The Effect of High Hydrostatic Pressure on Physicochemical Properties and Microbial Load of Lime Juice in Comparison with Thermal Techniques

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Abstract

This study investigates the effects of high hydrostatic pressure (HHP) processing applying at 600 MPa for 10 min on pH, total soluble solids, total acidity, total phenolic content, vitamin C, pectin methyl esterase activity, and microbial load of lime juice (*Citrus Latifolia*, Persian variety) immediately after treatment and during 45 days of storage at 20°C in comparison with conventional thermal pasteurisation at 72°C for 20 s. HHP treatment decreased pH and °Brix of lime juice, while thermal treatment had no effect on the mentioned parameters. The influence of HHP on pH and °Brix was not practically considerable. Total acidity was changed insignificantly by both treatments. Storage time caused a significant reduction in the pH and °Brix values of all samples. Total acidity of thermal and HHP of the treated samples remained stable until the 14th and 29th days, respectively. But then, there was a significant increase until the end of storage. The best retention of total phenolic (82.51%) and ascorbic acid (91.61%) content was observed by high-pressure processing during storage. Pectin methyl esterase was significantly inactivated by thermal and HHP processing, immediately after treatments. The residual activities were 5.69% and 3.59% at the end of the storage period, respectively. Initial contamination of control lime juice with acidophilic bacteria and total moulds and yeasts was 5.7 ± 0.51 and 4.79 ± 0.28 log CFU mL^{-1}, respectively. Immediately after treatment, high-pressure processing decreased the microbial load beyond detectable levels. The population of microorganisms remained stable until the 14th day (for acidophilic) and during the whole time of the storage period (for total moulds and yeasts) under HHP processing.

Keywords: High Hydrostatic Pressure, Lime Juice, Microbial Load, Thermal Pasteurisation**,** Vitamin C

Introduction

Postharvest decay is among the most critical factors limiting the shelf life of citrus fruits, significantly increasing product losses. Cold storage is considered the primary strategy for extending the long-term storage of citrus; however, the high sensitivity of certain commodities to chilling injury has complicated their storage under low temperature conditions (Kaur, Dhillon, & Mahajan, 2014). Therefore, emphasis on the development of processing industries and the production of various products not only contributes to waste reduction but also plays an important role in enhancing exports and generating foreign exchange revenue for countries.

Lime (*Citrus Latifolia*) fruit is widely consumed due to its nutrient-rich juice, which is utilised in various forms, including fresh, beverages, flavouring agents, and even as a food preservative. Lime juice provides a favourable environment for the growth and survival of pathogenic microorganisms that may pose significant risks to consumer health. Microbial spoilage in lime juice is primarily associated with naturally acidophilic bacteria, moulds, and yeasts (Bevilacqua, Speranza, Campaniello, Corbo, & Sinigaglia, 2013). Physicochemical changes resulting from microbial spoilage adversely affect the marketability of this product. Therefore, in order to reduce/inhibit spoilage, extend shelf life, and deliver a product with the highest standards of sensory and nutritional quality, there is a need for technologies capable of meeting these objectives.

Thermal pasteurisation (TP) is the

conventional method to inactivate microorganisms and inhibit deleterious enzymatic reactions in lime juice. A major concern associated with this method is the variability reported in numerous studies regarding the degradation of nutritionally valuable compounds (e.g., colour, aroma, flavour, and texture) across different food products (Geraldi *et al.*, 2021; Rios-Corripio, Welti-Chanes, Rodríguez-Martínez, & Guerrero-Beltrán, 2020). The degradation of key nutritional attributes of lime juice by thermal pasteurisation has prompted some producers to engage in adulteration to offset/conceal quality losses. Common adulterations include the addition of preservatives, water, colourants, organic acids (e.g., citric, malic, and ascorbic acids), or even blending with lower-cost fruit juices. Such practices not only obscure product authenticity and mislead consumers but also raise significant public health concerns due to the potential adverse effects of these additives (Wang & Jablonski, 2016).

Numerous studies have investigated emerging non-thermal technologies aimed at providing microbial stability while minimising damage to nutritionally valuable compounds. These technologies include the application of chemical and biochemical agents, pulsed electric fields, light pulses, cold plasma, high-power ultrasound, and high hydrostatic pressure. Their reported advantages are reduced greenhouse gas emissions, lower energy consumption, and diminished environmental impact (Erandya, Mihiri, & Jagath, 2019). HHP is an emerging technology primarily applied for the inactivation of microorganisms and enzymes in fruit juice production (Aghajanzadeh & Ziaiifar, 2018). The U.S. Food and Drug Administration recommended in 2010 the application of this technology at approximately 580 MPa for a minimum of 3 minutes to ensure the safety of acid (pH< 4.6) and low-acid foods stored under refrigeration (Augusto, Tribst, & Cristianini, 2018). In commercial applications, food products are packaged in flexible, impermeable materials and placed inside an insulated pressure vessel. High pressure is then transmitted to the product at room temperature through a pressure-transmitting fluid (typically distilled water) (Huang, Hsu, & Wang, 2020). This processing can induce structural modifications in cellular components, leading to changes in physicochemical properties (Hu *et al.*, 2020). Previous studies demonstrate that hydrostatic pressures within the range of 340–680 MPa do not disrupt covalent bonds but can interfere with hydrogen bonds. The former implies retention of essential nutritional and flavour compounds, whereas the latter results in the loss of membrane integrity and enzymatic activity in proteins. One consequence of hydrogen bond disruption is the inactivation of moulds, yeasts, and bacteria present in food matrices (Farkas, 2016). Physicochemical properties of orange juice (pH, total soluble solids, and acidity) showed stability after HHP (400 MPa and 600 MPa for 3, 6, and 9 min) and during 60 days of storage (Ambreen, Arshad, Imran, Afzaal, & Madilo, 2023). Significant increase in phenolic content (35%), anthocyanins (103%), and antioxidant capacity (99%) was observed by application of HHP (600 MPa/ 25°C/ 5 min) in jubuticaba juice under refrigerated conditions, and microbial growth was inhibited (Geraldi *et al.*, 2021). A comparative study by Rios- Corripio *et al.* (2014) showed similar efficacy of thermal pasteurisation (60°C/ 30 min) and HHP (600 MPa/ 5min) treatments in reducing aerobic mesophilic bacteria of fresh and fermented sour cherry juice. They indicated that fresh juices required higher pressures (at least 600 MPa) than fermented juices to achieve equivalent microbial reduction. Moussa- Ayoub *et al.* (2017) reported a slight decrease in pH value, no change in total soluble solids, and approximately 96% preservation of ascorbic acid using HHP (600 MPa, 15°C/ 10 min) in *opuntia dillenii* cactus juice. The authors also found that the initial microbial population (mould, yeast, and acid tolerance bacteria) was reduced from higher than 10^3 to less than 10 CFU mL^{-1} (Moussa-Ayoub et al., 2017). According to Fernández, Denoya, Agüero, Vaudagna, and Rosa (2019),

HHP treatment (630 MPa/ 6 min/ 20°C) produced an 83.9% reduction in the initial pectin methyl esterase activity of a fruit and vegetable smoothie. Another study investigated the effect of HHP (500 to 900 MPa/ 3 to 9 min) in comparison with thermal (98°C/ 15 min) treatment on tomato juice. The authors observed that increasing the processing time and pressure caused higher total pectin in the same way as after applying heat treatment (Porretta, Birzi, Ghizzoni, & Vicini, 1995). The effectiveness of HHP varies depending on the processing conditions (pressure level, holding time, and temperature) (Perez-Lamela, Franco, & Falque, 2021).In the literature, several studies have evaluated the effect of HHP treatment on different parameters of acidic juices (e.g., orange, mandarin, and tomato) in comparison with conventional pasteurisation.

Finally, the concerns over adulteration, health risks associated with preservative use, and unfavourable physicochemical changes in lime juice during processing and storage have cast doubt on the growing demand for this vitamin C-rich product. These issues underscore the necessity of adopting advanced processing technologies to mitigate such adverse effects. To date, most studies on the application of HHP to acidic juices have focused on orange juice, and no published study has investigated the HHP pasteurisation of lime juice. In addition, the majority of available studies have evaluated HHP-treated juices under refrigerated storage conditions, and information regarding the stability of HHP-treated lime juice during ambient storage remains limited. Evaluation of processed lime juice under these conditions is industrially relevant because storage temperature is a major determinant of juice quality and shelf life. Assessing product quality at ambient temperature provides valuable information regarding its commercial distribution and storage potential, particularly for shelf stable juice products. Nevertheless, because storage temperature strongly influences microbial growth, enzymatic activity, and physicochemical changes (Petruzzi *et al.*, 2017), results obtained under ambient conditions should not be directly compared with those from refrigerated storage. Accordingly, the present study aimed to evaluate the effects of high hydrostatic pressure (600 MPa for 10 min) on the physicochemical properties and microbial load of lime juice during 45 days of storage at 20 ºC and to compare its performance with conventional thermal pasteurisation (72 ºC for 20 s).

Materials and Methods

The overall experimental design and analytical workflow adopted in this study are illustrated in Figure 1. The schematic summarises the sequence of sample preparation, processing, and analytical procedures performed throughout the experiment.

Lime Juice Preparation

Fresh limes (*Citrus latifolia*, Persian variety) were purchased from the Citrus and Subtropical Fruits Research Centre (Ramsar, Iran) and stored at 4°C until use. After sorting and washing, the limes were juiced using a laboratory-scale juicer, and the juice was filtered through a No. 170 mesh sieve to remove pulp and impurities. To minimise the risk of secondary contamination during subsequent treatments, the filtered juice was packaged in manually fabricated three-layer polymer sachets (polyethylene/aluminium/polyethylene terephthalate). The prepared juices were transported to the Mashhad Research Institute of Food Science and Technology for further experiments, within less than one hour (to minimise potential enzymatic reactions).

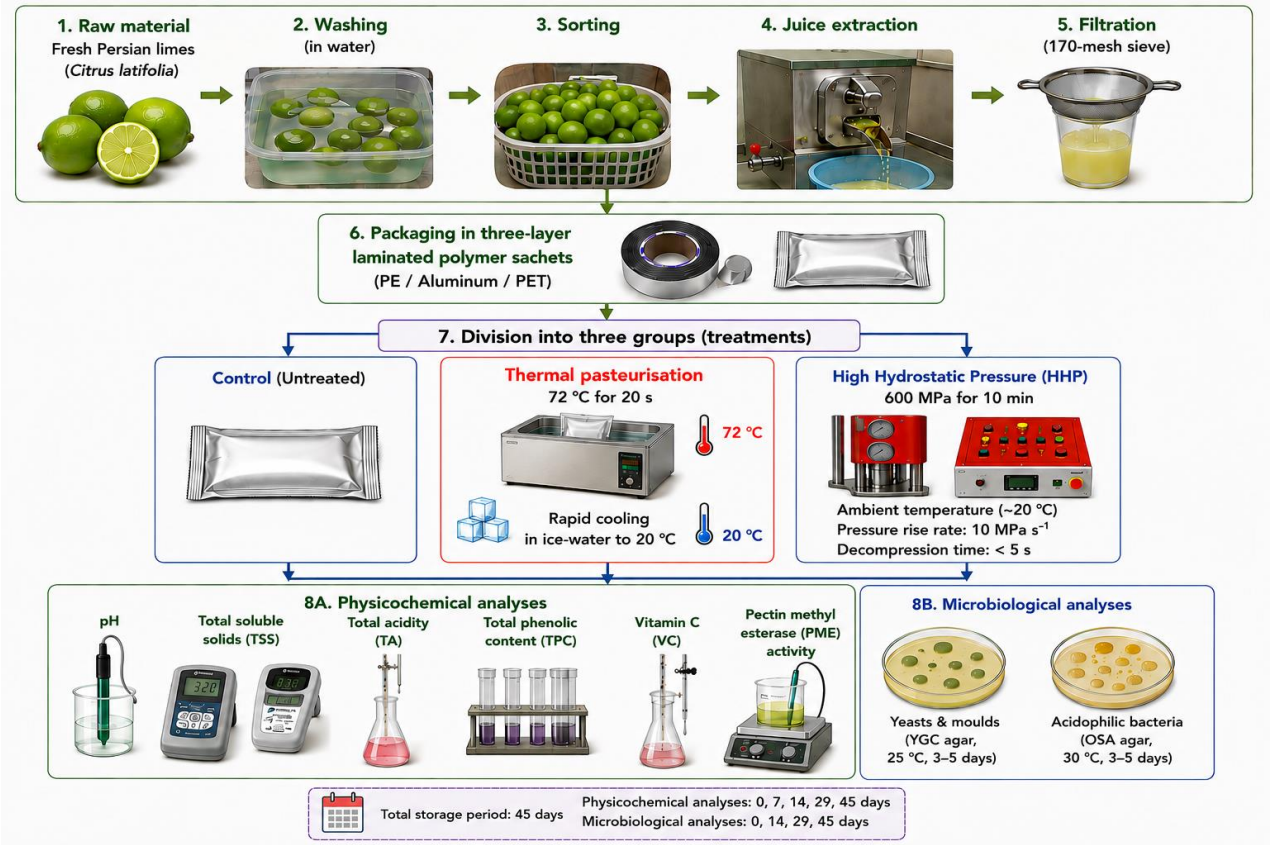

Fig. 1. Schematic representation of the experimental workflow for lime juice preparation, packaging, thermal pasteurisation, high hydrostatic pressure, and subsequent physicochemical and microbial analysis

High Hydrostatic Pressure

In commercial applications, hydrostatic pressure in the range of 200 to 600 MPa with a holding time of 5 to 10 min is used at ambient or chilled temperature (Aganovic *et al.*, 2021). Therefore, in this study, a high hydrostatic pressure of 600 MPa for 10 min at ambient temperature was chosen as a pasteurisation condition. High hydrostatic pressure processing was performed using an HHP unit (LS-6000B-60C, Iran), located in the Research Institute of Food Science and Technology. Lime juice sachets were directly loaded into the chamber, and the remaining volume of the chamber was filled with distilled water (as pressure-transmitting fluid). Hydrostatic pressure of 600 MPa was applied for 10 min. The rate of pressure rising was 10 MPa s^{-1}, and the decompression time was less than 5 s. After treatment, the samples were removed from the chamber and immediately prepared for storage.

Thermal Pasteurisation

Based on FDA guidelines, the minimum temperature- time for juices with a pH in the range of 3.6 to 4.0 is 71.1°C for 3 s. This specific condition is insufficient to inactivate spoilage microorganisms (Timmermans *et al.*, 2011). Furthermore, higher temperatures have a negative impact on the quality of juices (Amine *et al.*, 2023). Therefore, in this study, 72°C for 20 s was used as a condition for mild pasteurisation of lime juice. Thermal pasteurisation was carried out using a hot water bath (WNB10, Memmert, Germany). Lime juice sachets were placed into the bath chamber, and the remaining volume was filled with distilled water up to the marked level. Once the water temperature reached 72°C, the samples were held at this temperature for 20 seconds. After completion of the heat treatment, the samples were removed from the bath and rapidly cooled in an ice-water mixture until reaching ambient temperature (20°C).

Storage Conditions

Treated and not treated (control) lime juices were stored at 20°C for 45 days. Physicochemical analyses were performed on days 0, 7, 14, 29, and 45, while microbial assessments were conducted on days 0, 14, 29, and 45.

pH and Total Soluble Solids

pH and total soluble solids (TSS) were measured at 20°C using a pH meter (DR359TX-ION Meter) and a refractometer (RX-5000α, Atago, Japan), respectively.

Total Acidity

Total acidity (TA) was determined by the titration method with sodium hydroxide with slight modifications. Briefly, 3 g of the sample was diluted in 50 mL of distilled water, and phenolphthalein was added as an indicator. The mixture was titrated with sodium hydroxide (0.1 N) to a persistent pink endpoint. Finally, total acidity was calculated and expressed as gram of citric acid per 100 mL of sample as follow (Wibowo *et al.*, 2015):

$$TA = \frac{V \times 0.0064}{m} \times 100 \quad (1)$$

where V is the volume of sodium hydroxide consumed (mL), m is the sample mass (g), 0.0064 represents the gram of anhydrous citric acid equivalent to 1 mL of sodium hydroxide (0.1 N), and TA is the total acidity expressed as g of citric acid per 100 mL of sample.

Total Phenolic Content

Total phenolic content (TPC) was determined according to the method described by (Cao *et al.*, 2011) with slight modifications. In short, 150 µL of the sample was mixed with 1.5 mL of Folin–Ciocalteu reagent and 1.5 mL of 6% (w/v) sodium carbonate solution. The mixture was incubated in the dark for 60 min, and absorbance was measured at 765 nm using a "UV–Vis" spectrophotometer (DR 5000, HACH LANGE GmbH, Germany). Total phenol content was quantified using a gallic acid standard curve and expressed as mg gallic acid equivalents per L of the sample.

Vitamin C

Vitamin C (VC) concentration was determined by 2, 6-dichlorophenolindophenol titration with slight modifications (Feng *et al.*, 2020). Briefly, 5 g of the sample was diluted to 25 mL with standard oxalic acid solution, and a 10 mL aliquot was titrated with 2, 6-dichlorophenolindophenol dye solution until a light pink endpoint was achieved. The dye solution was standardised using 1 mg of ascorbic acid in 1 L of oxalic acid solution. Results were expressed as mg ascorbic acid per 100 g of the sample.

Pectin Methyl Esterase Activity

Pectin methyl esterase (PME) activity was determined according to the method described by Kimball. 40 mL of 1% (w/v) pectin–salt solution was added to 10 mL of sample. The pH was adjusted to 7.6–7.8 using 0.05 N sodium hydroxide. The reaction mixture was maintained at 30°C throughout the assay. Following the addition of 0.1 mL of 0.05 N sodium hydroxide, the increase in pH was recorded, then the time required for the pH to return to its initial value (7.6–7.8) was recorded. PME activity was subsequently calculated using Eq. (2) (Kimball, 1999).

$$PEU\ (Pectin\ Esterase\ Unit) = \frac{N \times V_{NaOH}}{V_{Sample} \times T} \quad (2)$$

where N is the normality of sodium hydroxide (0.05 N), V_{NaOH} is the volume of sodium hydroxide added (0.1 mL), V_{Sample} is the volume of the sample (10 mL), and T is the time (minutes) required for the pH to return to its initial value (7.6–7.8).

Microbiological Analysis

Microbiological analyses were performed in accordance with the Iranian National Standard No. 8788 (Lime Juice— Microbiological Specifications and Test Methods). Mould and yeast counts, as well as acidophilic bacteria, were evaluated in control and treated samples using the pour plate method. Yeasts and moulds were enumerated on yeast extract glucose chloramphenicol agar (YGC; Merck, Germany), while acidophilic bacteria were counted on orange serum agar (OSA; Merck, Germany). Following sample dilution and plating, YGC and OSA plates were incubated

in an inverted position for 3–5 days at 25°C and 30°C, respectively. Microbial counts were determined from plates containing 30–300 colonies and expressed as the logarithm of colony-forming units per mL of lime juice (Log CFU mL^{-1}) (Institute of Standards and Industrial Research of Iran, 2005).

Statistical Analysis

The data were analysed using Minitab software (version 17; Minitab Inc., USA) and described as the mean of all repetitions together with the standard deviation. All experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was performed separately for each response variable to compare the experimental groups. Prior to analysis, the assumptions of normality and homogeneity of variances were evaluated. Differences among means were assessed using Tukey's test. In all cases, a 95% confidence level was considered to determine significant differences.

Results and Discussion

pH and Total Soluble Solids

The changes in pH are described in Table 1. The highest pH was observed in the control sample on the first day of storage, whereas the lowest value was recorded in the TP-treated sample at the end of storage. Statistically, there was a significant difference between HHP- and control-treated juice ($P < 0.05$). However, this reduction of pH caused by HHP treatment cannot practically influence the quality of lime juice. No difference was detected between control and TP samples on the first day. The results of ANOVA demonstrated that storage time had a significant effect on pH, resulting in a decreasing trend in all treatments ($P < 0.05$). However, mean comparisons across storage days indicated no significant difference between the TP and control samples ($P > 0.05$). In contrast, HHP processing led to a significant reduction in pH compared to the control. The observed decline in pH during storage is consistent with previous reports on acidic fruit juices (Altemimi *et al.*, 2021; Drood, Daneshi, & Nateghi, 2019; Khoshnoudi & Hosseini, 2010; Zeina, 2000).

Table 1- The mean values of pH, TSS (°Brix), and TA (g citric acid/100mL juice) of treated and non-treated lime juice during storage

Parameter	Treatment	Day 0	Day 7	Day 14	Day 29	Day 45
pH	Control	2.69 ± 0.01 ^{aa}	2.68 ± 0.01 ^{aba}	2.67 ± 0.005 ^{bca}	2.65 ± 0.005 ^{cda}	2.63 ± 0.005 ^{da}
	TP	2.68 ± 0.005 ^{aa}	2.67 ± 0.005 ^{aba}	2.66 ± 0.005 ^{ba}	2.63 ± 0.005 ^{cab}	2.61 ± 0.01 ^{da}
	HHP	2.66 ± 0.005 ^{ab}	2.65 ± 0.005 ^{ab}	2.64 ± 0.00 ^{abb}	2.62 ± 0.005 ^{bcb}	2.61 ± 0.01 ^{ca}
TSS	Control	9.71 ± 0.01 ^{aa}	9.67 ± 0.005 ^{abab}	9.6 ± 0.01 ^{bc}	9.27 ± 0.07 ^{cb}	8.89 ± 0.03 ^{db}
	TP	9.69 ± 0.01 ^{aab}	9.68 ± 0.1 ^{aba}	9.67 ± 0.04 ^{aba}	9.66 ± 0.02 ^{bca}	9.64 ± 0.04 ^{ca}
	HHP	9.66 ± 0.01 ^{ab}	9.65 ± 0.01 ^{abb}	9.64 ± 0.01 ^{abb}	9.63 ± 0.01 ^{ba}	9.63 ± 0.05 ^{ba}
TA	Control	6.94 ± 0.12 ^{ca}	7.02 ± 0.09 ^{ca}	7.11 ± 0.06 ^{bca}	7.3 ± 0.05 ^{aba}	7.47 ± 0.03 ^{ab}
	TP	7.03 ± 0.1 ^{ba}	7.09 ± 0.09 ^{ba}	7.23 ± 0.07 ^{ba}	7.43 ± 0.03 ^{aa}	7.62 ± 0.02 ^{aa}
	HHP	7.08 ± 0.07 ^{ca}	7.18 ± 0.08 ^{ca}	7.31 ± 0.12 ^{bca}	7.46 ± 0.1 ^{aba}	7.62 ± 0.03 ^{aa}

* Values are expressed as mean ± standard deviation (n = 3).
Different lowercase letters indicate differences between treatments and different capitals indicate differences over time according to Tukey's multiple comparison test ($P < 0.05$).

Total soluble solids (TSS) constitute an important quality parameter in fruit juice grading, as they reflect the concentration of soluble sugars (Timmermans *et al.*, 2011). The TSS content of the control sample on the first day was 9.71 ± 0.01 °Brix (Table 1). HHP treatment immediately reduced this value to 9.66 ± 0.01 °Brix. Although, the reduction resulting from HHP was statistically significant ($P < 0.05$), it did not have a considerable effect on the quality of lime juice (the change did not exceed 1%). No significant difference was observed between TP and control. Previous studies have reported inconsistent findings regarding the effects of HHP and TP on TSS in fruit juices. For

instance, HHP (600 MPa/5 min) and TP (82°C/2 min) increased the TSS content of jaboticaba juice (Geraldi *et al.*, 2021). In contrast, HHP (600 MPa/60 s) and TP (65°C/1 min) had no significant effect on *Valencia* and *Navel* cultivars of orange juice (Bull *et al.*, 2004). TSS content decreased significantly ($P < 0.05$) in all samples during storage. The control sample exhibited the greatest reduction (0.82 °Brix), reaching 8.89 ± 0.03 °Brix. This could be related to the higher microbial load of control samples at the end of storage time (Rios-Corripio, Welti-Chanes, Rodríguez-Martínez, & Guerrero-Beltrán, 2024). Microorganisms can change the °Brix value by the consumption of soluble solids (Yeom, Streaker, Zhang, & Min, 2000). The TSS content of untreated bayberry decreased rapidly after 2-day storage at 25°C (Yu, Lin, Zhan, He, & Zhu, 2013), and for mango juice decreased from 15 to 14.1 by day 14 of storage at 4°C (Mandha, Shumoy, O. Matemu, & Raes, 2023). This finding is consistent with previous reports indicating that prolonged storage leads to a reduction in TSS content of lime juice (Kaddumukasa, Imathiu, Mathara, & Nakavuma, 2017).

Total Acidity

As shown in Table 1, TP and HHP treatments caused a slight increase in the total acidity (TA) compared to the control; however, this increase was not statistically significant ($P > 0.05$). Comparison of TA across storage days revealed no significant changes ($P > 0.05$) until day 14, followed by a significant increasing trend toward the end of the storage period ($P < 0.05$). The increase in total acidity observed during the latter stage of storage may be associated with microbial proliferation and the accumulation of organic acids. Similarly, Varela-Santos *et al.* (2012) reported that HHP (550 MPa/150 s) had no significant effect on TA of pomegranate juice during the first 15 days of storage at 4°C; however, significant changes were observed thereafter until the end of storage (35 days). In another study, HHP (600 MPa/5 min) and TP (72°C/15 s) did not significantly affect the TA of fermented pomegranate juice during 42 days of storage at 4°C (Rios-Corripio *et al.*, 2020). Likewise, Ambreen *et al.* (2023) reported that HHP treatment (600 MPa-9 min) did not impact the TA of orange juice during 60 days of storage at 4°C (Ambreen *et al.*, 2023). However, because neither microbial metabolites nor organic acid profiles were analysed in the present study, the above explanation should be regarded as a plausible hypothesis rather than a definitive conclusion. Additional studies are needed to clarify the mechanisms underlying the observed changes in acidity during storage.

Total Phenolic Content

The average values of total phenolic content (TPC) for the control, TP, and HHP samples were 97.65, 86.52, and 102.52 mg L^{-1}, respectively. The highest and lowest values, 112.71 and 69.37 mg L^{-1}, were observed in the HHP sample on the first day and the TP sample at the end of storage, respectively. The mean values of TPC in lime juice (treated and not treated) are presented in Table 2. TP treatment caused an immediate, non-significant reduction in TPC ($P > 0.05$). Similarly, HHP led to a slight, non-significant increase on the first day compared to the control ($P > 0.05$). This increase may be attributed to enhanced extractability of antioxidant compounds resulting from the application of high hydrostatic pressure (Chen *et al.*, 2013). Application of high pressure enhances cell permeability, thereby allowing the extraction/release of phenolic compounds (Pokhrel *et al.*, 2022). According to Le Chatelier's principle, the volume of the system decreases under high hydrostatic pressure, facilitating solvent penetration into cells to interact with bioactive compounds. Additionally, cell wall disruption and the breakdown of hydrophobic membrane interactions accelerate mass transfer, thereby increasing cellular permeability (Berkel Kaşıkcı, 2015). (Rios-Corripio *et al.*, 2020) reported that hydrostatic pressure (500 MPa/10 min; 550 MPa/10 min; 600 MPa/5 min) slightly enhanced the total phenol content of

fermented pomegranate beverage. Furthermore, HHP-treated whey-lime beverage exhibited higher total phenol content compared with thermally processed samples (Bansal, Jabeen, Rao, Prasad, & Yadav, 2018).

Table 2- The mean values of TPC (mg gallic acid/1 L juice) and VC (mg ascorbic acid/100g juice) of treated and non-treated lime juice during the storage

Parameter	Treatment	Day 0	Day 7	Day 14	Day 29	Day 45
TPC	Control	107.61 ± 2.26 ^{aab}	104.53 ± 0.98 ^{abab}	98.07 ± 4.47 ^{ba}	93.49 ± 3.24 ^{ca}	84.53 ± 0.81 ^{db}
	TP	104.01 ± 2.98 ^{ab}	94.27 ± 6.57 ^{abb}	87.08 ± 5.79 ^{bcb}	77.08 ± 2.56 ^{cdb}	70.15 ± 0.89 ^{dc}
	HHP	110.61 ± 2.4 ^{aa}	108.07 ± 2.63 ^{aa}	104.86 ± 1.8 ^{aba}	97.8 ± 2.05 ^{bca}	91.27 ± 3.97 ^{ca}
VC	Control	57.16 ± 0.76 ^{aa}	55.92 ± 0.83 ^{aba}	54.52 ± 0.84 ^{ba}	51.45 ± 0.87 ^{ca}	49.96 ± 0.94 ^{ca}
	TP	54.23 ± 0.78 ^{ab}	52.43 ± 0.82 ^{abb}	50.34 ± 0.84 ^{bb}	46.01 ± 0.89 ^{cb}	44 ± 1.15 ^{cb}
	HHP	55.95 ± 0.85 ^{aab}	55.08 ± 0.83 ^{aa}	54.06 ± 0.78 ^{aba}	52.21 ± 0.68 ^{bca}	51.26 ± 0.77 ^{ca}

* Values are expressed as mean ± standard deviation (n = 3).
Different lowercase letters indicate differences between treatments and different capitals indicate differences over time according to Tukey's multiple comparison test ($P < 0.05$).

TPC decreased non-significantly in the control and TP sample until day 14, whereas in the HHP sample, this trend persisted until day 29. Beyond these periods, a significant decline was observed in all samples until the end of the storage period ($P < 0.05$). The greatest and least TPC retention with values of 82.51% and 67.44%, respectively, were observed under HHP and TP treatments. Unlike thermal pasteurisation, HHP primarily disrupts non-covalent interactions while preserving the covalent structures. Therefore, pressure processing minimises thermal degradation and oxidation of phenolic compounds. In addition, the lower processing temperature reduces the activity of oxidative enzymes, thereby limiting enzymatic oxidation of phenolics during storage (Zhang, Wang, & Xiao, 2022). Consistent with these findings, Ambreen *et al.* (2023) reported that thermal pasteurisation reduced TPC in orange juice by up to 30% over 60 days of storage, whereas HHP preserved to a greater extent. Moreover, the greater TPC stability was observed in shorter HHP durations. The authors attributed this finding to the more extensive cellular disruption resulting from prolonged exposure to high pressure. Cao *et al.* (2011) illustrated that HHP at 400 MPa did not affect TPC in strawberry juice compared to the control, regardless of treatment duration.

Vitamin C

Vitamin C (VC) or ascorbic acid is one of the most important nutritional components of lime juice. It is inherently unstable and highly sensitive to temperature, pressure, light, pH, and other factors (Cheng, Jia, Gui, & Ma, 2020). The changes in VC concentration are described in Table 2. The initial value of VC concentration in the control sample was 57.16 ± 0.76 at day 0. Based on the results from the analysis of variance, TP treatment significantly reduced the VC of lime juice, while the observed difference between the HHP and control samples was not significant ($P > 0.05$). Previous studies have reported that TP treatment (above 60°C) causes ascorbic acid degradation in citrus juices (Basak, Shaik, & Chakraborty, 2023; Rabie, Soliman, Diaconeasa, & Constantin, 2015).

Storage time resulted in a significant decrease in all three treatments ($P < 0.05$). At the end of the storage period, VC concentrations in the control, TP, and HHP samples were 49.96 ± 0.94, 44.15 ± 1.15, and 51.26 ± 0.77, respectively. Therefore, high pressure demonstrated superior efficacy compared with TP in preserving VC, indicating its greater potential (91.61 % retention) for maintaining the stability of this bioactive compound. The higher retention of vitamin C under HHP can be attributed to the absence of severe thermal exposure. Ascorbic acid is highly susceptible to heat-induced oxidation, whereas high hydrostatic pressure causes minimal disruption to its molecular structure (Giannakourou & Taoukis, 2021).

These findings are consistent with previous studies on HHP-mediated ascorbic acid preservation. (Yu *et al.*, 2013) reported that more than 96% of ascorbic acid was retained in bayberry juice after HHP treatments at 500 and 600 MPa at room temperature for 10 min, which was stored at 25°C. HHP (200-800 MPa- 15 min- 20 to 22.5°C) followed by storage at 5ºC, 20ºC, and 30°C was carried out for blackcurrants. Authors showed that HHP treatment at 600 MPa had the best preservation for all the samples stored at 5ºC and 20°C (Kouniaki, Kadja, & Zabetakis, 2004). While there was no significant difference between the vitamin C concentration of thermal, pulsed electric field, and high hydrostatic pressure treated orange juice immediately after treatments, its reduction during storage was lower in HHP treated juices (Esteve & Frígola, 2009). (Polydera, Stoforos, & Taoukis, 2005) reported that HHP (600 MPa/4 min/40°C) preserved 84% of ascorbic acid in *Navel* orange juice stored at 5°C for 30 days. Similarly, Fernández García, Butz, Bognàr, and Tauscher (2014) found that HHP processing at 500 and 800 MPa (5 min-25°C) preserved 83% and 76% of ascorbic acid, respectively, in an orange-carrot-lime beverage stored at 4°C for 21 days.

Pectin Methyl Esterase Activity

Ensuring the stability of fruit juices during storage requires the inactivation, or at least reduction, of certain enzymatic activities. PME (Pectin Methyl Esterase) is one of the key endogenous enzymes in citrus juices. It catalyses the de-esterification of pectin, which can affect pectin functionality, cloud stability, and overall juice quality during storage. PME can affect the structure, flavour, colour, and even nutritional quality of juice upon oxidation (Basak, Ramaswam, & Simpson, 2001). Its inactivation is particularly important in acidic fruit juices because it exhibits higher thermal resistance than most microorganisms (Aghajanzadeh & Ziaiifar, 2018).

The changes in PME activity (as a percentage of residual activity) of control and treated samples during storage are described in Table 3.

Table 3- The mean values of residual PME (as percent) activity of treated and non-treated lime juice during the storage

Treatment	Day 0	Day 7	Day 14	Day 29	Day 45
Control	100 ± 0.0 ^{aa}	38.10 ± 6.9 ^{ba}	8.93 ± 0.44 ^{ca}	5.02 ± 0.08 ^{ca}	3.3 ± 0.19 ^{ca}
TP	5.69 ± 0.48 ^{ab}	2.84 ± 0.48 ^{bb}	3.55 ± 0.03 ^{bb}	3.38 ± 0.10 ^{bb}	2.76 ± 0.12 ^{bb}
HHP	3.59 ± 1.15 ^{ac}	2.26 ± 0.22 ^{ab}	3.61 ± 0.48 ^{ab}	3.23 ± 0.69 ^{ab}	3.35 ± 0.16 ^{aa}

* Values are expressed as mean ± standard deviation (n = 3).
Different lowercase letters indicate differences between treatments and different capitals indicate differences over time according to Tukey's multiple comparison test ($P < 0.05$).

TP and HHP markedly decreased the activity of PME immediately after treatment, with residual activity of 5.69% and 3.59%, respectively. Both treatments had a significant effect on reducing PME activity compared to the control ($P < 0.05$). Subsequently, no significant difference was observed between the two treatments throughout the storage period ($P > 0.05$). The observed reduction in PME activity may be associated with pressure-induced conformational changes that decrease the enzyme's catalytic efficiency, although this mechanism was not directly investigated in the present study (Aghajanzadeh & Ziaiifar, 2018). (Fernández, Denoya, Agüero, Vaudagna, & Rosa, 2019) reported a 70.7% reduction in pectin methyl esterase activity of a vegetable smoothie on the first day, with no significant changes throughout storage (28 days). These authors attributed the activity reduction on day 28 compared to the first day of the control sample to the influence of the acid environment that hinders the regeneration of enzymes. Storage time caused a significant reduction in PME activity of control samples ($P < 0.05$), whereas changes in HHP and TP samples were not statistically significant ($P > 0.05$). The PME residual activity of control samples decreased from an initial value of 100% to 38% after 7 days of storage. The marked reduction in PME activity may be related to changes in the juice matrix during

microbial proliferation. One possible explanation, as suggested in previous studies, is that microbial activity may contribute to the degradation or utilisation of proteinaceous compounds, including enzymes such as PME (Agcam, Akyildiz, & Evrendilek, 2014). However, because the underlying mechanisms were not investigated in the present study, this interpretation should be considered hypothetical.

Previous studies have also demonstrated the effectiveness of TP in reducing PME activity. For instance, residual PME activity in orange juice subjected to thermal treatment (90°C/10 and 20 s) decreased to 6.8% and 4.2%, respectively (Agcam *et al.*, 2014). Conflicting reports have been presented regarding the impact of storage time on PME activity in thermally and high-pressure treated juices. Vervoort *et al.* (2011) found a gradual decline in thermally pasteurised (72°C-20s) orange juice over 60 days at 4°C. In contrast, the application of HHP processing (50 to 250 MPa in 50 MPa increments) increased PME activity in orange juice stored at 4°C for 12 days. The authors attributed this increase to the formation of new isoenzymes during storage as a result of high pressure (Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009). Hydrostatic pressure in the range of 430–570 MPa for 1–8 minutes completely inactivated PME in apple juice, and no reactivation was observed during 34 days of storage at 4ºCand 20°C (Juarez-Enriquez, Salmeron-Ochoa, Gutierrez-Mendez, Ramaswamy, & Ortega-Rivas, 2015).

Microbiological Analysis

In the present study, the contamination of control and treated lime juice by acidophilic bacteria as well as total moulds and yeasts was evaluated on days 0, 14, 29, and 45 of storage. The residual counts of the mentioned microorganisms in all treated and non-treated samples during storage are presented in Table 4. Initial contamination of control samples by acidophilic bacteria was 5.7 ± 0.51 log CFU mL^{-1} on day 0, increasing to 7.06 log CFU mL^{-1} at the end of storage at 20°C. Its population was reduced to 1.57 log CFU mL^{-1} immediately after TP treatment, while HHP processing reduced it until below the detectable limit (< 1 log CFU mL^{-1}). This result is consistent with previous studies. HHP processing (350 MPa-150 s; 450, 500, and 550 MPa-30, 90, and 150 s), reduced all microorganisms (moulds, yeasts, and mesophilic aerobic bacteria) in fresh pomegranate juice below detectable levels (Varela-Santos *et al.*, 2012). The application of 400 MPa for 1 min resulted in a 7-log reduction in microbial counts of apple juice (Shahbaz *et al.*, 2015). Microbial proliferation was prevented by HHP (600 MPa for 5 minutes) in jabuticaba juice (Geraldi *et al.*, 2021). Storage time led to a significant increase in the population of acidophilic bacteria in all samples ($P < 0.05$). In HHP samples, the counts remained below the detectable limit until day 14 and reached 1.31 log CFU mL^{-1} by the final day. It reached 2.15 log CFU mL^{-1} at the end of storage in TP-treated samples.

Table 4- Residual counts of acidophilic and total moulds and yeasts (log CFU mL^{-1}) in treated and non-treated lime juice during storage

Treatment	Day 0	Day 14	Day 29	Day 45
Acidophilic bacteria				
Control	5.7 ± 0.03 ^{eab}	6.41 ± 0.02 ^{ca}	6.84 ± 0.03 ^{ba}	7.06 ± 0.03 ^{ab}
TP	1.53 ± 0.04 ^{db}	1.68 ± 0.01 ^{cb}	1.9 ± 0.03 ^{bb}	2.15 ± 0.03 ^{ac}
HHP	ND	1.01 ± 0.12 ^{abca}	1.19 ± 0.08 ^{aba}	1.31 ± 0.06 ^{aa}
Total moulds and yeasts				
Control	4.79 ± 0.28 ^{da}	5.31 ± 0.24 ^{bca}	5.71 ± 0.14 ^{aba}	6.16 ± 0.04 ^{aa}
TP	1.09 ± 0.09 ^{cb}	1.23 ± 0.1 ^{bcb}	1.43 ± 0.12 ^{bb}	1.77 ± 0.13 ^{ab}
HHP	ND	ND	ND	1.05 ± 0.13 ^{aa}

* Values are expressed as mean ± standard deviation (n = 3).
Different lowercase letters indicate differences between treatments and different capitals indicate differences over time according to Tukey's multiple comparison test ($P < 0.05$).

ND represents not detected (detection limit < 1 log CFU mL^{-1}).

Initial contamination of control by total moulds and yeasts was 4.79 log CFU mL^{-1}, rising to 6.16 log CFU mL^{-1} by the end of storage. Yeasts were the predominant microorganisms in lime juice during storage. HHP processing immediately reduced total mould and yeast counts below the detectable limit and maintained their stable population throughout most of the storage period, with only a minor increase to 1.05 log CFU mL^{-1} on day 45. TP treatment reduced mould and yeast counts by 3.7 log CFU mL^{-1}, with counts reaching 1.77 log CFU mL^{-1} by the end of storage. HHP inactivates microorganisms mainly through disruption of all cell membranes, alteration of membrane permeability, protein denaturation, and impairment of essential metabolic functions. Unlike thermal processing, these effects are achieved without extensive heating, allowing microbial safety to be improved while minimising quality deterioration (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015; Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008). In accordance with the results of this study, HHP treatment (200–300 MPa) in fresh orange juice decreased the counts of all bacteria (mesophilic bacteria, lactic acid bacteria, moulds, yeasts, coliforms, and enterobacteria) by approximately 4 log CFU mL^{-1} (Velázquez-Estrada, Hernández-Herrero, Guamis-López, & Roig-Sagués, 2012). Additionally, HHP (250 MPa-5 min-30°C) reduced microbial loads in white and red grape juices by 4 and 2.5 log CFU mL^{-1}, respectively (Mert, Buzrul, & Alpas, 2013). A comparative study of thermal (60°C/30 min) and hydrostatic pressure (200 and 400 MPa/10 min; 600 MPa/5 min) processing of black cherry juices showed that at least 600 MPa for 5 min was necessary to maintain mesophilic aerobic bacteria of fermented juice below a detectable level (<1 log CFU mL^{-1}) throughout the 49 days at 4°C. Fresh juice required higher pressures to achieve equivalent microbial inactivation. Both treatments had the same impact in reducing mesophilic bacteria (Rios-Corripio *et al.*, 2024). Optimal HHP conditions for microbial inactivation of strawberry juice were recognised at 600 MPa for 1 min, whereas treatment at 200 MPa required a longer processing time for the same effectiveness (Yildiz, Pokhrel, Unluturk, & Barbosa-Cánovas, 2019).

Conclusion

The results of the present study demonstrated that HHP (600 MPa/10 minutes) effectively controlled microbial growth during storage, keeping microbial populations significantly lower than those observed in thermally pasteurised and untreated samples. Although low levels of acidophilic bacteria and yeasts/moulds were detected at the end of storage, HHP provided superior microbiological stability throughout the storage period. Physicochemical analysis revealed no significant differences between TP and HHP treatments in terms of pH and total soluble solids. HHP treatment preserved 15.07% more total phenol content and 10.48% more ascorbic acid than TP over the storage period. Both treatments had a comparable effect on PME activity, significantly reducing enzyme activity immediately after treatment and during storage. The findings of this study demonstrated that HHP is a promising alternative to conventional thermal pasteurisation for lime juice processing, particularly due to its superior preservation of nutritional quality and microbial stability during storage at 20ºC. However, because the present investigation was conducted under ambient storage conditions, future studies under refrigerated storage are recommended to facilitate direct comparisons with previous reports and to support broader industrial implementation. Future studies may expand findings by incorporating sensory evaluation, packaging permeability characterisation, and techno-economic assessment of HHP processing.

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Author Contributions

R. Babagoltabar samakoosh: Conceptualisation, Methodology, Data acquisition, Data pre- and post-processing, Statistical analysis, Validation, Visualisation.

H. Sadrnia: Supervision, Conceptualisation, Methodology.

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All authors read and approved the final version of the manuscript.

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تأثیر فشار هیدرواستاتیک بالا بر خواص فیزیکوشیمیایی و بارمیکروبی آبلیمو در مقایسه با روش‌های حرارتی

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چکیده

مطالعه‌ی حاضر تأثیر فشار هیدرواستاتیک بالا (۶۰۰ مگاپاسکال- ۱۰ دقیقه) را بر pH، مواد جامد محلول، اسیدیته‌ی کل، فنل کل، ویتامین ث، فعالیت آنزیم پکتین متیل استراز و بار میکروبی آبلیمو رقم پرشین، بلافاصله پس از تیمار و طی ۴۵ روز نگهداری در دمای ۲۰ درجه‌ی سانتی‌گراد در مقایسه با پاستوریزاسیون حرارتی (۷۲ درجه سانتی‌گراد- ۲۰ ثانیه) مورد بررسی قرار می‌دهد. فشار هیدرواستاتیک بالا pH آبلیمو را به‌طور معنی‌دار کاهش داد. تیمارهای فشار هیدرواستاتیک بالا و حرارتی تأثیری روی مواد جامد محلول نداشتند، اما اسیدیته‌ی کل به‌طور غیرمعنی‌دار دستخوش تغییر شد. گذشت زمان موجب کاهش معنی‌دار pH و مواد جامد محلول در همه‌ی نمونه‌ها شد. اسیدیته‌ی کل در نمونه‌های تیمار حرارتی و فشار بالا به‌ترتیب تا روز ۱۴ و ۲۹ انبارداری بدون تغییر باقی ماندند، اما پس از آن تا پایان انبارداری به‌طور معنی‌دار افزایش یافتند. بیشترین محافظت از فنل کل (۸۲/۵۱ درصد) و ویتامین ث (۹۱/۶۱ درصد) طی انبارداری توسط فرایند فشار بالا به‌دست آمد. فعالیت آنزیم پکتین متیل استراز بلافاصله پس از هر دو تیمار به‌طور معنی‌دار کاهش یافت. باقیمانده‌ی فعالیت این آنزیم در پایان دوره‌ی انبارداری در نمونه‌های تحت تیمار حرارتی و فشار هیدرواستاتیک به‌ترتیب ۵/۶۹ و ۳/۵۹ درصد بود. آلودگی اولیه‌ی آبلیمو به باکتری‌های اسیددوست و مجموع کپک و مخمر به‌ترتیب ۰/۵۱ ± ۵/۷ و ۰/۲۸ ± ۴/۷۹ (واحد لگاریتم CFU در میلی‌لیتر) بود. تیمار فشار بالا شمار این میکروارگانیسم‌ها را بلافاصله پس از تیمار به زیر محدوده‌ی قابل‌مشاهده رسانید. شمار باکتری‌های اسید دوست تا روز ۱۴ انبارداری و شمار مجموع کپک و مخمر تا پایان انبارداری بدون تغییر باقی ماندند.

واژه‌های کلیدی: آبلیمو، بار میکروبی، پاستوریزاسیون حرارتی، فشار هیدرواستاتیک بالا، ویتامین ث

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