Shelf-life extension of multi-vegetables smoothies by high pressure processing compared with thermal treatment. Part I: Microbial and enzyme inhibition, antioxidant status and physical stability.

Shelf-life of multi-vegetables smoothies (part I)

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Abstract

Consumer demand for minimally processed food products based on fruits and vegetables is associated with their "fresh-like" qualities and a desire for convenience. Smoothies could help meet these needs and contribute to increasing fruit and vegetable intake. The first part of this study assesses microbial and enzyme inactivation, antioxidant status and physical stability of a vegetable smoothie (apple, carrot, zucchini, pumpkin and leek) stabilized (for up to 28 days at 4ºC) by high-pressure processing (HPP) (350MPa/5min/10ºC). Compared with mild heating (85ºC/7min), HPP ensured microbial quality (aerobic mesophilic and psychotropic bacteria, yeasts and moulds), inhibited peroxidase and slightly enhanced polyphenol oxidase and pectinmethylesterase enzymes. Consequently, the pressurized smoothies underwent earlier clarification and oxidation as reflected in their values of turbidity, browning index, viscosity and antioxidant capacity. Therefore, the pressurizing conditions and/or raw material selection need to be improved to achieve better stabilization by HPP.

Practical applications

High Pressure Processing allows fresh-like vegetable smoothies to be obtained with an extended shelf-life from the microbiological point of view. A handicap for industry is to choose the pressurization conditions able to maintain vegetable smoothies stable during a suitably long shelf time, without altering other properties of interest. This requires establishing pressurization patterns more adapted to the properties (enzyme activities, antioxidant status, colour, viscosity, turbidity, etc.) of the raw materials used in the homogenates.

Keywords

Like-fresh products, pasteurization, high hydrostatic pressure, vegetable smoothie.

Abbreviations

HPP: High-pressure processing, MH: Mild heating, AMB: Aerobic Mesophilic Bacteria, PYS: Psychrophilic Bacteria, YM: Yeasts & Moulds, PPO: polyphenol oxidase, POD: peroxidase,

PME: pectinmethylesterase, NTU: nephelometric turbidity unit, FRAP: ferric ion reducing antioxidant power.

1 INTRODUCTION

Consumers are increasingly demanding minimally processed products that retain the properties of fresh fruit and vegetables as long as possible (Huxley et al., 2004; Oey et al., 2008). In this context, the consumption of smoothies made of fruits and vegetables is associated with healthy and natural diets. When dehydrated fruit pulps are used to make smoothies, they are reconstituted with water or milk. Especially in the case of milk, the smoothie is nutritionally very interesting (Scarpin Guazi et al., 2019).

One of the current objectives of the food industry is to manufacture products that are readily associated by consumers with products containing fresh fruit and vegetables, as an alternative to conventional juices, jams and other fruit and vegetable products processed by pasteurization, sterilization or blanching, which can alter their sensory and nutritional properties (Deliza et al., 2005). One of the main concerns during the shelf-life of fresh smoothies is their stability during chilled storage, because they are prone to degradation by microorganisms, enzymes and oxidative reactions.

High pressure processing (HPP) is used as an alternative technology for processing fresh-like food products because it increases food safety and has a low impact on nutritional and sensory quality. Pressurizing at low or moderate temperatures causes the inactivation of microbial vegetative cells and enzymes without promoting major changes in the sensory and nutritional properties of food. HPP affects the viability of microbial cells (Patterson et al., 2012) and the structure of proteins/enzymes (Rastogi et al., 2007), while leaving mostly unaffected low molecular weight food compounds, such as vitamins, pigments, flavouring agents and other compounds (Barba et al., 2013; Butz et al., 2003; Fernández-García et al., 2001; Oey et al., 2008). Studies on HPP involving products based on fresh vegetables are becoming more numerous. Some authors have studied the effect of HPP in cabbage pieces (Alvarez-Jubete et al., 2014), turnip slices (Clariana et al., 2011), pumpkin slices (Zhou et al., 2014), carrot juice (Jabbar et al., 2014), two vegetable meals composed of pumpkin and broccoli, and of eggplant,

zucchini, chard and spinach (Masegosa et al., 2014) or green beans and broccoli pieces (Mc Inerney et al., 2007). However, less information is available on pressurized smoothies in which mixed vegetables are the main ingredient. In particular, there are few studies dealing with their microbial, physical-chemical, nutritional and sensory quality traits. A recent study performed on a fruit-based smoothie that incorporated carrot and beet established 627.5MPa/6.4min/21ºC as the optimal pressurizing conditions to inactivate altering microorganisms and altering enzymes, without affecting other physical and chemical properties (Fernández et al., 2018). However, it has been seen that these pressurizing conditions would have produced off-flavours in a fruit smoothie treated at 600 MPa (Hurtado et al., 2015), which is against the aim of obtaining "fresh-like" products. This limitation led to applying mild pressurizing conditions to pasteurize fruit smoothies without producing sensory alterations, although some risk of enzymatic deterioration remains (Picouet et al., 2016), which also needs to be evaluated in vegetable smoothies.

The objective of the present study was to compare the effects of a HPP mild treatment (350MPa/5min/10°C) and a mild heat treatment (85°C/7min) on the quality and stability of mixed fruit and vegetables based smoothies during their shelf-life under refrigeration, following the processing conditions of HPP and MH previously established for multi-fruits smoothies (Hurtado et al., 2015; Picouet et al., 2016; Hurtado et al., 2017a,b). The impact of smoothie processing on microbial and enzyme inactivation, antioxidant status and physical stability is presented in this publication, while the second part deals with the relevant nutrients and the sensory quality.

2 MATERIALS AND METHODS

2.1 Sample preparation

The smoothie formulation was based on commercial smoothies selected for their sensory properties. Smoothie composition by weight consisted of 20 % blanched carrot (*Daucus carota*), 20 % apples (*Pyrus malus* golden delicious), 20 % *Citrus* pectin solution 1 %,

19.9 % zucchini (*Cucurbita pepo*), 15 % pumpkin (*Cucurbita moschata* butternut), 5 % blanched leek (*Allium ampeloprasum* porrum) and 0.1 % salt. Fruit and vegetables were purchased at a local market.

Juices were obtained using a C40 juicer (Robot Coupé, Montceau-en-Bourgogne, France) and blended in a tank to achieve the above-mentioned composition. During sample preparation room temperature was stabilized at 14 ºC. Smoothies subjected to HPP were packaged in 250 mL polyethylene terephthalate (PET) bottles (Sunbox, Madrid, Spain), while a specific HT300 pouch (Seal Air Cryovac, Milano, Italy) was used in MH samples. Both packages were selected for high pressure and heat processing, respectively, to avoid the effect of packaging materials on the quality of the smoothie.

2.2 Thermal and high-pressure treatments

For the MH treatment, the samples were introduced into an Ilpraplus autoclave (Ilpra Systems, Mataró, Spain) and heated at 85 ºC for 7 min, including the initial ramp of 5.7 ºC/min, the total heating lasted 27 min. The conditions of mild heat treatment were selected in previous works with fruit smoothies (Picouet et al., 2016) to find, under mild heat conditions, microbiological safety (destruction of pathogens). HPP stabilisation consisted of the pressurization at 350 MPa for 5 min at an initial temperature of 9 - 10 ºC in a 120 l HPP system Wave 6500/120 (Hyperbaric, Burgos, Spain). The pressure ramp was 200 MPa/min and the total processing time was 7.3 min. The HPP treatment was selected based on the results obtained in a previous study with a fruit smoothie, in which three HPP treatments ranging from 350 to 600 MPa (350MPa/5min/10ºC, 450MPa/5min/10ºC, 600MPa/3min/10ºC) were tested (Hurtado et al., 2015), and in a preliminar HPP treatment of the multi-vegetable smoothie. After the respective treatments, samples were cooled and stored for up to 28 days at 4±1 ºC in darkness.

2.3. Experimental design

The studied parameters were measured in untreated products (only on day 1), after MH and HPP treatment (day 1), as well as throughout the refrigerated storage period at 4±1

ºC (day 1, 7, 14, 21 and 28) in darkness, representing the retail conditions used for these smoothies. Microbiological, physical-chemical, enzymatic analyses and/or measurements were taken in three independent samples (3 different 250 mL bottles/pouches) per day of sampling. Two replicates of the full experiment were performed.

2.4 Microbiological analyses

Microbiological analyses to enumerate spoilage microorganisms were performed before (unprocessed multi-vegetable smoothie) and after the HPP and MH treatments and after 7, 14, 21 and 28 days of storage at 4±1 ºC. For each sample, an aliquot of 10 mL was diluted (1/10, v/v) with sterile saline peptone water, which contained 1 g L^{-1} Bacto Peptone (Difco Laboratories, Detroit, MI, USA) and 8.5 g L^{-1} NaCl (Merck, Darmstadt, Germany). Further decimal dilutions were made using the same diluent. Total aerobic mesophilic bacteria (AMB) and psychrophilic bacteria (PSY) were determined on Plate Count Agar (PCA, Merck) after incubation at 30 ºC for 72 h and 4 ºC for 10 days, respectively. Yeasts and moulds were counted on Yeast Extract Glucose Chloramphenicol Agar (YGC, Merck) after incubation at 25 ºC for 5 days. Counts were expressed in log CFU mL^{-1}. The detection limit was 1.0 CFU mL^{-1} (0 log CFU mL^{-1}]).

2.5 Determination of the enzymatic activities

2.5.1 Peroxidase activity

Peroxidase (POD) was first extracted from smoothies by mixing a 10 mL sample with 10 mL 0.2 M sodium phosphate buffer pH 6.5. The mixture was centrifuged for 10 min at 15430 x g. The extraction of POD was performed in triplicate. The POD activity was spectrophotometrically measured by adding 1.1 mL 0.2 M sodium phosphate buffer pH 6.5, 0.5 mL enzyme extract, 1 mL O-phenylenediamine solution (10 g L^{-1} in 0.2 M sodium phosphate buffer pH 6.5) as substrate (proton donor) and 0.5 mL hydrogen peroxide solution (15 g/l in 0.2 M sodium phosphate buffer pH 6.5) as oxidant to a 1 cm path cuvette. The formation of the coloured oxidation product (2,3-diaminophenazine)

was measured as the change in absorbance at 485 nm and 25 °C for 20 min (Vervoort et al., 2011). The results were expressed as the percentage of relative activity (%), which was calculated as the ratio between the values of the treated (HPP or MH) and the untreated smoothies.

2.5.2 Polyphenol oxidase activity

The enzymatic activity of polyphenol oxidase (PPO) was assessed according to the procedure of Wang et al. (2014) with slight modifications. Samples (3 g) were homogenized in 6 mL 0.2 M sodium phosphate buffer, pH 7.0, containing 10 g L^{-1} insoluble polyvinyl pyrrolidone (PVP) and 5 g L^{-1} Triton X-100. Homogenates were centrifuged at 15430 x g for 10 min and PPO activity was determined by measuring the rate of linear increase in absorbance at 420 nm and 25 ºC. The reaction material contained 2 mL of 7 mM 4-t-butyl catechol solution, 1 mL of distilled water and 0.2 mL of the extract supernatant, containing the active enzyme. The reference cuvette contained only the substrate solution and distilled water. PPO activity was defined as the change in absorbance under conditions of the assay (Δ absorbance min^{-1}). The final results were expressed also as percentage.

2.5.3 Pectinmethylesterase activity

The enzymatic activity of pectinmethylesterase (PME) was determined according to the method of Li et al. (2015) with slight modifications. PME activity was measured by monitoring the release of free carboxylic groups of galacturonic acid during pectin methyl ester hydrolysis. PME activity was assayed reacting 5 mL of sample with 50 mL of a 1% (w/v) citrus pectin solution containing 0.2 M NaCl. During pectin hydrolysis, the pH was maintained constant by the addition of 0.01 N NaOH using a GLP21 pHmeter (Crison, Barcelona, Spain). The results of PME activity (expressed as PME units g^{-1}) were calculated using the equation:

$$PME\ units\ g^{-1} = \frac{ml\ NaOH * normality\ of\ NaOH}{weight\ of\ sample\ (g) * 30\ min}$$

2.6 Antioxidant capacity

The antioxidant status of the smoothies was quantitatively assessed by using the ferric ion reducing antioxidant power (FRAP) method (González-Hidalgo et al., 2012). The FRAP assay (μmol equivalents Fe^{2+} 100 mL^{-1}) was used to measure the antioxidant ability against all the reactive oxidative species. The antioxidant ability was determined from the blue compound formed as a result of the reaction between the sample solution and the FRAP reagent (acetate buffer, 2,4,6-Tripyridyl-S-Triazine and ferrous chloride) at 37 ºC. The absorbance of this compound was measured at 593 nm after 2 min.

2.7 Physical measurements and analyses

The degree of clarification in samples (bottle or pouch) kept in vertical position was measured. Transmittance (%) was read at 660 nm using pure water as a blank (100% transmittance) and a UV2 Series UV/Vis spectrophotometer (UNICAM, Cambridge, UK). Nephelometric turbidity (NTU) was measured using a 2100N Hach turbidimeter (Loveland, CO, USA). Pure water was the blank (0 NTU). The total soluble solids (TSS), CIELAB colour and pH (GLP21 pHmeter (Crison, Barcelona, Spain)) were determined in shaken samples. Total soluble solid content was determined using an ATC-1e hand refractometer (Atago, Minato-ku, Tokyo, Japan) and expressed as g 100 g^{-1} or ºBrix. Accuracy was ±0.1 ºBrix and the measuring range was 0-32 ºBrix according the Association of Official Analytical Chemists (1990) (AOAC 932.14). CIELAB colour was measured with a CR-200/08 Chroma Meter II (Minolta Ltd., Milton Keynes, UK) with D65 illuminant, 2º observer angle and 50 mm aperture size and calibrated with a standard white reflector plate (González Hidalgo et al., 2019). The results (CIE L* a* b* units) were expressed as L* or lightness (0 black to 100 white), Chroma ($C^* = \sqrt{(a^{*2} + b^{*2})}$) and Hue angle ($H^* = \tan^{-1} b^*/a^*$).

Samples were centrifuged at 15430 g for 10 min in a 5804 Eppendorf centrifuge (Hamburg, Germany) to determine viscosity in the supernatant and total insoluble solids as described by Ros et al. (2004). Absolute viscosity, expressed in centipoises (cP), was measured at 40 ºC using a No. 100 Ostwald Cannon-Fenske viscometer tube (Proton, Madrid, Spain). Total insoluble solids (g 100 g^{-1}) were calculated as the relative weight difference between the shaken sample and the resulting supernatant after centrifugation. Finally, the browning index (absorbance units) was determined according to the method of Ting and Rouseff (1986). A solution of the sample in methanol at 1:1 (v:v) was kept in an ice bath for 15 min and then the solution was centrifuged at 15430 g for 10 min in a 5804 Eppendorf centrifuge. The absorbance of the supernatant was measured at 420 nm in a UV2 Series UV/Vis spectrophotometer. Three repetitions of each measurement were made for each sample.

2.8 Statistical analysis

Data were analysed by ANOVA using the GLM procedure of SAS 9.01 (SAS Institute Inc, Cary, USA). The model for microbiological, physical, enzymatic activities and antioxidant capacity data included the treatment (Untreated, MH, HPP), storage time (1, 7, 14, 21 and 28 days) and the replica (1,2) as fixed effects. No significant interactions ($P>0.05$) were removed from the model. The mean differences were tested using the Tukey test ($P<0.05$).

3 RESULTS AND DISCUSSION

3.1 Previous HPP treatment

The Table 1 shows the residual enzymatic activities of a first multi-vegetal smoothie, subjected to three different conditions of processing by high pressures, and also subjected to moderate heat treatment, in order to confirm that in the multi-vegetable smoothie, in the same way as in that of fruits (Hurtado et al., 2015), the most favorable treatment is 350 MPa (5min, 10ºC). These conditions are chosen to carry out the HPP treatment of a second multi-vegetable smoothie and to carry out the whole study of the

shelf-life as the evolution of the multi-vegetable smoothies during the storage under refrigeration conditions (4ºC). The composition of both multi-vegetable smoothies is described in Materials and methods (2.1 Sample preparation). The treatment at 350 MPa was chosen (Table 1) because is the high pressure at which the PPO and PME enzymes are least activated, responsible for the loss of quality of the vegetable smoothie during storage.

3.2 Microbial load and pH

The microbial counts and pH values are shown in Table 2. In non-treated smoothies, the levels of AMB, PSY and YM were 4.9, 4.1 and 3.6 log CFU mL^{-1}, respectively, and were significantly reduced by both HPP and MH treatments. HPP decreased AMB counts by 2.6-3.0 log units while the reductions after MH were slightly higher (2.9-3.2 log units). After both treatments (day 1), the counts of PSY and YM fell to below the limit of detection, with reductions higher than 4.1 log units for PSY and higher than 3.3 log units for YM. During storage, there was a slight increase in PSY and YM from day 14, which was more evident in the HPP than in the MH smoothies. At the end of storage (day 28), the counts for the three evaluated microbial groups were lower in thermally treated than pressurized smoothies although in both cases the growth of spoilage microorganisms was inhibited by the moderately low pH of the smoothie and the effect of refrigerated storage.

The effectiveness of HPP has been demonstrated in several studies dealing with fruit juices, such as apple, orange, apricot and cherry (Bayindirli et al., 2006), pomegranate (Varela-Santos et al., 2012), cape gooseberry pulp (Vega-Gálvez et al., 2016), strawberry purée (Marszalek et al., 2015), apple puree (Landl et al., 2010), multifruit smoothies (Hurtado et al., 2015), red-fruits smoothies (Hurtado et al., 2017a, 2017b), and in vegetable juices (Pilavtepe-Celik, 2013), carrot juice (Kim et al., 2001), cucumber juice (Zhao et al., 2013), sliced pumpkin (Zhou et al., 2014) or pumpkin purée (García-Parra et al., 2016). Most results concerning the microbiological inactivation by pressurization refer to acidic products, including fruit juices. The vegetable smoothies

evaluated in this study, although less acidic than most usual fruit juices or smoothies, maintained a satisfactory microbiological quality (AMB<4 log CFU/g) until the end of the shelf-life. At levels below 6 log CFU/g, AMB are usually associated with a mixed microbiota while at higher levels there is usually a predominant microorganism and the organoleptic quality of the product may be compromised (Health Protection Agency, 2009).

3.3 Enzyme activities

The relative activities for PPO, POD and PME are shown in Table 3. PPO activity was enhanced as result of the HPP treatment (days 1 and 7) and then fell by less than 50% until day 28, while, after MH treatment (day 1), PPO activity was reduced by less than 50% and completely disappeared at the end of storage (day 28). Both treatments reduced POD activity by about 12-14% (day 1) although, unlike PPO, POD more or less maintained these levels throughout storage, with a slight loss of activity on day 28 in the HPP product. PME activity strongly increased after HPP treatment (day 1) and then gradually decreased but maintained considerable activity in the refrigerated product. By contrast, it decreased by around 60% after the MH treatment, remaining at these levels throughout storage. These results suggest that pressurized smoothies were more prone to enzymatic degradation than the thermally treated product, in particular, to cloud clarification.

Our results are in agreement with those previously reported, in which PME and POD activities were seen to increase in carrot juice after applying 500-600MPa and 350-500MPa/10min/70ºC/, respectively (Anese et al., 1994; Kim et al., 2001). The activation of PME (200-600MPa/10min/15-40°C) (Barón et al., 2006) and of PPO (450MPa/15min/25ºC) (Bayindirly et al., 2006) was also reported in pressurized apple. However, others found contrary results; for example, treatment at 100-200MPa/13min/20ºC decreased POD activity by 50% in carrot pieces (Van Buggenhout et al., 2006). Furthermore, in a study of different HPP treatments in pumpkin (300-

900MPa/1 min/60-80ºC), PPO partial inactivation was achieved for all treatments except for 300MPa/1min/70ºC, which, as occurred in our study, enhanced this enzyme (García-Parra et al., 2016). High intensity HPP treatments lead to protein denaturation and, depending of the pressurizing conditions applied, many enzymes can irreversibly lose their functionality. However, HPP mild treatments could activate these enzymes due to the reversible configuration of the enzyme after inactivation, structural changes that favour enzyme-substrate interaction (Anese et al., 1994) or the release of a second active centre (Butz et al., 2003). These findings might explain why HPP enhanced PPO and PME enzymes in our study. Whatever the case, the resulting clarification and discolouration also depends on the fruit and vegetables used in smoothies and juices, since different enzymes are involved (Hurtado et al., 2015; 2017a, b; Picouet et al., 2016; Ludikhuyze et al., 2003; McInerney et al., 2007). Indeed, no activation or inactivation for POD and PPO was observed when the same pressurizing conditions were applied in two different fruit smoothies (Picouet et al., 2016; Hurtado et al., 2017a). On the other hand, it has also been seen that MH treatments may not completely inactivate these enzymes; for example, Neves et al. (2012) had to apply high pasteurization temperatures (85ºC/3min) to achieve partial inactivation of POD in zucchini, while, in other study performed on carrot juice, PPO and PME activities decreased by up to 2% and 28%, respectively, after applying a "high temperature short time" treatment (105°C/30s) (Kim et al., 2001). Another treatment (627.5MPa/6.4min/22ºC) led to reductions of 85%, 45% and 10% in PME, POD and PPO, respectively, in a mixed fruit and vegetable smoothie (Fernández et al., 2018). Thus, the degree of inactivation for these enzymes in pressurized products may vary depending of the treatments and raw materials used, since the same type of oxidase or esterase enzyme, depending of its origin, might differently respond to the same treatment (Swami Hulle et al., 2017).

3.4 Antioxidant status

The antioxidant capacity of the vegetable smoothies was low, since the response in the FRAP assay was between 30-40 μmol Fe^{2+} 100 mL^{-1} (Table 3), which suggests that the

smoothies retain low level of antioxidants, as reported in the second part of this study, and also compared with the results of Abountiolas and Nunes (2018), who found a minimum value of 100 μmol Fe^{2+} 100 mL^{-1} in commercial berry smoothies bottled in aseptic conditions.

FRAP values were higher ($P < 0.05$) for MH smoothies than for HPP smoothies at all storage times, which agrees with the results reported for other fruit-based smoothies and juices (Keenan et al. 2010; Fernandez Garcia et al. 2003), although other authors reported the opposite results in orange juice (Polydera et al., 2005). This loss of antioxidant capacity may be explained by the lower degree of enzyme inactivation, in particular, of oxidase enzymes, because of HPP. The loss of antioxidant capacity found for both HPP and MH smoothies in our study is coherent with the data reported for their fresh ingredients, such as pumpkin (381 μmol Trolox 100 g^{-1}) (Zhou et al., 2014), carrot (27 μmol Fe^{2+} 100 g^{-1}) and apple (121 μmol Fe^{2+} 100 g^{-1}) (Araya et al., 2006). Besides the effects of the preservation treatment, the loss of antioxidant capacity in smoothies may be due to other factors, such as differences in the raw materials (maturation degree, storage conditions, etc.) and pre-treatments (blanching, mincing, etc.) used. For example, Mazzeo et al. (2011) reported a loss of antioxidant capacity after a blanching pre-treatment in carrot, cauliflower and spinach. Similarly, Araya et al. (2006) observed a decrease of 45% in the FRAP value (15 μmol Fe^{2+} 100 g^{-1}) after cooking carrot. However, in our study, there was no relevant decrease in FRAP values in either pressurized (from 32 to 29 μmol Fe 100 mL^{-1}) or thermally treated (from 43 to 37 μmol Fe 100 mL^{-1}) smoothies during storage, as reported for fruit smoothies with a tenfold higher antioxidant capacity processed in similar conditions to those of our study (Picouet et al., 2016). This seems to indicate that this type of vegetable smoothie with apple, once pressurized, retains its antioxidant mechanisms quite intact, a question that will be addressed in the second part of this study.

3.5 Physical stability

The physical assessment of smoothie discolouration is shown in Table 4. A decrease in L* often reflects greater darkness as a consequence of oxidizing treatments in this type of product, while a browning tendency is often associated with an increase in H* angle and browning index. L* was initially increased by the HPP treatment (day 1) and then remained constant throughout storage, with minor differences in L* between treatments during storage (day 28). HPP did not modify H* and C* compared with the untreated product, while, in contrast, MH provided a slightly brown smoothie (higher H*) on day 1. Chromatic coordinates were more stable in the HPP smoothies than in the MH smoothies during storage. In addition, the browning index was lower in the HPP than in the MH smoothies from day 14 onwards. The colour changes observed in smoothies may be due to a variety of causes, such as thermal degradation of chlorophyll or carotenoid pigments, enzymatic or non-enzymatic browning reactions and microbial spoilage (Oey et al., 2008; Sadilova et al., 2009; Zhou et al., 2014). According previously published reports, it is not clear whether pressurizing or heating has a greater impact on the colour. Several studies performed on fruit smoothies (Keenan et al., 2012a, 2012b), turnip slices (Clariana et al., 2011) and shredded cabbage (Alvarez-Jubete et al., 2014) found that MH (90-95 ºC/3 min) improves colour retention compared with HPP (400-450MPa/5min/20ºC), while the opposite results have been reported for other products, such as pumpkin slices (HPP conditions) (Zhou et al., 2014), cucumber juice (400MPa/4min/15ºC) (Zhao et al., 2013), tomato purée (400MPa/15min/25ºC) (Sánchez-Moreno et al., 2006) and vegetable juice (400MPa/2-9min/15ºC) (Barba et al., 2013), where the pressurized product had higher L* than the thermally treated product, as in our study. In general, mild pressurization did not alter the pigments responsible for colour in vegetable products, such as pumpkin purée (400-600MPa/5min/20ºC) (Contador et al., 2014), cucumber juice (400MPa/4min/20ºC) (Zhao et al., 2013) or carrot purée (600MPa/15min/20ºC) (Patras et al., 2009).

The physical assessment of smoothie clarification is shown in Table 5. Total soluble solids were not affected by any preservation treatment or storage time, while the

content of insoluble solids was higher in the HPP than in the MH smoothies from day 14 onwards, which appears to indicate that the reactions involving polysaccharide´s insolubilization were more intense in the former. As expected, smoothies presented the highest turbidity (the lowest transmittance and the highest NTU) on day 1, with slight differences between treatments. Small differences between treatments concerned the cloud clarification rate during storage: HPP and MH smoothies showed earlier clarification and turbidity loss after 7 days, which is consistent with the values of soluble solids as well as the residual enzyme activities reported above. A decrease in smoothie viscosity is another consequence of cloud clarification, due to the hydrolysis reactions and aggregation phenomena occurring with polysaccharides. In our study, viscosity was similar for all the treatments on day 1, although then decreased in the HPP smoothies from day 14 onwards. This gradual loss of viscosity was clearly associated with a higher content in soluble solids in the pressurized product, as seen in other studies made in orange (600MPa/1min/20ºC) (Bull et al., 2004), apple (350MPa/room temperature/10min) (Abid et al., 2014) and blueberry (400MPa/5min/20ºC) (Barba et al., 2013) juices. A loss of viscosity in smoothies and juices has been attributed to pectin degradation through the action of PME and non-enzymatic mechanisms (Sila et al., 2009). PME hydrolyzes methyl esters of galacturonic acid, generating low methoxyl pectin, which can react with the Ca^{2+} ions present in the medium to form calcium pectate and other water insoluble compounds that precipitate, involving a gradual loss of turbidity in juices (Wicker et al., 2003). A certain amount of pulp may also be degraded by other enzymes present in smoothies, such as polygalacturonase, which is able to hydrolyse low-methoxyl pectin, which, like PME, would not have been inactivated by the HPP treatment (Eisenmenger and Reyes de Corcuera, 2009; Van Buggenhout et al., 2009). In our study, the fact that HPP enhanced PME in a smoothie containing pectin-rich ingredients could have promoted further clarification. In addition, sucrose has a higher molecular weight than glucose or fructose and provides a higher viscosity than other sugars at the same concentration (Chirife and Buera, 1997), which may also explain our results for MH smoothies.

4 CONCLUSION

HPP allows fresh-like vegetable smoothies to be obtained with an extended shelf-life from the microbiological point of view. However, pressurized smoothies show earlier clarification and oxidation, so, it will be necessary to carefully select raw materials and improve pressurization conditions to achieve a better stabilization during further chilled storage. Accordingly, the use of varieties of fruits and vegetables with a high antioxidant content, rich in soluble polysaccharides and/or low enzyme activity, could provide smoothies with longer shelf-life.

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TABLE 1 Effect of the treatments (HPP *vs* MH) on the enzymatic activity of vegetable smoothies kept at 4 ºC for one day after treatment

Treatment	PPO (%)	POD (%)	PME (u g^{-1})
Untreated	100.0±1.0^{a}	100.0±2.1^{a}	0.08±0.02^{a}
HPP 350 MPa	167.0±1.0^{b}	95.3±0.7^{b}	0.13±0.02^{b}
HPP 450 MPa	333.0±1.0^{d}	95.4±1.1^{b}	0.13±0.03^{b}
HPP 600 MPa	283.0±2.4^{c}	101.0±1.6^{a}	0.13±0.02^{b}
MH	26.7±0.6^{e}	65.0±0.8^{c}	0.08±0.02^{a}

Treatments:
Untreated: Untreated smoothie (raw)
HPP 350 MPa: High Pressure Processing (350MPa/5min/10ºC)
HPP 450 MPa: High Pressure Processing (450MPa/5min/10ºC)
HPP 600 MPa: High Pressure Processing (600MPa/3min/10ºC)
MH: Mild Heating (85ºC/7min)
M±SEM: Mean ± Standard Error of Mean.
PPO: polyphenol oxidase; POD: peroxidase; PME: pectinmethylesterase.
^{abcde} treatment effects for P≤0.05.

TABLE 2 Effect of the treatments (HPP *vs* MH) on the microbial counts (log CFU mL^{-1}) and pH in vegetable smoothies kept at 4ºC for up to 28 days of storage

	Storage	AMB	PSY	YM	pH
Untreated	1	4.9±0.2 ^{x}	4.1±0.2 ^{x}	3.6±0.2 ^{x}	4.96±0.03 ^{x}
HPP	1	2.3±0.2 ^{y}	0.4±0.2 $^{b\ y}$	0.2±0.2 $^{b\ y}$	4.93±0.03 ^{y}
MH	1	1.9±0.2 ^{y}	0.2±0.1 $^{b\ y}$	< L.D. $^{c\ y}$	4.98±0.03 ^{x}
HPP	7	2.0±0.3	0.7±0.4 ^{ab}	0.8±0.5 ^{b}	4.94±0.01 ^{y}
MH	7	1.9±0.4	0.4±0.2 ^{ab}	0.1±0.1 ^{b}	4.97±0.01 ^{x}
HPP	14	3.5±0.4 ^{x}	2.2±0.8 $^{ab\ x}$	2.4±0.4 $^{a\ x}$	4.94±0.01
MH	14	1.8±0.5 ^{y}	0.9±0.1 $^{a\ y}$	0.8±0.2 $^{a\ y}$	4.96±0.01
HPP	21	3.6±0.6	1.2±0.1 ^{ab}	0.6±0.2 ^{b}	4.89±0.03
MH	21	2.5±0.1	0.6±0.3 ^{ab}	< L.D. ^{c}	4.92±0.04
HPP	28	2.9±0.8	2.6±0.8 ^{a}	0.6±0.5 ^{b}	4.90±0.00 ^{y}
MH	28	1.2±0.7	0.8±0.1 ^{ab}	< L.D. ^{c}	4.96±0.00 ^{x}

Treatments:
Untreated: Untreated smoothie (raw)
HPP: High Pressure Processing (350MPa/5min/10ºC)
MH: Mild Heating (85ºC/7min)
M±SEM: Mean ± Standard Error of Mean.
AMB: Aerobic Mesophilic Bacteria; PYS: Psychrophilic Bacteria; YM: Yeasts & Moulds.
Counts below the limit of detection (L.D.) were considered as 0.1 log CFU mL^{-1} for statistical analysis.
^{xy} treatment effects (within time) for $P \leq 0.05$.
^{abc} storage time effects (within treatment) for $P \leq 0.05$.

TABLE 3 Effect of the treatments (HPP *vs* MH) on the enzymatic activity and antioxidant status (FRAP) in vegetable smoothies kept at 4 ºC for up to 28 days of storage

Storage day		PPO (%)	POD (%)	PME (u g^{-1})	FRAP (μmol Fe^{2+} 100 mL^{-1})
Untreated	1	100.0±6.9 ^{xy}	100.1±1.7 ^{x}	0.26±0.03 ^{xy}	35.8±3.6 ^{xy}
HPP	1	171.5±36.9 $^{a\ x}$	86.7±4.1$^{a\ y}$	0.47±0.10 $^{a\ x}$	31.7±3.2 ^{y}
MH	1	41.9±11.2 $^{a\ y}$	88.1±0.8^{y}	0.11±0.02 ^{y}	43.0±1.7 ^{x}
HPP	7	114.5±36.6 $^{ab\ x}$	76.9±1.9 $^{ab\ y}$	0.32±0.05 $^{ab\ x}$	31.4±1.0 ^{y}
MH	7	33.7±16.1$^{a\ y}$	87.1±2.9 ^{x}	0.08±0.02 ^{y}	39.6±1.3 ^{x}
HPP	14	61.7±25.4 $^{b\ x}$	80.1±2.4 ^{ab}	0.28±0.03 $^{ab\ x}$	31.7±1.4 ^{y}
MH	14	16.7±7.6 $^{ab\ y}$	85.0±3.4	0.11±0.01 ^{y}	39.6±3.1 ^{x}
HPP	21	48.2±18.9 $^{b\ x}$	80.2±3.1 ^{ab}	0.26±0.03 $^{ab\ x}$	29.1±2.4 ^{y}
MH	21	0.0±0.0 $^{b\ y}$	88.1±3.6	0.10±0.01 ^{y}	36.3±3.2 ^{x}
HPP	28	46.6±18.4 $^{b\ x}$	74.0±1.6 $^{b\ y}$	0.19±0.02 $^{b\ x}$	29.5±3.4 ^{y}
MH	28	0.0±0.0 $^{b\ y}$	88.5±4.7 ^{x}	0.08±0.03 ^{y}	37.0±3.0 ^{x}

Treatments:
Untreated: Untreated smoothie (raw)
HPP: High Pressure Processing (350MPa/5min/10ºC)
MH: Mild Heating (85ºC/7min)
M±SEM: Mean ± Standard Error of Mean.
PPO: polyphenol oxidase; POD: peroxidase; PME: pectinmethylesterase.
^{xy} treatment effects (within time) for P≤0.05.
^{abc} storage time effects (within treatment) for P≤0.05.

TABLE 4 Effect of the treatments (HPP *vs* MH) on the colour parameters in vegetable smoothies kept at 4ºC for up to 28 days of storage

Storage day		L*	Hue	Chroma	Browning Index
Untreated	1	33.9±0.1^{y}	75.9±0.1^{x}	17.6±0.5	0.08±0.00
HPP	1	34.5±0.1^{x}	75.3±0.4^{x}	17.3±0.6	0.07±0.01^{ab}
MH	1	33.7±0.2^{y}	73.2±0.3$^{a\ y}$	15.9±1.0^{a}	0.08±0.00^{c}
HPP	7	34.0±0.3	74.8±0.2^{x}	17.9±0.3^{x}	0.07±0.01^{ab}
MH	7	33.9±0.1	71.2±0.4$^{ab\ y}$	15.5±0.5^{ab}	0.08±0.00^{bc}
HPP	14	34.2±0.3	72.8±2.5	16.5±1.4	0.07±0.01$^{b\ y}$
MH	14	33.8±0.3	69.8±0.6^{bc}	13.9±0.5^{bc}	0.09±0.00^{abcx}
HPP	21	33.9±0.7	75.4±1.3^{x}	16.8±1.1^{x}	0.08±0.00$^{a\ y}$
MH	21	33.8±0.1	68.5±1.3$^{c\ y}$	13.3±0.4$^{c\ y}$	0.10±0.00$^{ab\ x}$
HPP	28	34.5±0.1^{x}	74.0±0.7^{x}	16.9±0.5^{x}	0.08±0.00$^{ab\ y}$
MH	28	34.0±0.1^{y}	66.1±0.4$^{c\ y}$	12.4±0.2$^{c\ y}$	0.10±0.01$^{a\ x}$

Treatments:
Untreated: Untreated smoothie (raw)
HPP: High Pressure Processing (350MPa/5min/10ºC)
MH: Mild Heating: 85ºC/7min
M±SEM: Mean ± Standard Error of Mean.
^{xy} treatment effects (within time) for P≤0.05.
^{abc} storage time effects (within treatment) for P≤0.05.

TABLE 5 Effect of the treatments (HPP *vs* MH) on the physical properties related with clarification in vegetable smoothies kept at 4ºC for up to 28 days of storage

	Storage day	Soluble solids (ºBrix)	Insoluble solids (g 100 g^{-1})	Transmittance (%)	Turbidity (NTU)	Absolute viscosity (cP)
Untreated	1	6.8±0.0	9.6±1.1	0.3±0.0^{xy}	2843±148^{xy}	1.61±0.21
HPP	1	6.9±0.0	9.7±1.3^{c}	0.5±0.1$^{b\ x}$	2541±309$^{a\ y}$	1.55±0.19^{a}
MH	1	6.8±0.0	9.9±1.9	0.3±0.1$^{b\ y}$	2985±121$^{a\ x}$	1.63±0.21^{a}
HPP	7	6.9±0.1	16.7±2.0^{b}	47.8±21.3^{a}	1546±687^{b}	1.64±0.24^{a}
MH	7	6.8±0.1	14.5±4.7	4.4±2.1^{b}	1084±225^{b}	1.64±0.21^{a}
HPP	14	6.9±0.0	23.7±0.7$^{a\ x}$	48.6±21.6$^{a\ x}$	1548±689^{b}	1.11±0.05$^{b\ y}$
MH	14	6.9±0.0	13.9±4.1^{y}	5.7±2.8$^{b\ y}$	915±150^{b}	1.53±0.23$^{b\ x}$
HPP	21	6.8±0.0	17.7±2.4$^{ab\ x}$	48.3±21.5^{a}	1450±646^{b}	1.10±0.03$^{b\ y}$
MH	21	6.8±0.0	9.87±2.5 ^{y}	16.2±5.8^{ab}	653±114^{bc}	1.51±0.20$^{b\ x}$
HPP	28	6.8±0.0	24.4±1.1$^{a\ x}$	47.8±21.3$^{a\ x}$	1576±701^{b}	1.10±0.03$^{b\ y}$
MH	28	6.9±0.0	13.5±4.0 ^{y}	47.0±20.7$^{a\ y}$	299±133^{c}	1.57±0.22$^{ab\ x}$

Treatments:
Untreated: Untreated smoothie (raw)
HPP: High Pressure Processing (350MPa/5min/10ºC)
MH: Mild Heating (85ºC/7min)
M±SEM: Mean ± Standard Error of Mean.
NTU: Nephelometric turbidity unit.
^{xy} treatment effects (within time) for P≤0.05.
^{abc} storage time effects (within treatment) for P≤0.05.