5 ºC until their analysis. The

122 microstructure, rheological properties and loss of stability were analysed within 24 h of
123 milk-based beverage preparation.
124
125 **Extraction and quantification of carotenoids**
126 The total carotenoids were determined according to the method described by Hornero-
127 Méndez & Mínguez-Mosquera [31] with modifications. Freeze-dried persimmon (5 g) was
128 extracted with 25 mL of cool acetone using a homogenizer (IKA T25 Basic Ultra-
129 Turrax) and vacuum filtered until no more colour was extracted. The extract was added
130 gradually to 50 mL ethyl ether in a decanting funnel. With each addition of extract,
131 enough NaCl solution (100 g L^{-1}) was added to separate the phases and transfer the
132 pigments to the ether phase; the aqueous phase was removed. This process was
133 carried out in several steps to ensure maximum elimination of the aqueous phase. The
134 ether phase was treated several times with anhydrous Na_2SO_4 to remove residual
135 water and finally evaporated to dryness in a rotary evaporator (model RII; Büchi
136 Labortechnik, Flawil, Switzerland) at a temperature below 35 ºC. Finally, the pigments
137 were collected with acetone to a volume of 100 mL and the absorbance was measured
138 at 450 nm using a spectrophotometer (model Helios Zeta UV Visible; Thermo Fisher
139 Scientific Inc., Cambridge, UK). The calibration curve was constructed with different
140 concentrations of β carotene (Sigma Aldrich, Madrid, Spain) in acetone (Panreac,
141 Barcelona, Spain). The results were expressed as mg β carotene/100 g of dry weight
142 freeze-dried persimmon. Three separate carotenoid extractions were made for each
143 type of persimmon treatment and for untreated persimmon and the measurements
144 were performed in triplicate.
145
146 **Total soluble tannin content**
147 The total soluble tannin content of the freeze-dried persimmons was determined with a
148 spectrophotometer (Helios Zeta UV Visible) using the Folin Denis colorimetric method
149 as described by Arnal and Del Río [32]. Freeze-dried persimmon (5 g) was homogenised

with 25 mL of 800 g kg^{-1} methanol-water blend in a homogenizer (IKA T25 Basic Ultra-Turrax). The homogenate was centrifuged (14500 rpm, 20 min, 4 ºC) and filtered. The supernatant was kept. More supernatant was extracted from the pellet with 25 mL of 800 g kg^{-1} methanol and added to the first supernatant. The total supernatant was brought up to 100 mL with 800 g kg^{-1} methanol. In a test tube, 1 mL of the extract and 6 mL of distilled water were mixed and vortexed, then 0.5 mL of Folin Ciocalteu reagent (Panreac, Barcelona, Spain), was added. After 3 min, 1 mL saturated Na_2CO_3 was added, the mixture was vortexed and 1.5 mL distilled water was added. Absorbance was measured after 90 min at 725 nm. The calibration curve was constructed with different concentrations of gallic acid (Panreac, Barcelona, Spain) in 800 g kg^{-1} methanol. The results were expressed as g gallic acid/100 g of dry weight. Three separate total soluble tannin extractions were made for each persimmon treatment and for untreated persimmon and the measurements were performed in triplicate.

Confocal Laser Scanning Microscopy (CLSM)

Equipment and dyes. CLSM was selected as the most appropriate microscopy technique for studying the microstructure of the freeze- dried persimmons and the milk-based beverages, due to the ability of carotenoid compounds to emit fluorescence when excited by a laser line. This makes it possible to locate them using CLSM without staining the sample. Moreover, protein and fat can also be identified by using specific dyes such as Rhodamine and Nile Red, respectively. A Nikon confocal microscope C1 unit fitted on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) was used. An Ar laser line (488 nm) was employed as the light source to excite the Rhodamine B and Nile Red fluorescent dyes. Rhodamine B (Fluka, Sigma-Aldrich, Missouri, USA) with $\lambda_{ex\ max}$ 488 nm and $\lambda_{em\ max}$ 580 nm was dissolved in distilled water at 2 g L^{-1}. This dye was used to stain proteins and carbohydrates. Nile Red (Fluka, Sigma-Aldrich, Missouri, USA) with $\lambda_{ex\ max}$ 488 nm and $\lambda_{em\ max}$ 515 nm was dissolved in polyethylene glycol (PEG) 200 at 0.1 g L^{-1} and was used to stain fat. The autofluorescence of the samples

was observed using the Ar laser line without any dye. A 60x/1.40NA/Oil/ Plan Apo VC Nikon objective lens was used.

Sample viewing. A drop of freeze-dried persimmon or milk- based beverages was placed on a slide and 20 µL of Rhodamine B solution and 20 µL of Nile Red solution were added. The observations were made 10 min after diffusion of the dyes into the sample or beverage. The images were obtained and stored with 1024 x 1024 pixel resolution using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

Rheological measurements

Both the flow behaviour and the viscoelastic properties of each milk-based beverage were measured in triplicate. The measurements were carried out in a RS1 controlled stress rheometer (Thermo Haake, Karlsruhe, Germany), using a parallel plate geometry of 6 cm in diameter with a 1mm gap, and monitored by the RheoWin software package (version 2.93, Thermo Haake). A temperature of 10 ± 1ºC, selected as representative of the usual consumption temperature of dairy desserts; it was maintained throughout the measurements by means of a Phoenix P1 Circulator device (Thermo Haake). The milk-based beverages were allowed to rest on the rheometer plate for 5 min before measurement and a fresh milk-based beverage was loaded for each measurement.

Flow behaviour. The flow of milk and milk-based beverages was measured by recording the shear stress values when shearing them with a linear increasing shear rate from 0 to 200 s^{-1} for a period of 60 s and in reverse sequence for the same time. The areas under the upstream data point curve (A_{up}) and under the downstream data point curve (A_{down}), as well as the hysteresis area ($A_{thix}= A_{up}–A_{down}$), were obtained using RheoWin Pro software (version 2.93, Thermo Haake). The data from the

ascending segment of the shear cycle were fitted to the Ostwald-de Waele model (Eq. (1)) using RheoWin Pro software (version 2.93, Thermo Haake):

$$\sigma = K \cdot \gamma^{n} \quad (1)$$

where K (Pa s^n) is the consistency index and n is the flow index. In addition, apparent viscosity values at 10 s^{-1} and 100 s^{-1} (η_{10} and η_{100}, respectively) were also calculated as follows.

$$\eta_{app} = K \cdot \gamma^{n-1} \quad (2)$$

Viscoelastic properties. In order to determine the linear viscoelastic region (LVR), stress sweeps (0.01–100 Pa) were run at 1 Hz. Frequency sweeps were then performed within the LVR over the range *f* = 0.01-10 Hz; the values of the storage modulus (G') and the loss modulus (G'') as a function of frequency (mechanical spectra) were obtained using RheoWin Pro software (version 2.93, Thermo Haake).

Sedimentation

Sedimentation is very negative for the quality of food products and may cause consumers to reject the product. The loss of stability (sedimentation) of the milk-based beverages was measured by placing the milk-based beverages in 10 mL test tubes and leaving them to stand until the amount of sediment remained constant. This state was reached after 90 min. The % of sedimentation was calculated as (volume of sediment/total volume) x 100.

Statistical analysis

The effect of the persimmon treatment (untreated, HHP treatment and pasteurization) on the carotenoid and total soluble tannin content was analysed by one-way ANOVA. The effects of the type of milk (whole milk, semi-skimmed milk, and skimmed milk) on the flow parameters (K, n, $\eta_{10,}$ $\eta_{100,}$ and A_{thix}) and viscoelastic parameters (G' and G'') at

1Hz were analysed by one-way ANOVA for each type of milk-based beverage (prepared with freeze-dried untreated, freeze-dried HHP-treated, and freeze-dried pasteurized persimmon). The least significant difference (LSD) test with a 95% confidence interval was used to compare the mean values obtained. All the calculations were made with Statgraphics Plus 5.1 software.

Results and discussion

Carotenoid and total soluble tannin content of the freeze-dried persimmons.

Fig. 1A shows the carotenoid content of the different persimmons analysed in this study. The untreated and the pasteurized persimmons did not show statistically significant differences ($P > 0.05$) in the extracted carotenoid content. However, the HHP-treated persimmons presented a significantly higher extracted carotenoid content ($P < 0.05$). Similar results have been published for persimmon and orange juice, in which the authors reported increases in total carotenoids extracted after HHP processing [30, 33-34].

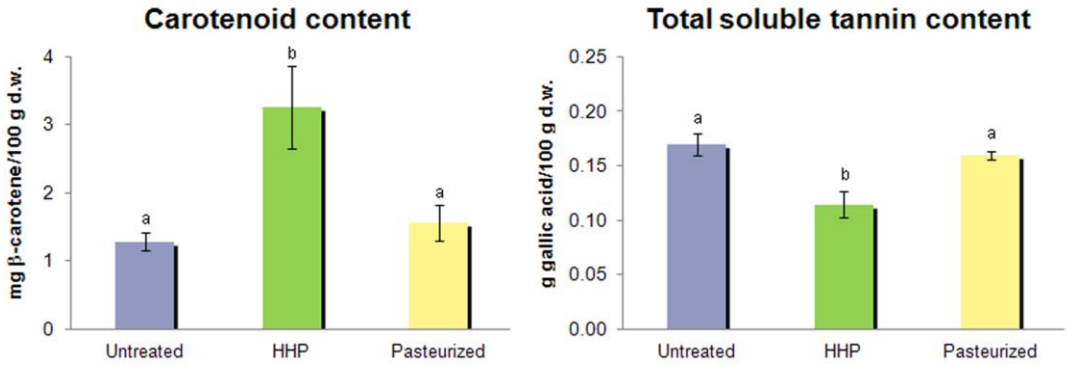

Fig. 1. Carotenoid content and total soluble tannin content of untreated, HHP-treated and pasteurized freeze-dried persimmon. Different letters indicate significant differences ($P < 0.05$) between the samples.

No statistically significant differences in total soluble tannin content were found between the untreated and pasteurized persimmons ($P > 0.05$). The HHP-treated persimmons presented significantly lower total soluble tannin content ($P < 0.05$). It has been proved that this treatment decreases the total soluble tannin content in

persimmon, as this treatment favours the precipitation of soluble tannins [35]. The fact that HHP treatment seems to favour tannin precipitation could be related to a decreased astringency of the HHP-treated fruit. So, this treatment could be used to develop new, less astringent and more versatile persimmon derivatives.

Confocal laser scanning microscopy (CLSM)

Freeze-dried persimmon. In order to understand the structure of the freeze-dried persimmon without the interference of the milk structure, samples were prepared by dispersing the corresponding amount of freeze-dried persimmon in deionised water and their microstructure was analysed. Fig. 2 shows the CLSM images of the freeze-dried persimmon dispersed in water. All the persimmon samples showed significant autofluorescence (Figs. 2A, 2E, and 2I) due to the presence of carotenoids. According to Vázquez-Gutiérrez et al. [36], carotenoids are associated mainly with cell walls, forming spherical bodies (chromoplasts). In the untreated samples (Fig. 2A) most of the carotenoids tended to aggregate into interconnected clusters, forming a network. In the HHP-treated and pasteurized samples (Fig. 2E and 2I) the carotenoid aggregates were smaller than in the untreated ones, probably because the carotenoids were more dispersed throughout the plant tissue. HHP treatments are known to induce morphological changes in plant cells which result in the rupture of cell walls [36-38]. These structural modifications could cause leaching of cellular constituents into the food matrix, and so the spread of carotenoids throughout the tissue. Other authors have established that thermal processing can affect functionalities such as carotenoid bioaccessibility due to its effect on the barrier properties of the cell wall polysaccharide network [39]. When the samples were stained with Nile Red, a specific dye for fat, (Figs. 2B, 2F and 2J) a green network was observed, consisting of unstructured fat from the plant persimmon tissues and carotenoid compounds, as these pigments were excited by the same wavelength as Nile Red. This network was denser and more widely-dispersed in the HHP and pasteurized samples (Figs. 2F and 2J) than in the untreated

ones (Fig. 2B); this fact could be related to the structural modifications occurred on the treated tissue (HHP and pasteurized) in comparison with the untreated one. Staining with Rhodamine dye (Figs. 2C, 2G and 2K) made it possible to view the plant persimmon structures in the samples because Rhodamine allows visualizing proteins and carbohydrates as those present in the cell wall. When Rhodamine and Nile Red (Figs. 2D, 2H and 2L) were used to stain protein and fat respectively, the samples showed a protein network (red) in which carotenoid pigments (green) were dispersed. The HHP-treated samples seemed to present the highest carotenoid content (Fig. 2H), as reported above (Fig. 1A).

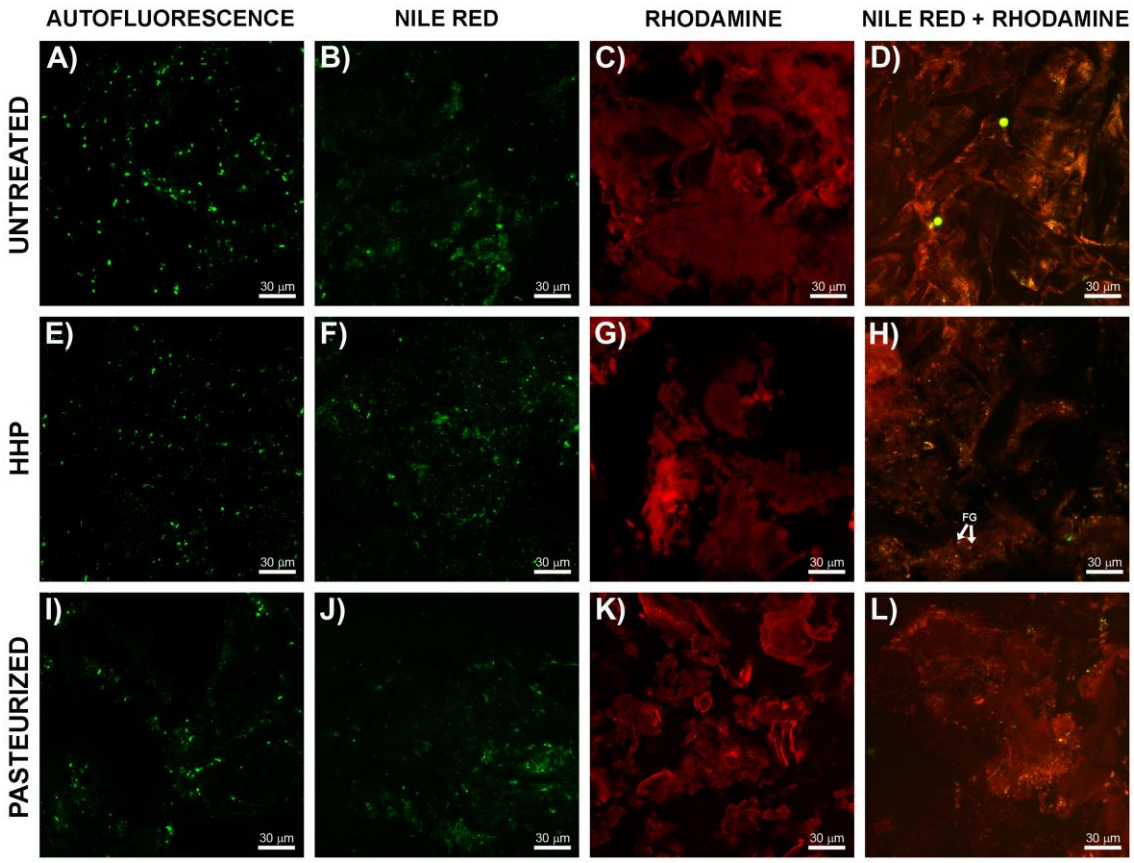

Fig. 2. Confocal Laser Scanning Microscopy (CLSM). Samples prepared with untreated, HHP-treated and pasteurized freeze-dried persimmon dispersed in water. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG: Fat globules. Magnification 60x.

Milk. Fig. 3 shows the CLSM images of the three different types of milk (whole, semi-skimmed and skimmed) employed for formulating the milk-based persimmon

beverages. The fat globules did not display intrinsic fluorescence, despite β-carotene being normally present in their composition (Figs. 3A, 3E and 3I). To study these different milks by fluorescence, an extrinsic fluorescent dye was therefore required [40]. Figs. 3B, 3F and 3J show the fat globules coloured green due to Nile Red staining. In Figs. 3C, 3G and 3K, the proteins can be seen as a continuous phase stained red by Rhodamine. Fig. 3D shows how the protein and lipid phases interacted, forming a homogenous network in whole milk. In semi-skimmed milk (Fig. 3H) this mixture was not as homogenous as in whole milk, as in these milks the protein acted as a continuous phase and the fat globules as a dispersed phase. In skimmed milk (Fig. 3L) the distinction between the two phases was more evident.

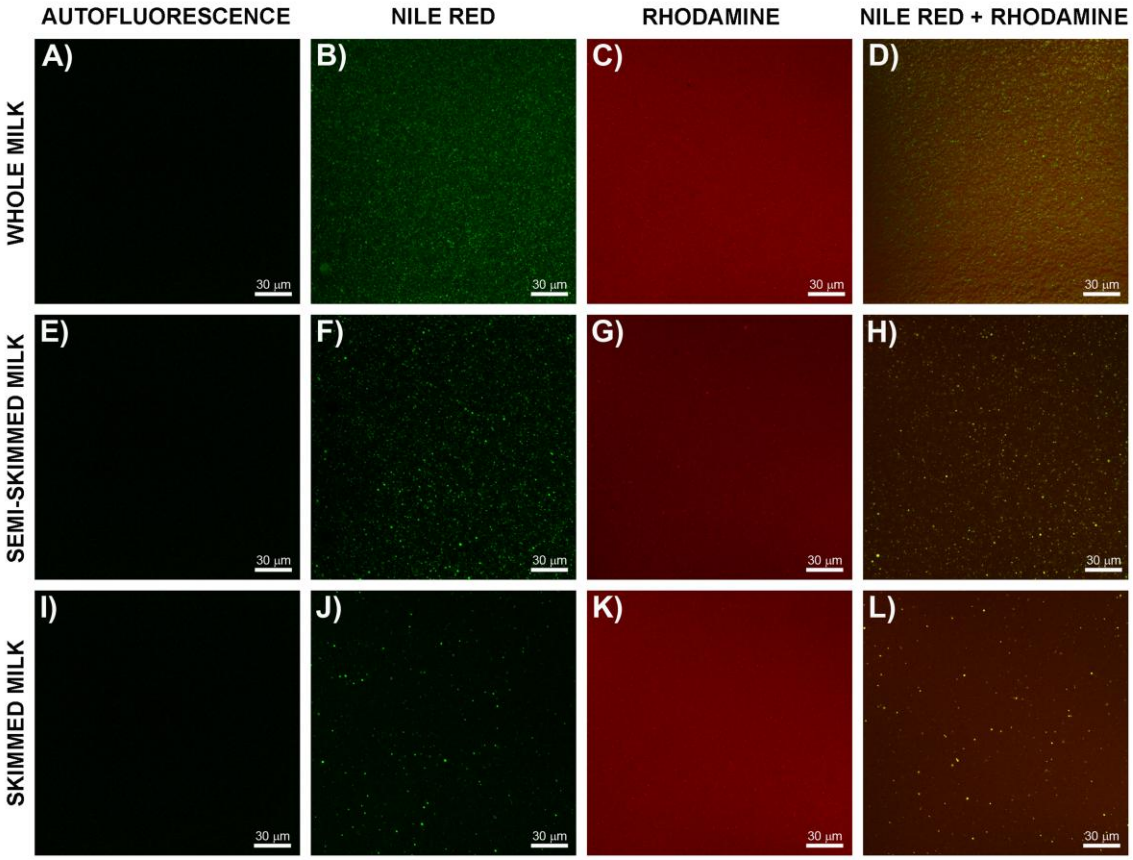

Fig. 3. Confocal Laser Scanning Microscopy (CLSM). Different milk matrixes (whole, semi-skimmed, and skimmed milk) used in the formulation of the milk-based persimmon beverages. Green: carotenoids and fat globules. Red: proteins and carbohydrates. Magnification 60x.

Milk-based persimmon beverage. Fig. 4 shows the CLSM images of the untreated persimmon milk-based beverages prepared with milk with different fat contents. All the beverages studied presented considerable intrinsic autofluorescence (Figs. 4A, 4E and 4I) due to the presence of carotenoids, which appeared as groups of spherical bodies. These bodies appeared to be linked up and arranged in a pattern. The milk-based beverages stained with Nile Red (Figs. 4B, 4F and 4J) showed a green network made up of fat globules and unstructured fat from the milk and unstructured fat and carotenoid compounds from the persimmon. Consequently, the fat from the milk was found to form two networks: one made up of globules connected together forming bright green clusters and another made up of unstructured fat and carotenoids coloured dark green.

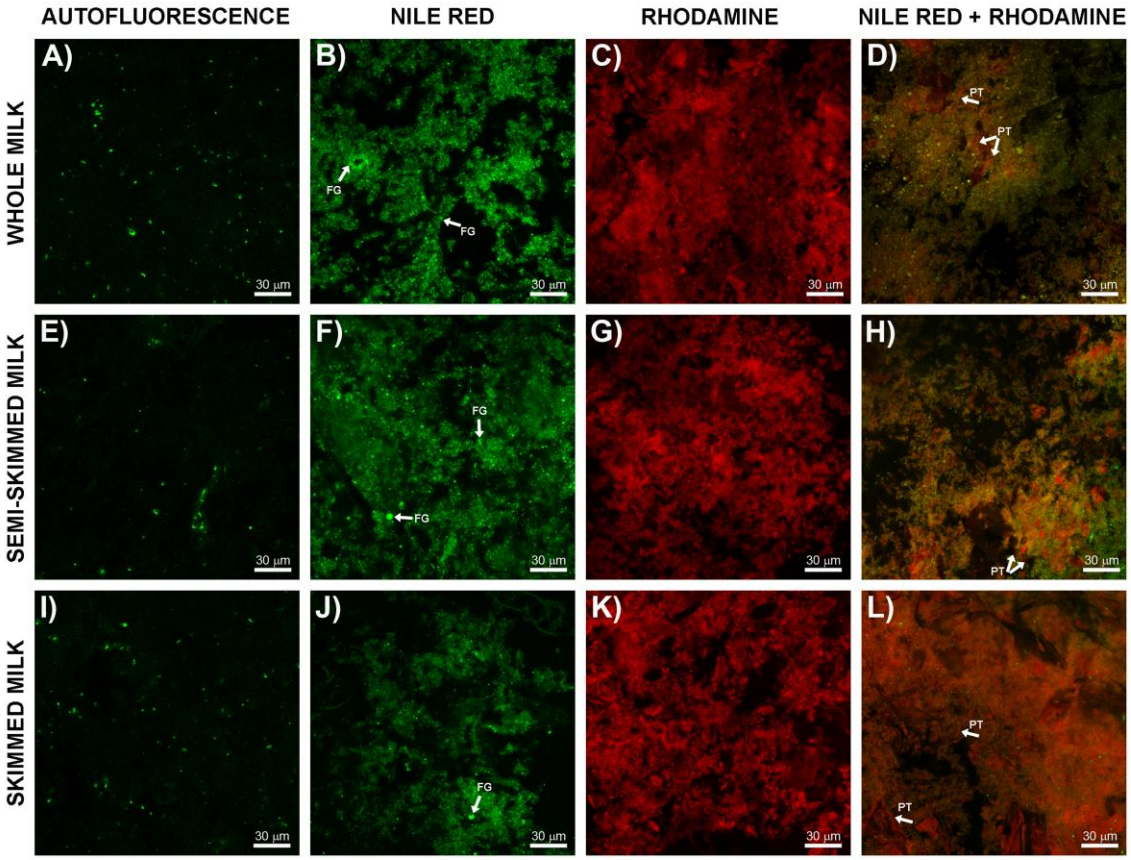

Fig. 4. Confocal Laser Scanning Microscopy (CLSM). Milk-based beverages prepared with freeze-dried untreated persimmon dispersed in whole, semi-skimmed and skimmed milk. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG: Fat globules, PT: Plant tissue. Magnification 60x.

In the milk-based beverages stained with Rhodamine (Figs. 4C, 4G and 4K), a red protein network was observed. This distribution was similar in all the milk-based persimmon beverages studied. This protein network, in which part of the plant tissue is fused, seemed to behave as a continuous phase that kept some fragments of plant tissue dispersed throughout. In the micrographs of the milk-based persimmon beverages with a high fat content stained with Nile Red and Rhodamine (Figs. 4D and 4H), two different phases could be observed: a continuous phase consisting of a dense lipoprotein network stained a yellow-green colour and a dispersed phase formed by green groups, probably carotenoids and fragments of plant tissue, with red contours. When skimmed milk was used in the formulations (Fig. 4L) the continuous phase was coloured orange and was mainly composed of protein, while the dispersed phase consisted of plant material, with red contours.

Effect of the persimmon treatment. The milk-based persimmon beverages prepared with freeze-dried HHP-treated persimmon (Fig. 5) differed from the milk-based beverages prepared with freeze-dried untreated persimmon (Fig. 4). The former presented higher autofluorescence (Figs. 5A, 5E and 5I) than the latter despite containing a lower quantity of persimmon in their formulation. This high autofluorescence made it possible to see fragments of persimmon, probably due to the dispersion of carotenoids throughout the tissue. Consequently, HHP treatment could encourage the release and extraction of carotenoids. Nile Red staining (Figs. 5B, 5F and 5J) showed a network made up of fat from the milk matrix and fat and carotenoids from the plant material. This network formed a continuous film in the HHP-treated milk-based beverages and clusters in the untreated ones which could be related, again, to higher carotenoid extractability obtained in the HHP-treated persimmons compared to the untreated ones. When Rhodamine (Figs. 5C, 5G and 5K) was used to stain the milk-based beverages a continuous and homogeneous red-coloured protein phase was observed, with red- and black-coloured fragments of plant tissue dispersed throughout

the milk matrix. Milk-based beverages with a high fat content stained with Nile Red and Rhodamine (Figs. 5D and 5H) showed two different phases, a continuous phase consisting of a dense lipoprotein network coloured yellow-green and a dispersed green-stained phase formed by carotenoids and fat with dark fragments of plant tissue. When skimmed milk was used in the formulation of the milk-based beverages (Fig. 5L) the continuous phase was composed basically of protein. The carotenoid compounds were better identified in these milk-based beverages (Figs. 5D, 5H and 5L) than in those made with freeze-dried untreated persimmon (Figs. 4D, 4H and 4L).

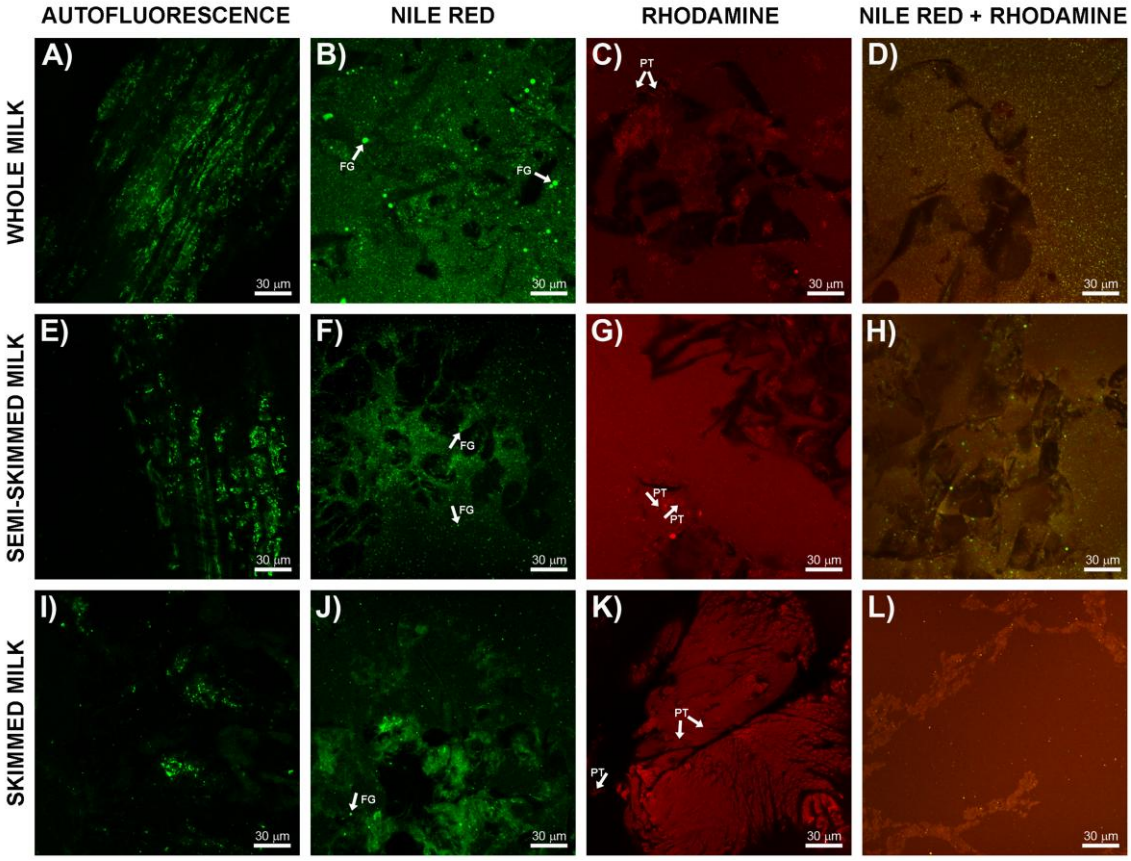

Fig. 5. Confocal Laser Scanning Microscopy (CLSM). Milk-based beverages prepared with freeze-dried HHP-treated persimmon dispersed in whole, semi-skimmed and skimmed milk. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG: Fat globules, PT: Plant tissue. Magnification 60x.

When the milk-based beverages were prepared with freeze-dried pasteurized persimmon (Fig. 6) considerable autofluorescence was also observed (Figs. 6A, 6E and 6I), although less than in the milk-based beverages prepared with freeze-dried

HHP-treated persimmon (Figs. 5A, 5E and 5I). It should be noted that the formulation of these milk-based beverages contained less persimmon than in those made with freeze-dried untreated persimmon, but more than when prepared with the freeze-dried HHP-treated persimmon, although containing the same carotenoid content. The milk-based beverages stained with Nile Red (Figs. 6B, 6F and 6J) showed a less dense network of fat and carotenoids than those with the freeze-dried HHP-treated persimmon.

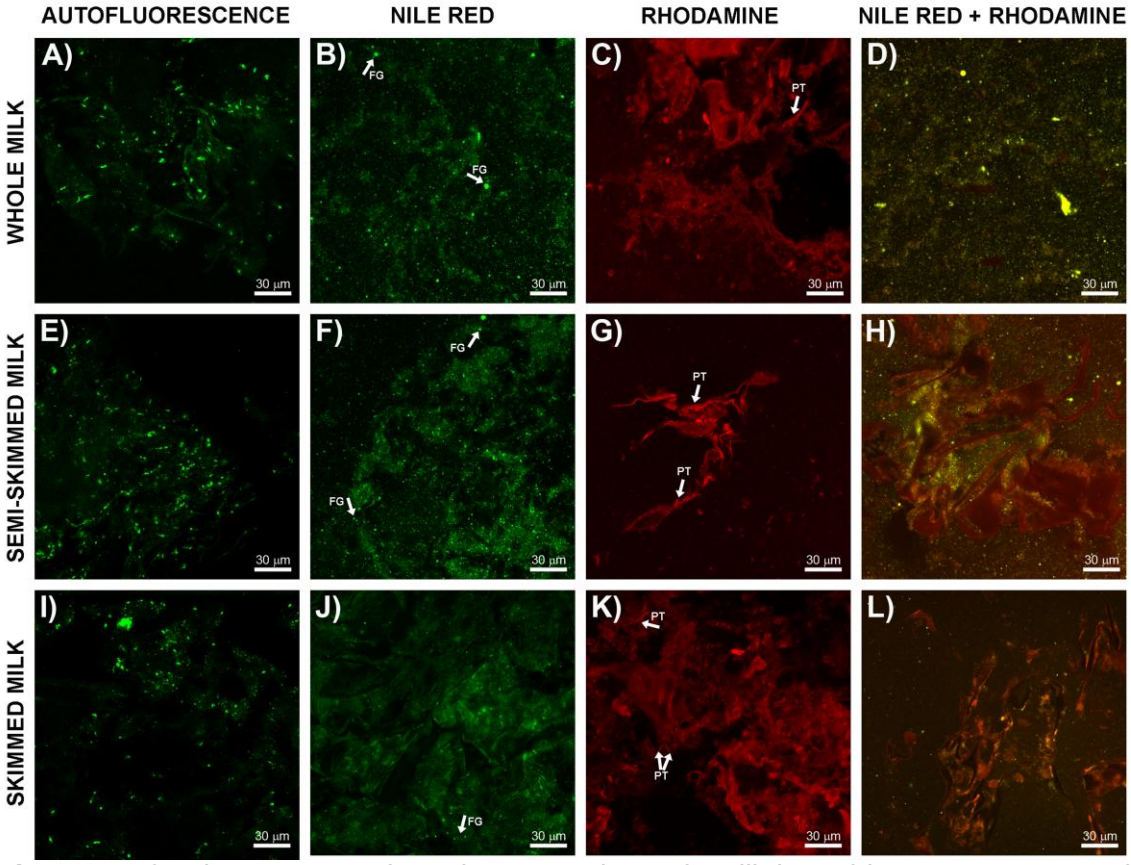

Fig. 6. Confocal Laser Scanning Microscopy (CLSM). Milk-based beverages prepared with freeze-dried pasteurized persimmon dispersed in whole, semi-skimmed and skimmed milk. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG: Fat globules, PT: Plant tissue. Magnification 60x.

When Rhodamine was used to stain the milk-based beverages (Figs. 6C, 6G and 6K), red- and black-coloured fragments of plant tissue were observed as a dispersed phase. When the milk-based beverages were stained with Nile Red and Rhodamine (Figs. 6D, 6H and 6L), the carotenoids appeared as a green dispersed phase. They were

observed more clearly than in the beverages with freeze-dried untreated persimmon (Figs. 4D, 4H and 4L) but less so than in those made with the freeze-dried HHP-treated persimmon (Figs. 5D, 5H and 5L).

Rheological behaviour of milk-based persimmon beverages

Flow behaviour. Fig. 7 shows the flow curves for the different milk-based persimmon beverages analysed in this study. The milk-based beverages prepared with the different types of persimmon presented different flow behaviour.

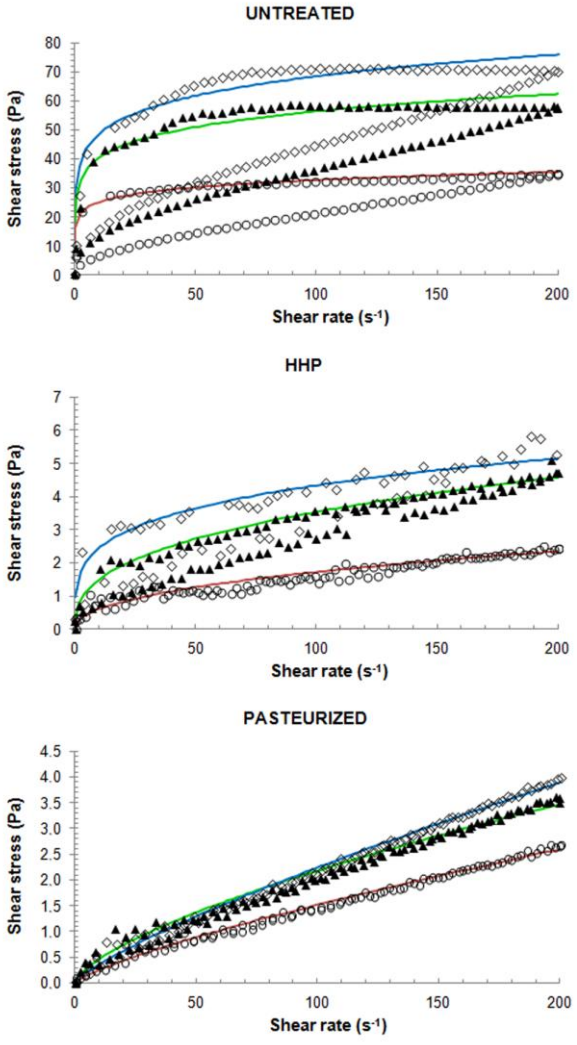

Fig. 7. Flow behaviour of milk-based persimmon beverages prepared with: freeze-dried untreated persimmon, freeze-dried HHP-treated persimmon, and freeze-dried pasteurized persimmon. Whole milk (◊), semi-skimmed milk (▲) and skimmed milk (○). Lines represent the curves fits to Ostwald models.

Those made with freeze-dried untreated persimmon were semisolid systems which could be attributed to the gelation of pectins in the presence of divalent ions like Ca^{+2} from the persimmon fruit and the milk. The gelation mechanism of persimmon has been studied previously in acidified and natural persimmon purée with and without the addition of ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA) [4]. Table 1 and 2 show the flow parameters of milk without persimmon and milk-based persimmon beverages, respectively. The flow curves for the milk-based beverages made with freeze-dried untreated persimmon, regardless of the type of milk used, showed pseudoplastic, time-dependent flow behaviour that was characterised by determining Ostwald–de Waele parameters and the thixotropic area (Table 2). The milk-based beverages prepared with freeze-dried untreated persimmon presented higher consistency, pseudoplasticity and thixotropy than milk without persimmon (Table 1). No statistically significant differences ($P > 0.05$) were observed in the consistency index (K), flow index (n), apparent viscosity at 10 s^{-1} (η_{10}), or thixotropic area (A_{thix}) when different types of milk were used (Table 2). It would therefore seem to be the addition of persimmon rather than the fat content of the milk that defines the flow behaviour of the milk-based beverages. However, the apparent viscosity at 100 s^{-1} (η_{100}) decreased significantly when skimmed milk was used ($P < 0.05$).

Table 1. Ostwald de Waele fit of milks used to formulate the milk-based persimmon beverages (R^2>0.9979)

Milk type	K (Pa·s^n)	n	η_{10} (Pa·s)
Whole milk	0.0079	0.8254	0.0053
	(0.0002)	(0.0005)	(0.0001)
Semi-skimmed milk	0.0066	0.8459	0.0046
	(0.0010)	(0.0260)	(0.0004)
Skimmed milk	0.0046	0.9015	0.0037
	(0.0004)	(0.0191)	(0.0001)

Values between parentheses are the standard deviations

Table 2. Ostwald de Waele fit of milk-based persimmon beverages ($0.915 < R^2 < 0.9957$)

	Milk type	K (Pa·s^n)	n	η_{10} (Pa·s)	η_{100} (Pa·s)	A_{thix} (Pa·s^{-1})
Untreated	Whole milk	28.275^a (8.719)	0.191^a (0.058)	4.313^a (0.770)	0.664^a (0.030)	3860.500^a (962.372)
Untreated	Semi-skimmed milk	31.630^a (3.847)	0.146^a (0.001)	4.423^a (0.544)	0.619^a (0.077)	4258.500^a (656.902)
Untreated	Skimmed milk	20.710^a (2.093)	0.125^a (0.013)	2.764^a (0.362)	0.369^b (0.059)	2597.000^a (576.999)
HHP	Whole milk	1.258^a (0.177)	0.253^a (0.007)	0.225^a (0.028)	0.040^a (0.004)	148.850^a (2.333)
HHP	Semi-skimmed milk	0.730^b (0.139)	0.353^b (0.029)	0.164^a (0.020)	0.037^a (0.002)	139.700^a (26.022)
HHP	Skimmed milk	0.225^c (0.011)	0.458^c (0.012)	0.065^b (0.005)	0.019^b (0.002)	34.055^b (21.970)
Pasteurized	Whole milk	0.050^a (0.011)	0.813^a (0.023)	0.032^a (0.005)	0.021^a (0.002)	9.639^a (4.145)
Pasteurized	Semi-skimmed milk	0.090^a (0.001)	0.670^a (0.003)	0.046^a (0.001)	0.022^a (0.000)	27.675^a (2.001)
Pasteurized	Skimmed milk	0.065^a (0.032)	0.714^a (0.085)	0.032^a (0.010)	0.016^a (0.002)	17.620^a (7.750)

Values between parentheses are the standard deviations.

All values shown are averages of three measurements. For each milk-based beverage, values within a column with different letters are significantly different

The HHP-treated persimmon milk-based beverages (Fig. 7B) were liquid systems that also showed pseudoplastic and thixotropic behaviour. As in the case of freeze-dried untreated persimmon, when compared the milks without persimmon (Table 1) with those of the milk-based beverage elaborated with freeze-dried HHP-treated persimmon, an increase in the consistency, pseudoplasticity and thixotropy could be observed (Table 2). However, these effects were much smaller than those observed with the freeze-dried untreated persimmon, indicating that gelation did not occur or was less intense with the persimmon obtained by HHP treatment. So the freeze-dried HHP-treated persimmon was more appropriate for formulating this kind of product since a gel-like texture would be not adequate for beverages. The ANOVA results showed that with the HPP-treated persimmon the flow parameter values varied significantly depending on the type of milk. As expected, as the fat content of the milk decreased, the consistency index decreased significantly ($P < 0.05$) and the milk-based beverages formulated with skimmed milk presented significantly the lowest values ($P < 0.05$). The opposite occurred with the flow index: as the fat content decreased, the flow index increased significantly ($P < 0.05$), which corresponded to a more Newtonian flow. No significant differences ($P > 0.05$) in apparent viscosity at 10 s^{-1} and 100 s^{-1}, and thixotropic area were found between the HHP-treated milk-based beverages made with whole and semi-skimmed milk, but the milk-based beverages formulated with skimmed milk presented significantly the lowest values ($P < 0.05$). It therefore seems that the flow behaviour of these milk-based beverages is governed by both the addition of persimmon and the fat content of the milk used in the formulation. Freeze-dried untreated persimmon has a gelling ability very high; so the presence of fat globules in the system structure does not significantly affect the response to deformation. In contrast, in the case of freeze-dried HHP-treated persimmon, which has less gelling ability, the presence of fat or not in the structure, does influence its response to deformation.

When freeze-dried pasteurized persimmon (Fig. 7C) was used in the formulation of the milk-based beverages, non-homogeneous dispersion of persimmon particles in a liquid matrix were obtained. This fact could probably correspond to the persimmon already being gellified and particles of persimmon gel remaining undissolved. As commented above, gelation of the persimmon could have taken place during pasteurization: the gel formation of pectins caused by heating process has been observed previously by other authors in olives [41] and persimmons [4]. A noticeable sedimentation was observed in these milk-based beverages (43 ± 1% for whole milk; 45 ± 4% for semi-skimmed milk, and 42 ± 3% for skimmed milk). This sedimentation could be responsible for the lower consistency index (K) and apparent viscosity at 10 s^{-1} and 100 s^{-1} obtained for these milk-based beverages compared to the values obtained when freeze-dried untreated and HHP-treated persimmon were used to prepare the milk-based beverages (Table 2). Before flow measurement, the milk-based beverages that showed sedimentation were stirred manually to favour homogenisation. In these milk-based beverages, Newtonian-like flow was observed, with more linear flow curves, without thixotropy (Fig. 7C) and flow index (n) values closer to 1 (Table 2) compared to the milk-based beverages prepared with freeze-dried untreated and HHP-treated persimmon. No statistically significant differences ($P > 0.05$) were observed (Table 2) in any of the flow parameters studied when different types of milk were used. The heterogeneous final product, with sedimented particles, could explain the fact that the type of milk used did not affect the rheological behaviour of the milk-based beverages. The flow response of the milk-based beverages made with freeze-dried pasteurized persimmon therefore seems to be mainly governed by the gelified persimmon particles.

Viscoelastic properties. Fig. 8 shows the storage modulus (G') and loss modulus (G'') values as a function of frequency. The mechanical spectra of the untreated persimmon milk-based beverages showed a response typical of weak gels, with G' higher than G'' (Fig. 8A). Although small differences in rheological parameters were noticed when

using different types of milk (Table 3), these differences were not significant (P > 0.05). As with flow behaviour, it was observed that the fat content did not affect the viscoelastic properties of the milk-based beverages.

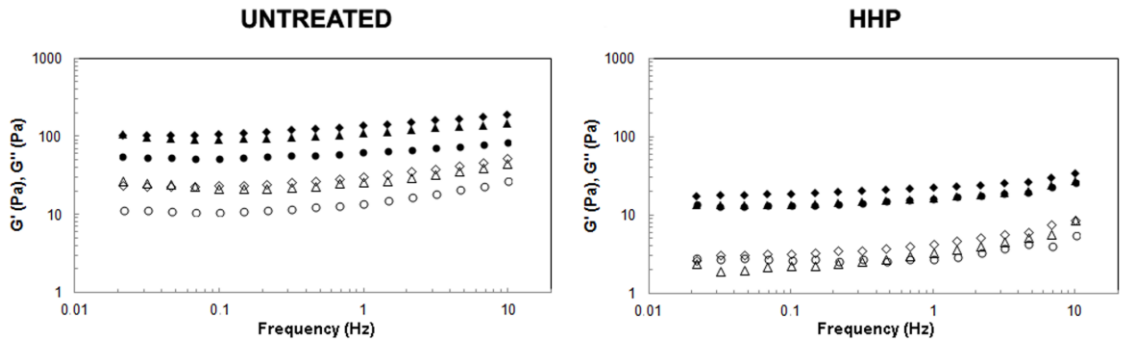

Fig. 8. Viscoelastic properties of milk-based persimmon beverages (G' *full symbols*, G'' *empty symbols,* whole milk, ◊; semi-skimmed milk, △; skimmed milk, ○).

Table 3. Storage modulus (G') and loss modulus (G'') values at 1 Hz for milk-based persimmon beverages

	Milk type	G' (Pa)	G''(Pa)
Untreated	Whole milk	138.300[a] (31.961)	30.950[a] (8.910)
Untreated	Semi-skimmed milk	113.095[a] (22.210)	26.420[a] (2.291)
Untreated	Skimmed milk	62.435[a] (4.250)	14.220[a] (0.325)
HHP	Whole milk	23.230[a] (1.428)	4.360[a] (0.074)
HHP	Semi-skimmed milk	17.080[b] (0.792)	3.344[b] (0.227)
HHP	Skimmed milk	16.315[b] (0.884)	2.766[b] (0.346)

Values between parentheses are the standard deviations.

All values shown are averages of three measurements. For each milk-based persimmon beverage, values within a column with different letters are significantly different.

The mechanical spectra of the HHP-treated milk-based beverages (Fig. 8B) also showed G' values higher than G'' ones, but both of them were much lower than those obtained with freeze-dried untreated persimmon and G'' showed higher frequency dependence, indicating a much weaker structure and therefore confirming the lower gelling ability of these milk-based beverages. No statistically significant differences (P > 0.05) in either G' or G'' at 1 Hz were found between the milk-based beverages made

511 with semi-skimmed and skimmed milk (Table 3). However, the milk-based beverages
512 formulated with whole milk presented significantly the highest G' and G'' values ($P < 0.05$).
513
514 Due to the heterogeneity and low viscosity of the milk-based beverages made with
515 freeze-dried pasteurized persimmon, the linear viscoelastic region could be not found
516 and viscoelastic properties for these milk-based beverages could not be determined.
517

518 Conclusions

519 HHP treatment would encourage the release of carotenoids from the plant material
520 matrix and hence increase their extractability as could be seen via confocal microscopy
521 and when quantifying the carotenoid content. In addition, HHP treatment seems to
522 favour tannin precipitation and could therefore decrease the astringency of the fruit.
523 Freeze-drying processing demonstrate to be useful to extend the shelf life of
524 persimmon and to obtain derivatives that could be incorporated in formulations with
525 new functional features. HHP treatment provides persimmon to formulate milk-based
526 beverages with high carotenoid content using smaller quantities of freeze-dried
527 persimmon. These beverages possess suitable rheological properties because they do
528 not form a gel-like structure, unlike the milk-based beverages with freeze-dried
529 untreated persimmon, and do not present sedimentation, unlike the milk-based
530 beverages formulated with freeze-dried pasteurized persimmon. Further research
531 should be conducted to evaluate the sensory properties and consumers' liking of these
532 milk-based persimmon beverages prior to their commercial release.

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