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Characterisation of fresh-like orange juice prepared by ultrasound treatment followed by high-pressure processing under industrial setting

Boon-Seang Chu [a], M. Adilia Lemos [a], Slim Blidi [a], Branimir Kostic [b], Rossella Di Monaco [c], Nicoletta Antonella Miele [c], Elena Torrieri [c], Moira Ledbetter [a], Óscar Rodríguez [d], Alberto Fiore [a,*]

[a] Department of Built Environment and Life Sciences - Food Science and Nutrition Group, Faculty of Social and Applied Sciences, Abertay University, Dundee, DD1 1HG, UK
[b] Cold Pressok, Dobracina 23°, Belgrade, Serbia
[c] Food Science and Technological Division, Department of Agricultural Science, University of Naples Federico II, Gussone Park, H Building, 80055, Portici (Na), Italy
[d] IRIS Technology Solutions, S.L. Crta d'Esplugues 39-41, 08940, Cornellà de Llobregat, Spain

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ABSTRACT

The rising demand for minimally processed nutritious food has led to advancements in non-thermal food processing technologies. This study evaluated the effects of combined ultrasound (US) and high-pressure processing (HPP) on the quality of Navel orange juice. Fresh juice was treated with US at 40 W/mL (K1), HPP at 450 MPa for 3 min (CP), or both US and HPP (UH). This study found that none of the treatments significantly altered the pH, total soluble solids, colour attributes, or total phenolic content compared to the untreated control (K0). HPP effectively eliminated microorganisms, reducing microbial counts below detection limits. US showed greater effectiveness than HPP in deactivating pectin methylesterase (PME) and maintaining cloud stability for up to 7 days at 4 °C. PME activity decreased from 6.5×10^{-4} unit/mL in K0 to 4.6×10^{-4} unit/mL with US, 5.3×10^{-4} unit/mL with HPP, and 3.8×10^{-4} unit/mL with the combined treatment, extending cloud stability to 14 days. Sensory evaluations indicated that US-HPP-treated juice was rated significantly better than CP and K0 samples. However, both treatments increased polyphenol oxidase and peroxidase activities by 22 % and 17 %, respectively. Further research is needed to optimize conditions to reduce these browning enzymes while preserving freshness.

1. Introduction

The commercial production of fruit juices presents specific challenges due to the sensitivity of fruits to thermal processing conditions and the need to maintain the natural attributes of the raw ingredients. Standard thermal treatments, such as pasteurisation, can have detrimental effects on the organoleptic properties and micronutrient content of fruit juices (Aghajanzadeh, Ziaiifar, & Verkerk, 2023), while higher temperatures and extended processing times can induce chemical reactions, including Maillard browning and caramelisation, leading to the formation of processing contaminants such as acrylamide, furans and hydroxymethyl furfural (HMF) which are harmful due to their potential carcinogenic and genotoxic properties (Capuano & Fogliano, 2011). For these reasons, non-thermal food processing technologies have gained significant attention due to their potential to preserve the sensory, nutritional, and functional characteristics of foods while achieving microbial safety (De Corato, 2020). In this context, analysing HMF formation during ultrasound treatment of fruit juices becomes crucial, as it serves as a key indicator of processing intensity and potential quality changes.

Numerous studies on the development and application of non-thermal technologies for fruit and vegetable juices, including high-pressure processing (HPP) and ultrasound (US), have been reported in the literature with some promising results (Tsikrika et al., 2018, 2022; Bull et al., 2004). HPP involves subjecting food products to pressures ranging typically from 100 to 600 MPa. The isostatic nature of the process ensures that all parts of the food product experience the same pressure, leading to effective microbial inactivation. Thanks to its effectiveness in inactivating spoilage and pathogenic vegetative bacteria, HPP has been considered a promising alternative to pasteurisation.

* Corresponding author.
E-mail address: a.fiore@abertay.ac.uk (A. Fiore).

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For example, Usaga et al. (2021) demonstrated that greater than 5-log reductions of *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* were achieved in pH-adjusted apple juice when treated with HPP at 550 MPa for 1 min, while 600 MPa for 3 min provided an adequate safety margin for control of the vegetative pathogens in acid and acidified juices and beverages (pH < 4.5). However, the inactivation of spoilage and browning enzymes in juices by HPP is more challenging and produces mixed results, mainly because of the sensitivities of these enzymes towards HPP are unpredictable and very much specific to the fruit and cultivar (Chakraborty, Kaushik, Rao, & Mishra, 2014). For instance, Sulaiman et al. (2015) reported that HPP of pear puree at 600 MPa for 60 min did not cause any inactivation of polyphenoloxidase (PPO), one of the oxidoreductases involved in browning reactions that cause deterioration of colour and flavour. However, HPP under the same conditions led to 89 % and 8 % inactivation of PPO in apple and strawberry puree, respectively (Sulaiman et al., 2015). It is also notable that pectin methylesterase (PME), a pectin-destabilizing enzyme, from different varieties of orange juice showed varied resistance to HPP. Bull et al. (2004) demonstrated that the PME in Valencia orange juice was not inactivated by HPP at 600 MPa and 20 °C for 1 min, while the pressure treatment of Navel orange juice under the same conditions was able to reduce PME activity by approximately 45 %.

Various attempts have been taken to inactivate PPO, peroxidases (POD) and PME by combining HPP with other treatments such as mild thermal treatments (Tejada-Ortigoza et al., 2015; Terefe et al., 2016), enzyme inhibitors (Liu et al., 2024), thermosonication (Chen et al., 2022) and US (Abid et al., 2014). US treatment typically utilises low-frequency (20–25 kHz) sound waves to induce physical and chemical changes as a consequence of cavitation (Tsikrika et al., 2017). A time-resolved fluorescence study on horseradish POD has revealed that US cavitation causes the formation of di-tyrosine and removal of the haem within the enzyme, leading to the loss of its secondary structure and hence inactivation (Tsikrika et al., 2017). Abid et al. (2014) demonstrated the synergistic impact of combined US-HPP in inactivating of POD, PPO and PME in apple juice, although complete enzyme inactivation could not be achieved. Similarly, there was a clear trend in that the inactivation rate of PPO increased when high pressure was combined with US when treating blueberry juice (Zhu et al., 2017). Although promising results at laboratory scale, to the best of our knowledge, no published study focuses on the impact of combined US-HPP on juices at pilot scale. Thus, this study aimed to bridge the gap by characterising orange juice prepared by US and HPP at pilot scale under industrial setting. Fresh orange juice was subjected to sonication using an industrial US device followed by industrial HPP. The enzyme activities of POD, PPO and PME in the orange juice upon the treatment were determined, and the potential changes in the juice qualities and production of HMF were examined. The comprehensive analyses of the resulting juice should help informing the industry when considering adopting the technologies in the production of fresh-like quality fruit juices.

2. Materials and methods

2.1. Materials

Fresh oranges (*Citrus sinensis*, Navel variety), grown in Greece, were obtained from a supplier of Cold Pressok, Belgrade, Serbia. The fruits were stored at refrigerated temperature upon received until they were processed, with no sorting before processing except for manually removing mouldy or spoiled oranges. All reagents and chemicals either of analytical or HPLC grades were purchased from Merck (Gillingham, UK).

2.2. Extraction of orange juice and treatments

Fresh orange juice was prepared and treated at the premises of Cold Pressok in Belgrade, Serbia. The environment temperature in the factory was 16 °C. Briefly, fruits stored at 4 °C were pressed (using a Zummo Z40 juicer, Valencia, Spain) and the juice was filtered (pore size 0.4 mm). The orange juice was then divided into four 40-L portions and subjected to either US treatment (K1), HPP (CP) or US followed by HPP (UH), and a portion of no-treatment control (K0) for comparison purposes. The initial temperature of the juice before subjected to processing was 8 °C.

During the US treatment, the juice was sonicated using a UIP2000hdT (2000 W, 20 kHz) ultrasonicator (Hielscher Ultrasonic GmbH, Teltow, Germany). The acoustic energy was delivered using different constellations (booster-cascatrode) in order to achieve different acoustic amplitudes and intensities. The acoustic energy was applied using a flow cell FC2T600K. The power input was set at 40 W/mL with an external cooling system throughout the process to keep the temperature of the juice below 35 °C. The power input of US was optimised from a preliminary study using the same ultrasonicator working at varying specific energy (40–200 W/mL), acoustic power (1500–2000 W), treatment duration (15–20 min) and temperature (35–45 °C). The operating parameters were selected based on a balance between the inactivation of browning enzymes and native microbial flora versus the preservation of fresh-like qualities of orange juice.

Orange juice samples were manually bottled in 250-mL polyethylene bottles, following general HACCP food safety guidelines. For HPP, the bottled orange juice samples were treated using an industrial scale Hiperbaric 55 instrument (Hiperbaric, Burgos, Spain), which has a 55L capacity and a throughput of 270 kg/h. Samples underwent HPP immediately after US treatment (UH samples) or directly after juicing (CP samples). The pressure was gradually increased in 10-MPa increments from 0 to 450 MPa over 3 min to avoid drastic increase in the juice temperature and maintain the juice temperature below 24 °C throughout the process. This was the standard operating parameters of HPP by Cold Pressok for their commercial fruit juices. After the US and/or HPP treatments, the orange juice samples were kept at −20 °C until analysis. Another set of juice samples were kept undisturbed at 4 °C for 14 days for cloud stability test. The height of sediment of the juice samples was measured on Days 1, 3, 7 and 14.

2.3. Physio-chemical analyses

The colour of the orange juice samples was determined using a colorimeter (Minolta Chroma Meter, CR 300, Japan). The instrument was calibrated using a plate (L* = 96,64; a* = 0,23; b* = 1,85), and for each sample the L* (from 0 = black to 100 = white), a* (-a* = greenness to +a* = redness), and b* (-b* = blueness to + b* = yellowness) parameters were determined. The pH was measured using a pH meter (pH 8 series-vers 1.1 04/2015, Eutech Instruments Pte Ltd., Ayer Rajah Crescent, Singapore), while a digital refractometer (Atago PR32-Palette, Tokyo, Japan) was used for determination of total soluble solids (Kimball, 2012).

The determination of K, Na, Mg and Ca in the orange juice samples was performed using a Synchronous Vertical Dual View 5100 ICP-OES spectrophotometer (Agilent Technologies, Santa Clara, CA). The instrumental setting and operational conditions were as following: a sample flush time of 30 s, pump stabilisation time of 15 s, nebuliser gas flow of 0.7 L/min, auxiliary gas flow of 1.0 L/min, flush pump rate of 50 rpm, radiofrequency power of 1200 W, analysis pump rate of 12 rpm, plasma SVDV view and a coolant gas flow of 12 L/min. The following analytical lines were used for each element: K at 766.491 nm, Ca at 396.847 nm, Mg at 279.553 nm and Na at 589.592 nm.

The total phenolic content (TPC) of the samples was determined using Folin-Ciocalteu assay according to Singleton & Rossi (1965), and the results were expressed as g of gallic acid equivalent per L of juice (g GAE/L).

2.4. Liquid chromatography quadrupole time-of-flight (LC QToF) analysis

The main phytochemicals in the orange juice were determined by a LC QToF. To this end, the juice sample was centrifuged at 18,000 *g* for 5 min and the resulting supernatant (1 mL) was subjected to 10-mL methanol/water (60:40 v/v) extraction for 30 min with orbital shaking. The sample was then centrifuged at *ca.* 4000 *g* at 4 °C for 10 min and filtered through a 0.2 μm PTFE filter into HPLC vial for analysis using a high-resolution LC QToF. The system consisted of a 1290 Infinity II LC and a 6546 LC QToF detector (Agilent Technologies, Santa Clara, CA). The chromatographic separation was performed on a Synergi Hydro RP column (150 mm × 4.6 mm, 4 μm Phenomenex), maintained at 25 °C. Mobile phase A was water containing 0.1 g/100 mL formic acid and mobile phase B was acetonitrile containing 0.1 g/100 mL formic acid. The gradient was 2 % B at 0.4 mL/min increasing to 20 % B over 8 min, increasing to 40 % over 2 min, increasing to 95 % B over 3 min, held for 3 min, before re-equilibrating to initial conditions. The mass spectroscopy was equipped with an Agilent jet steam electrospray ionisation (AJS ESI) operated in negative ionisation mode. Both centroid and profile data was collected in scan from 50 to 1700 m/z at a rate of 1 spectra/s. The QToF parameters were as follows: capillary voltage 2.3 kV, drying gas temperature 350 °C, drying gas flow 8 L/min, nebuliser gas pressure 35 psi, sheath gas temperature 350 °C and sheath gas flow 11 L/min. The mass spectrometer was controlled using MassHunter Workstation Version 11.0, build 11.0.221.1 (Agilent Technologies, Santa Clara, CA). The qualitative data obtained was processed using MassHunter Workstation Qualitative Analysis Version 10.0, build 10.0.10305.0, and compounds were identified using MassHunter PCDL Manager.

2.5. Microbiological analysis

Twenty-five millilitre of each sample was aseptically diluted with 225 mL Buffered Peptone Water (BPW) (Liofilchem, Rosseto Degli Abruzzi, Italy), and four 10x serial dilutions were conducted. All counts of the detected microorganisms were recorded as $\log_{10}$ CFU per mL.

Using the previous BPW dilutions, the total plate count was carried out using the pour plate technique (Plate Count Agar, Liofilchem), according to the protocol of ISO 4833 (International Standard Organization, 2022). Plates were incubated at 30 °C for 72 h.

The enumeration of coagulase positive *Staphylococci* spp. was carried out according to the ISO 6888–1:2009 (International Standard Organization, 2021). The ISO 16649–2 (International Standard Organization, 2001) protocol was applied to enumerate *Escherichia coli*. Isolation and identification of *Salmonella* spp. was carried out according to the ISO 6579 2017 (International Standard Organization, 2017), while yeasts and moulds were counted according to the ISO 21527-2-1:2011 (International Standard Organization, 2011) and counting of *Listeria monocytogenes* was carried out according to the protocol ISO 11290–1:2017 (International Standard Organization, 2017; International Standard Organization, 2017).

2.6. Determination of antioxidant activities

The antioxidant activities of each juice sample were determined using 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays.

For ABTS assay, the antioxidant activity of juice samples was estimated as described in a previous work (Re et al., 1999) with minor modifications. Briefly, a 1:15 diluted juice sample or Trolox standard (100 μL) was added to 1.0 mL of diluted ABTS solution, an absorbance was recorded at 734 nm after 2.5 min using a UV–Vis spectrophotometer (Thermo Scientific Genesys 10S, Waltham, MA). The results were expressed as Trolox equivalent antioxidant capacity (TEAC) in g per L of juice (g TEAC/L).

The FRAP assay was adapted from the work of Benzie & Strain (1996). Briefly, 100 μL of diluted juice sample (1:15) was mixed with 900 μL of FRAP reagent. After incubation for 4 min at room temperature, absorbance was measured at 593 nm using a UV–Vis spectrophotometer (Thermo Scientific Genesys 10S, Waltham, MA). Results are expressed as gallic acid equivalent (GAE) in g per L of juice (g GAE/L).

DPPH radical scavenging ability of the juice samples was evaluated according to Blois (1958)with some modifications. Briefly, 1.0 mL DPPH working solution was added to 20 μL of sample. After 10 min, absorbance was recorded at 517 nm using a UV–Vis spectrophotometer (Thermo Scientific Genesys 10S, Waltham, MA). The results were expressed in g GAE/L of juice.

2.7. Determination of POD and PPO residual activities

POD and PPO residual activities were assessed by adapting the assays used by Tsikrika et al. (Tsikrika et al., 2018, 2022). To determine POD residual activity, in brief, 50 μL orange juice was mixed with 0.5 mL monopotassium phosphate buffer (100 mM or 0.136 g/L; pH 6.1), 0.25 mL guaiacol (96 mM or 11.917 g/L) and 0.25 mL hydrogen peroxide (12 mM or 0.408 g/L). To assess PPO residual activity, 70 μL orange juice was mixed with 0.233 mL catechol solution (0.05 M or 5.505 g/L in 0.1 M sodium phosphate buffer solution; pH 6.1). The oxidation of guaiacol by POD and catechol by PPO was measured by the increase in optical density of the sample mixture at 470 nm and 420 nm, respectively, over a minute in a UV–Vis spectrophotometer (Thermo Scientific Genesys 10S, Waltham, MA). The percentage of residual activity of both enzymes was calculated as below:

$$\text{POD or PPO residual activity } (\%) = \left(\frac{A}{A_0}\right) \times 100 \quad [1]$$

Where A_0 and A were the initial (before treatment) and final (after treatment) enzyme activity, respectively.

2.8. Determination of PME activity

The activity of PME in the juice samples (Day 1) was evaluated by titrating the free carboxylic groups of pectin, employing the method of Rouse & Atkins (1955) with the modification of alkali strength, pH and temperature. Juice sample (5 mL) containing 0.15 M NaCl and pH adjusted to 7.7 was added with exactly 0.1 mL 0.05 N (2.00 g/L) NaOH. The reaction was started when 20 mL of 1 % w/v pectin solution, also containing 0.15 M (6.00 g/L) NaCl and at pH 7.7, was added to the mixture. The mixture was incubated at 30 °C under magnetic stirring and the time taken for the pH to decrease back to 7.7 was recorded. The PME activity, expressed as unit per mL of sample, was derived from the following equation

$$\text{PME activity } \left(\text{unit} \Big/ \text{mL sample}\right) = \frac{\text{mL of NaOH} \times \text{N of NaOH} \times 1000}{\text{mL of juice} \times \text{time (min)}} \quad [2]$$

To follow the kinetics of PME activity titrimetrically, another set of experiments were conducted using a pH-stat autotitrator (Titrando 902, Metrohm AG, Herisau, Switzerland). A juice sample (5 mL) containing 0.15 M (6.00 g/L) NaCl, pH 7.7, was incubated at 30 °C under magnetic stirring. The reaction was started by the addition of 20 mL 1 % w/v pectin solution which also containing 0.15 M (6.00 g/L) NaCl and at pH 7.7. The free carboxylic groups of pectin released by PME were continuously titrated with 0.05 N (2.00 g/L) NaOH to maintain the pH at 7.7. The amount of NaOH required to maintain equilibrium was used to calculate the PME kinetics during the pectin hydrolysis.

2.9. Determination of HMF

HMF determination was performed following the HPLC method previously described by Yuan & Chen (1998) with minor modifications. In brief, juice aliquots (2 mL) were centrifuged at 7000 *g* (Centrifuge 5417R, Eppendorf, Germany) for 10 min at 4 °C. The supernatants were then filtered through 0.22 μm nylon filters into HPLC vials for injection. The HPLC system consisted of a Dionex autosampler ASI-100 (San Jose, CA), a Thermo Scientific Ultimate 3000 Pump (Loughborough, UK) and a Dionex DDA-100 diode array detector (San Jose, CA). Twenty μL were injected onto an Agilent Eclipse Plus C18 250 mm × 4.6 mm (5 μm) column (Santa Clara, CA). The mobile phase was methanol in water (10 % v/v). The chromatographic run was isocratic at a flow rate of 0.8 mL/min at 20 °C with a run time of 20 min. Chromatograms were recorded at 280 nm and HMF eluted at 10.21 min. HMF quantification was carried out by an external calibration in the range 200–1000 μg/L; $R^2 < 0.99$. Limit of detection (LOD) was 50 μg/L, and limit of quantification (LOQ) was 150 μg/L and the method detection limit was 109 μg/L.

2.10. Sensory evaluation

Quantitative Descriptive Analysis (QDA) of the orange juice samples was performed by 9 trained judges (8 females, 1 male; mean age = 27.4 ± 3 years), who provided the written informed consent according to the principles of the Declaration of Helsinki (1964 and its later amendments), Italian ethical requirements on research activities and personal data protection (D.L. 30.6.03 n. 196) and the ethical standards of the University of Naples Federico II. The training comprised of two sessions. In the first session, the judges were familiarised with the samples and eight sensory attributes associated with the juice were generated (two related to appearance, two related to flavour, and four related to taste). The judges were then trained on the corresponding standardised evaluation techniques to improve data reproducibility in the second training session. For the sensory test, frozen juice samples were thawed at 4 °C and filled in small transparent glasses, marked with three-digit numerical codes and served at room temperature. The judges evaluated the samples in individual sensory booths and the attributes were scored on 10-cm unstructured scales with references. Descriptive data were collected using the "Smart Sensory Solutions" software (Vers. 2.11.11; Italy), following a randomized design with three replications from three different sessions.

2.11. Statistical analysis

One-way ANOVA and post-hoc Tukey HSD test were performed on the data where appropriate (IBM SPSS Statistics 23). Unless specified otherwise, all experiments were repeated three times and samples from each repetition were analysed in triplicate. The values were considered significantly different when $p < 0.05$.

3. Results and discussion

3.1. Physico-chemical characteristics and microbial loads

The Navel oranges used to prepare the orange juice were a winter orange with thick, bright orange skin and sweet, juicy fruit. Table 1 showed some physico-chemical characteristics of the orange juice samples. The fresh untreated orange juice (K0) had a pH of 3.3 and total soluble solids of 11.2 °Brix which were similar to the reported values of Australian (Bull et al., 2004) and Greek Navel varieties (Zvaigzne et al., 2017). The untreated juice contained 124.3 mg/L of Na, 87.8 mg/L of Mg, 51.7 mg/L of Ca and 1560.9 mg/L of K. The unusually high sodium concentration might be attributed to a combination of orchard-specific factors (e.g. soil or irrigation salinity) and the filtration step prior to analysis, which likely increased the availability of soluble ions in the juice. While the colour L*, a* and b* values were 44.7, −3.7, and 15.3, respectively. There was little colour change in the orange juice after US treatment (ΔE = 1.02) compared to the untreated control K0, but the difference became more apparent after the combined US-HPP (ΔE = 8.28). The TPC of the untreated juice was 0.83 g/L (expressed as gallic acid equivalent), which was slightly lower than that of reported Greek Navel (1.06 g/L (Zvaigzne et al., 2017)) but higher than the values obtained from the same variety grown in the Western Cape region of South Africa (0.54 g/L) (Hunlun, de Beer, Sigge, & Van Wyk, 2017)). Such differences were likely owing to growing conditions of the oranges. There were no significant ($p > 0.05$) changes in pH, total soluble solids, colour attributes and TPC of the orange juice upon treatment with either US (K1), HPP (CP) or the combination of US-HPP (UH) (Table 1). This was consistent with previous studies (Bull et al., 2004).

Identification of phytochemicals in the orange juice samples was performed using the LC QToF analysis and the results were summarised in Table 2. The two main flavonoids found in the orange juice samples were hesperetin and naringin, while naringenin, kaempferol and quercetin were present in smaller amounts. The relative abundance of hesperetin decreased significantly ($p < 0.05$) upon the HPP (32.8 peak area/ng) and US-HPP combined treatments (37.1 peak area/ng) but remained the same after the US treatment (41.9 peak area/ng) when compared to the untreated sample (43.4 peak area/ng).

On the other hand, the processing methods resulted in a notable increase in naringin levels, rising from 11.8 peak area/ng in the control K0 to 38.6, 60.4 and 57.1 peak area/ng in K1, CP and UH, respectively ($p > 0.05$). Another phytochemical detected in the orange juice samples was citroside A, a terpene glycoside, which showed an increased concentration following both individual and combined applications of the US and HPP treatments compared to the control ($p > 0.05$). These results were in accordance with some previous studies (Guerrouj et al., 2016; De Ancos et al., 2020) which also reported that US or HPP increased flavonoids content in orange juice. This positive effect was attributed to an increase of the membrane permeability and disruption of cell walls and cell organelles that favoured the release of bioactive compounds from the tissues and improved their extractability (Vázquez-Gutiérrez et al., 2013).

In terms of microbiological quality, the total plate count, and yeast

Table 1
Physico-chemical characteristics of the orange juice samples without treatment (K0), treated by ultrasound (K1), high-pressure processing (CP), and combined ultrasound-high pressure processing (UH).

	K0	K1	CP	UH
pH	3.33 ± 0.36[a]	3.50 ± 0.31[a]	3.36 ± 0.34[a]	3.54 ± 0.31[a]
Total soluble solids, °Brix	11.20 ± 0.39[a]	11.25 ± 1.12[a]	11.00 ± 0.30[a]	11.30 ± 0.98[a]
Colour parameter L*	44.68 ± 1.57[a]	43.69 ± 2.10[a]	44.31 ± 1.92[a]	46.28 ± 7.28[a]
Colour parameter a*	−3.71 ± 0.90[a]	−2.94 ± 0.72[a]	−3.59 ± 0.64[a]	−3.42 ± 1.36[a]
Colour parameter b*	15.32 ± 2.62[a]	14.64 ± 2.03[a]	17.99 ± 6.97[a]	19.05 ± 7.08[a]
ΔE (in comparison to K0)	–	1.02	3.64	8.28
Total phenolic content, g GAE/L	0.83 ± 0.05[a]	0.82 ± 0.04[a]	0.81 ± 0.05[a]	0.81 ± 0.02[a]

Values presented are the mean ± standard deviation (n = 3). Different letters between the data of each parameter indicate statistically significant difference ($p < 0.05$).

Table 2
Major phytochemicals identified in the orange juice samples without treatment (K0), treated by ultrasound (K1), high-pressure processing (CP), and combined ultrasound-high pressure processing (UH).

Compound	Molecular formula	m/z calculated	t_R, min	Error, ppm	K0	K1	CP	UH
5,4′-Dihydrox-3-methoxy-6,7-methylenedioxyflavone-4′-glucuronide	$C_{23}H_{20}O_{13}$	504.0904	2.90	−3.97	2.87 ± 0.63a	3.40 ± 0.12a	3.22 ± 0.37a	2.33 ± 0.66a
Catechin	$C_{15}H_{14}O_6$	290.0790	12.71	−1.26	2.61 ± 0.08b	2.34 ± 0.18b	2.39 ± 0.03b	1.68 ± 0.16a
Citroside A	$C_{19}H_{30}O_8$	386.1941	13.05	−2.36	23.96 ± 1.06a	35.88 ± 2.03b	36.06 ± 3.73 ab	44.96 ± 2.88c
Deacetylnomilinic acid	$C_{26}H_{34}O_9$	490.2202	7.47	−4.28	3.58 ± 0.12a	3.56 ± 0.52a	5.19 ± 0.97b	3.48 ± 0.30a
Hesperetin	$C_{16}H_{14}O_6$	302.0790	13.83	−3.93	43.4 ± 2.09a	41.9 ± 2.27a	32.8 ± 0.65c	37.06 ± 1.88b
Kaempferol 3-(6′′-acetylglucoside)-7-rhamnoside	$C_{29}H_{32}O_{16}$	636.1690	1.17	−4.30	9.46 ± 0.10 ab	6.15 ± 0.82a	12.12 ± 0.28b	6.07 ± 3.89a
Kaempferol 3-(2′′-glucosyl-6′′-acetyl-galactoside)-7-glucoside	$C_{53}H_{56}O_{28}$	1140.2958	2.76	−0.46	3.24 ± 0.27a	4.54 ± 2.17a	4.25 ± 0.91a	3.70 ± 0.11a
Naringenin	$C_{15}H_{12}O_5$	272.0685	15.22	−1.21	n.d.a	10.19 ± 1.34c	6.92 ± 1.67b	8.13 ± 0.42bc
Naringin	$C_{27}H_{32}O_{14}$	580.1792	14.05	−2.50	11.78 ± 0.21a	38.60 ± 6.60b	60.39 ± 0.55c	57.12 ± 5.05c
Nomilinic acid	$C_{28}H_{36}O_{10}$	532.2308	2.97	−4.14	7.61 ± 1.62b	n.d.a	11.41 ± 12.71c	4.36 ± 1.38b
Quercetin 3-(6′′-malonylglucoside)	$C_{24}H_{22}O_{15}$	550.0959	2.92	−3.82	4.82 ± 0.89a	5.04 ± 0.34 ab	5.92 ± 0.35 ab	6.97 ± 1.23b
Sinapic acid	$C_{11}H_{12}O_5$	224.0685	10.86	−5.00	17.14 ± 3.51c	n.d.a	8.54 ± 5.29b	n.d.a
Uridine 5′-diphosphoglucose	$C_{15}H_{24}N_2O_{17}P_2$	566.0550	2.91	−4.80	27.11 ± 9.89a	36.81 ± 7.81a	38.53 ± 9.96a	17.02 ± 14.47a
Xanthosine	$C_{10}H_{12}N_4O_6$	284.0757	8.31	−4.59	4.65 ± 0.15a	5.13 ± 0.01b	4.88 ± 0.05 ab	4.99 ± 0.20b

The unit (relative abundance) of the phytochemicals is expressed as peak area/ng of juice sample. Values presented are the mean ± standard deviation (n = 3). Different letters between the data of each compound indicate statistically significant difference ($p < 0.05$). t_R, retention time; n.d., not detected.

and mould count of the untreated control (K0) were 3.63 $\log_{10}$ cfu/mL and 3.20 $\log_{10}$ cfu/mL, respectively. The US treatment (at 40 W/mL) reduced the population levels to below 2.48 $\log_{10}$ cfu/mL for both microbial counts. HPP alone (at 450 MPa for 3 min) could reduce the microbial population to below detection limit, so the US treatment prior to the HPP did not add beneficial effect on reducing the microbial activity of the orange juice. The effectiveness of HPP in inactivating microorganisms in juices is well documented in the literature. For example,

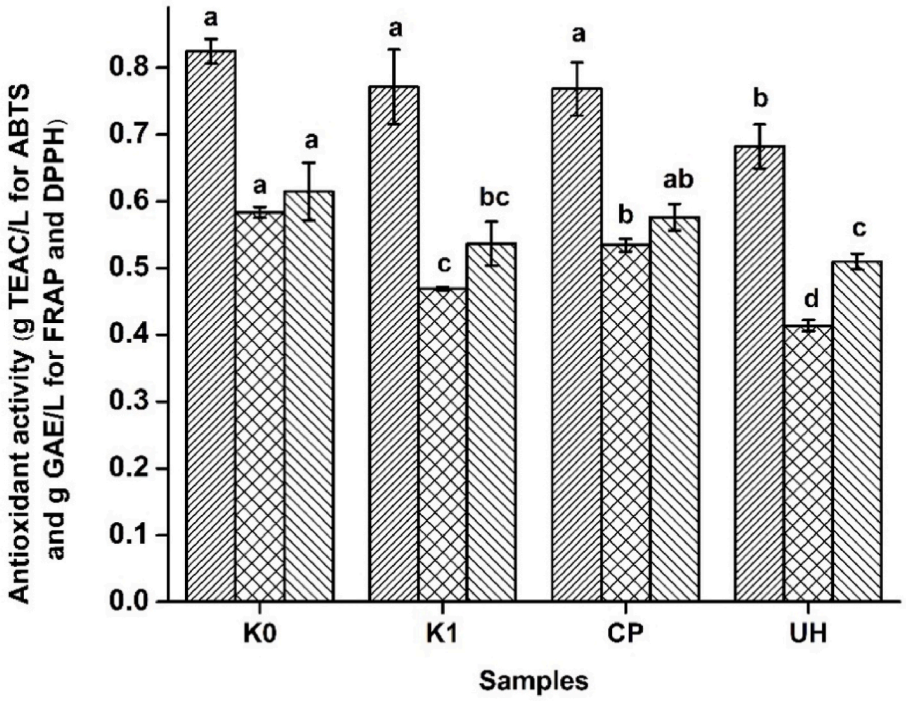

Fig. 1. Antioxidant activities (ABTS, FRAP and DPPH) of orange juices without treatment (K0), treated with ultrasound (K1), high-pressure processing (CP) and combined ultrasound-high pressure processing (UH). Values presented are the mean ± standard deviation (n=3). Different letters between the data of each antioxidant activity test indicate statistically significant difference ($p < 0.05$). ▨ ABTS, ▩ FRAP and ▧ DPPH.

Bull et al. (2004) reported that pressurisation at 600 MPa for 1 min reduced viable aerobic bacteria in Navel orange juice from 4.5 $\log_{10}$ cfu/mL to below detection limit and the juice was still microbiologically stable up to 12 weeks when stored at 4 °C. Similarly, Ambreen et al. (2023) demonstrated that yeast and mould of initial population 3.83 $\log_{10}$ cfu/mL in Valencia orange juice was reduced to below detection limit after treated at 400 MPa for 6 min. It was thought that HPP induced changes in microbial cells such as inhibition of key enzymes, alterations in cell morphology and disruption of cellular functions responsible for survival and reproduction that led to cell death (Podolak et al., 2020). In this study *E. coli*, *Staphylococcus* sp., *Salmonella* sp. and *Listeria monocytogenes* were not detected in all samples. The combination of ultrasound (US) and high-pressure processing (HPP) has demonstrated a synergistic effect on microbial load reduction, effectively inactivating *E. coli* and achieving undetectable levels of colony-forming units, as previously reported by Rodríguez et al. (2024).

3.2. Effects on antioxidant activities

The antioxidant activities of the orange juice before and after the treatments were assessed by ABTS, FRAP and DPPH and the results were shown in Fig. 1. Either US treatment or HPP significantly ($p < 0.05$) reduced the antioxidant activities of the orange juice as shown by K1 (US-only) and CP (HPP-only) samples when compared to K0 the untreated control, although the retention of the activities was still high, ranging from 81 % to 95 % in both samples. Coupling the two treatments further decreased the antioxidant activity in the juice samples (UH), with an antioxidant activity retention of 71–84 %. The results were in contrary with some previous studies which showed that US treatment enhanced the DPPH free radical scavenging activity and total antioxidant activity in fruit juices (Aadil, Zeng, Han, & Sun, 2013; Wang et al., 2019), probably due to the release of bound antioxidants such as phenolics in the fruit matrices into the juices by the cavitation of sound waves. As for HPP, the effect on the antioxidant activities appeared to depend on the processing conditions, with a shorter processing time and at a lower temperature resulting in a higher retention of antioxidant activity (Bisconsin-Junior et al., 2015). The literature showed that fruit type might also play a role in the retention of antioxidant activity in HPP-treated juices, with orange juice generally had a minor reduction or no change in antioxidant activities and radical scavenging parameters (Sánchez-Moreno et al., 2005; Vieira et al., 2018).

3.3. Effects on POD and PPO residual activities

Fig. 2 showed the POD and PPO residual activities present in untreated (control) and treated orange juice. Overall, the use of ultrasound and/or high-pressure processing increased the POD and PPO activities in juice samples although with no statistical significance ($p > 0.05$) between the different treatments. The increase of PPO and POD activity by non-thermal technologies, is not unknown and several works reported this before while at times the response to ultrasound treatment is affected also by the type of enzyme present. For instance, Sun, Zhong, Cao, Lin, & Ye, 2015 reported an increase of PPO activity while no changes were observed in the POD present in freshly apple juice. In fact, the cavitation process of ultrasound can alter enzyme conformation, making the enzyme's active site more accessible to the substrate, which helps explain the observed increase in enzyme activity (Li & Tang, 2021) . Additionally, ultrasound-induced cell and cell wall disruption can release previously entrapped enzymes into the surrounding medium, further enhancing enzyme activity. Ultrasound may also promote the dissociation of enzymes into monomers and activate isoenzymes, both of which contribute to the increase in activity (Zhu et al., 2019). According to Li & Tang (2021), ultrasound can make the active site of POD more accessible to the substrate, thereby enhancing its activity.

Both POD and PPO are responsible for enzymatic browning, however HPP and US are often unable to completely inactivate them. There were

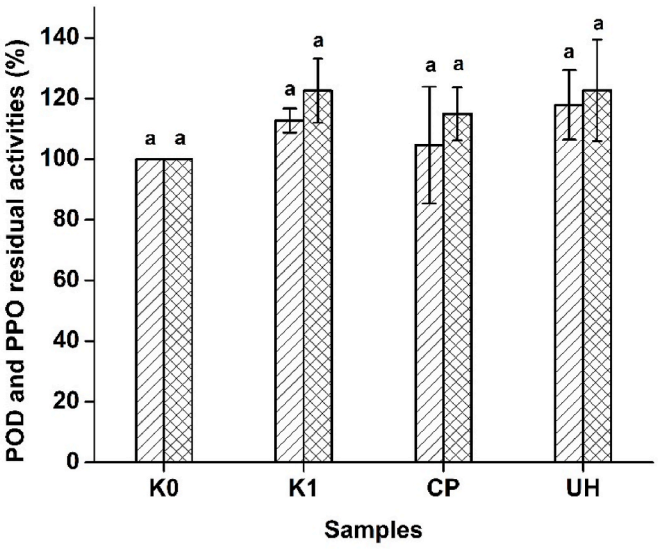

Fig. 2. Residual activity of peroxidase (POD) and polyphenol oxidase (PPO) present in the orange juice samples without treatment (K0), treated with ultrasound (K1), high-pressure processing (CP) and combined ultrasound-high pressure processing (UH). Values presented are the mean ± standard deviation (n=3). Different letters between the data of each enzyme indicate statistically significant difference ($p < 0.05$). ▨ POD and ▨ PPO.

inconsistent results in literature in relation to POD and PPO activity when using US (Cao, Cai, Wang, & Zheng, 2018; Santos et al., 2015; Silva & Sulaiman, 2022) that could be associated to the source of the enzyme and the use of different experimental conditions. In fact, a lack of consistency in indicating the US conditions was often observed in publications, making comparisons between different treatments very difficult. Enzyme inactivation is still an issue when using non-thermal technologies and more research is required in this area to better explore the possibility to overcome this. This pilot study used pre-established US and HPP parameters, so there are possibilities to adjust these conditions which may lead to POD and PPO inactivation, while keeping quality and safety of the juice.

3.4. Physical stability and PME activity

Fig. 3 showed the appearance of orange juice during storage at 4 °C on Days 1, 3, 7 and 14. It was apparent that cloud loss started to occur in K0 (untreated control), K1 (US-only) and CP (HPP-only) at the early stages of the storage. On the other hand, UH (combined US and HPP) remained largely homogeneous and significant juice clarification, or cloud loss, was only observed on the Day 14 samples.

The presence of PME residual in the samples explained the undesirable cloud loss. PME catalysed the C-O bond hydrolysis of the methoxylester groups of pectin, forming methanol and converting pectin into pectate (Gonzalez & Rosso, 2011). So PME demethoxylated pectin and once the degree of esterification reached a critical low, divalent cations (mainly calcium ions that naturally present in the juice) crossed-link free carboxylic groups belonging to adjacent pectin chains, giving insoluble macropolymers that entrapped other components of cloud (Carbonell, Contreras, Carbonell, & Navarro, 2006) and eventually leading to juice clarification as observed in the samples.

Conventionally PME in citrus juices was inactivated by heat treatment. Pasteurisation at ≥ 88 °C for 10–15 s was required to inactivate most of the PME activity (Kimball, 2012), but the residual activity would not be destroyed until around 90 °C (Atkins, 1953). PME comprised of several isozymes with very different thermal resistance, and the residual activity of PME played an important role in cloud stability of citrus juice. Cameron, Baker, & Grohmann, 1998 isolated four isoenzymes of PME in

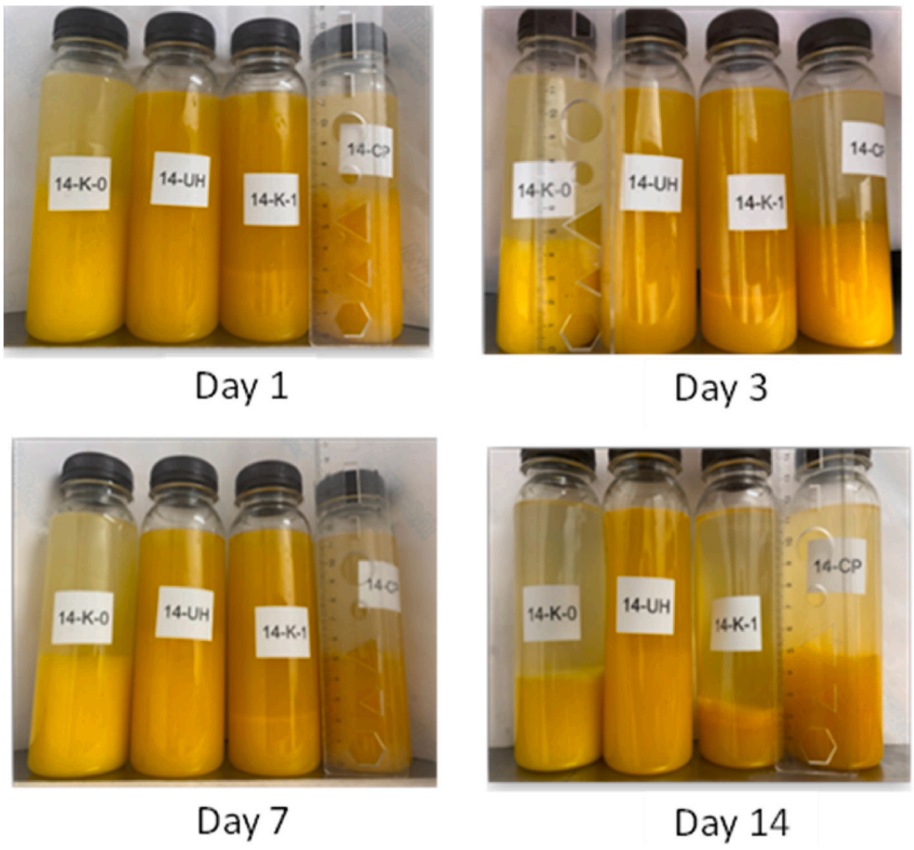

Fig. 3. Changes in cloudiness of the orange juice samples without treatment (K0), combined ultrasound-high pressure processing (UH), treated with ultrasound (K1), and high-pressure processing (CP) during refrigerated storage.

Valencia variety orange juice and found that the most heat-resistant form, although representing only 7.9 % of the total enzyme, recorded the largest cloud loss at refrigerated temperature.

It was evident that neither US nor HPP alone could sufficiently inactivate PME as seen in K1 and CP samples with PME activity of 4.6×10^{-4} and 5.3×10^{-4} unit/mL, respectively, in comparison to that of the untreated control 6.5×10^{-4} unit/mL. The results agreed with previous studies. For examples, Tiwari, Muthukumarappan, O'donnell, & Cullen, 2009 reported that a 62 % inactivation of PME in orange juice Valencia variety was observed after US treatment at acoustic energy density of 1.05 W/mL, ≤45 °C for 10 min, while HPP treatment of orange juice Navel variety at 600 MPa, 20 °C for 1 min was able to reduce the PME activity by approximately 45 % (Bull et al., 2004). The PME residual activity in CP and K1 samples manifested itself as significant cloud loss occurred on Days 3 and 7, respectively, during refrigerated storage (Fig. 3). This contrasted with other studies which reported that orange juice following HPP treatment at 700 MPa for 1 min was cloud stable for over 50 days stored at 4 °C (Goodner, Braddock, & Parish, 1998), and for over 60 days at 4 °C after treated at 800 MPa for 1 min (Nienaber & Shellhammer, 2001). However, it should be noted that the pressures used in these studies were higher than that of current study. Interestingly, the combined treatment of US and HPP although could not completely inactivate PME (enzyme activity of 3.84×10^{-4} unit/mL in UH), was able to prolong cloud stability of the orange juice for at least 14 days as observed in the UH samples (Fig. 3). The results demonstrated the combined effect of the two non-thermal treatments in preserving the cloud stability of orange juice.

To investigate the kinetics of PME activity in the samples, the release of H^+ ions due to PME action on pectin was followed by using a pH-stat and the results were shown in Fig. 4A. The PME activity was directly proportional to the rate of NaOH required to titrate the acid produced by PME (Fachin et al., 2002), and the PME activity was expressed as mole of H^+ ions titrated over time. The kinetics study demonstrated that all K0, K1 and CP samples had a very similar PME activity with a steep initial reaction rate, before the reaction reached to a plateau. On the other hand, UH sample showed a slower initial reaction rate and reached to a lower activity at the plateau (Fig. 4A). The amount of H^+ ions produced by PME action at the end of the pH-stat experiment was significantly ($p < 0.05$) higher in K0, K1 and CP in contrast to UH. The results were consistent PME activity determined manually using a pH meter to titrate the H^+ ions produced by the enzyme present in the juice sample (Fig. 4B). PME activity of UH was significantly lower ($p < 0.05$) than those of K1 and CP samples. The slower reaction kinetics and lower PME activity in UH explained why the sample was more cloud stable as observed in the storage study.

3.5. Production of HMF

HMF is a recognised indicator of nonenzymic browning, and it is often used as an index of deteriorative changes which take place during (thermal) processing and/or storage of foods. In this study, both the US (K1) and HPP-treated (CP) orange juice samples showed significantly ($p < 0.05$) higher HMF contents, increases of 12.7 % and 14.5 %, respectively, in comparison to that of the untreated control (K0) (Fig. 5). The combined treatment resulted in a further significant ($p < 0.05$) increase in HMF concentration, a 32.7 % increase compared to the control. The HMF increase after HPP treatment was lower than some previous studies. For instance, Liu et al. (2014) observed an increase of 58.8 % after HPP was applied at 600 MPa for 1 min in mango nectar. These differences could be due to the composition of the tested samples and/or to the treatment intensity. On the other hand, Cao, Cai, Wang, & Zheng, 2019 noted a HMF increase of 18.8 % after processing bayberry juice

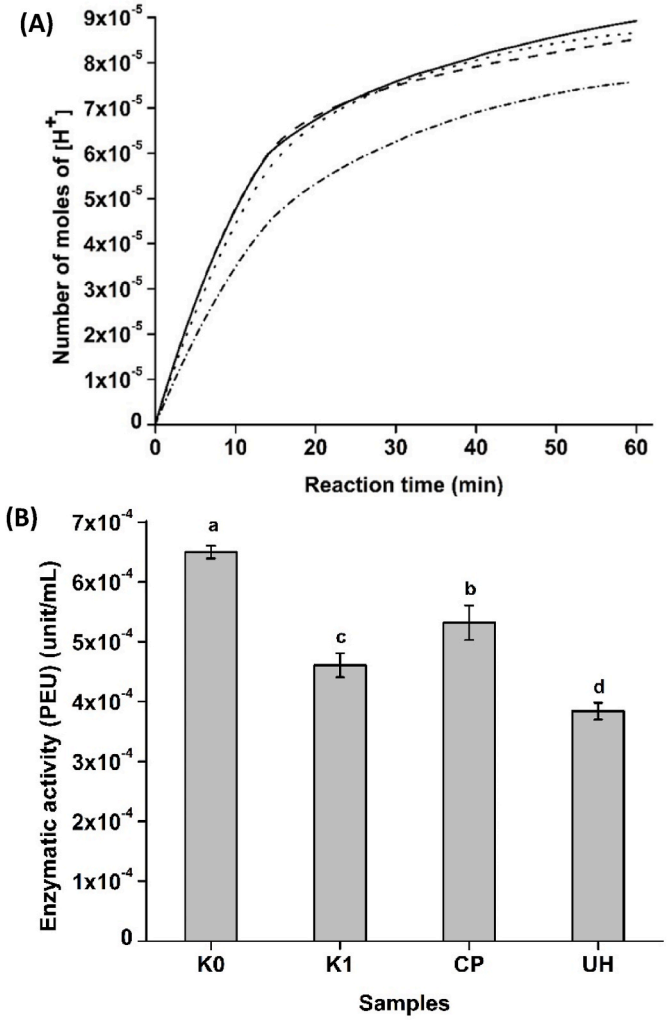

Fig. 4. (A) The kinetics and (B) enzyme activity of pectin methylesterase in the orange juice samples without treatment (K0), treated with ultrasound (K1), high-pressure processing (CP) and combined ultrasound-high pressure processing (UH) during refrigerated storage. Values presented in (B) are the mean ± standard deviation (n=3). Different letters between the data indicate statistically significant difference ($p < 0.05$). —— K0, – – K1, · · · · CP and -·-·- UH.

with ultrasound at 450 W/cm^2 for 10 min, which was in line with the current study. These results underscore the sensitivity of HMF formation to the specific conditions and characteristics of the juice being treated, and they highlight the importance of tailoring processing methods to the unique attributes of different juice types to minimize HMF formation. The increase in HMF (5-hydroxymethylfurfural) during HPP of juices can occur even in the absence of significant heat, primarily due to several non-thermal mechanisms. These include adiabatic heating – transient temperature spikes generated during compression (Nakornpanom et al., 2025), pressure-induced cell disruption that releases sugars for degradation (Gao et al., 2024; Lund & Ray, 2017), and acid-catalysed reactions, as the naturally low pH of juice can accelerate HMF formation under pressure (Konwar et al., 2024; Wibowo et al., 2015). Structural changes in sugars under pressure, such as the formation of reactive open-chain forms, also contribute to non-thermal HMF generation (Lund & Ray, 2017). Furthermore, processing conditions such as prolonged dwell times or pressures exceeding 500 MPa can intensify these effects, even when thermal input is minimal (Nakornpanom et al., 2025; Putnik et al., 2020).

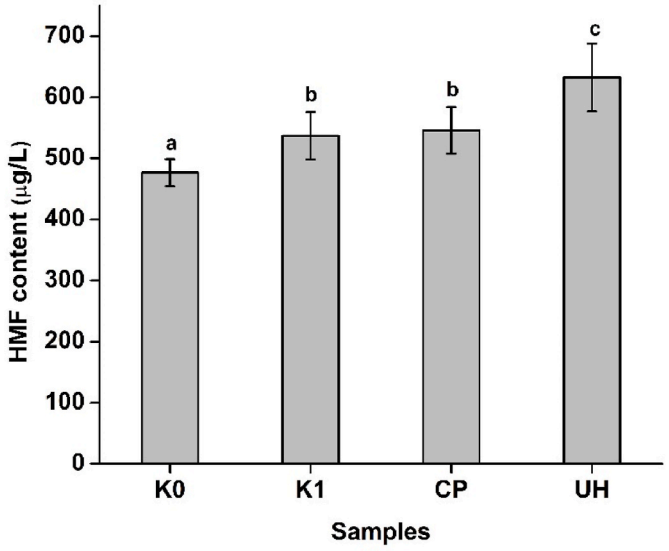

Fig. 5. Hydroxymethyl furfural (HMF) present in the orange juice samples without treatment (K0), treated with ultrasound (K1), high-pressure processing (CP) and combined ultrasound-high pressure processing (UH). Values presented are the mean ± standard deviation (n=3). Different letters between the data indicate statistically significant difference ($p < 0.05$).

3.6. Sensory evaluation

The judges evaluated the orange juice samples based on the eight attributes identified during the training and the results are shown in Fig. 6. No significant ($p > 0.05$) differences were detected among the orange juice samples in terms of colour, orange aroma, fermented odour, orange flavour, sourness and astringency, indicating that the US, HPP and the combined US-HPP did not convey noticeable changes in these sensory attributes. Note that the judges did not pick up the artificial "cooked" aroma normally associated with thermally processed juices in either of the samples, hence attaining the desirable fresh-like attributes of the orange juice. However, CP (HPP-only) and K0 (untreated control) were scored significantly higher than K1 (US-only) and UH (US-HPP) samples in terms of the presence of particulates in the appearance ($p < 0.001$) as well as when tasting ($p < 0.01$). The particulates were most likely the insoluble pectin macropolymers with other entrapped components of the orange juice due to the residual PME activity. The PME which was not deactivated at all (Control K0) or not deactivated sufficiently (as in the CP sample) (Fig. 4) precipitated the pectin in the juice within a few days after the juices were prepared (Fig. 3). On the other hand, US and US-HPP treatment decreased the PME activity to a larger extent and there were fewer insoluble pectin macropolymers in the samples. The results indicated that the combined treatment improve the cloud stability of orange juice, thus it could potentially enhance the appearance acceptability by consumers.

4. Conclusion

This study demonstrated the potential of the combined ultrasound and HPP in processing fresh orange juice at a pilot scale. These non-thermal treatments did not impart significant physico-chemical changes to the orange juice either applied individually or in combination. HPP alone (at 450 MPa for 1 min) was capable of reducing the microbial counts in the orange juice to undetectable limits. A key benefit of the combined US-HPP was that it reduced the PME activity in the juice to a level that could prolong the cloud stability for up to 14 days during refrigerated storage – an advancement could neither be achieved by US or HPP alone. A higher cloud stability was perceived as an important factor by consumers when purchasing the juice. However, combining US

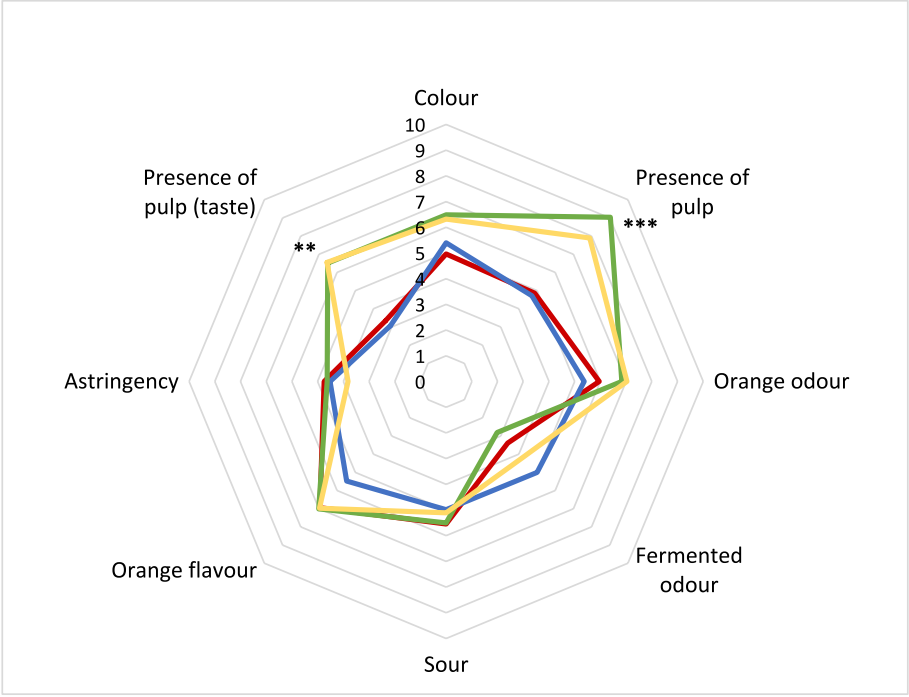

Fig. 6. Sensory Quantitative Descriptive Analysis results of the orange juice samples. untreated (K0), ultrasound (K1), high-pressure processing (CP), combined ultrasound and high-pressure processing (UH). **p<0.01; ***p<0.001.

and HPP did not reduce the activities of PPO and POD in the orange juice. It should be noted that the US and HPP were operated at predefined parameters and conditions in this trial, so the results only provided a snapshot of the combined treatment's performance. Further research is needed to optimize process parameters, further affirm microbial safety by inoculation tests, evaluate cost-effectiveness, and investigate the potential impact on sustainability and consumer acceptability. Continued exploration of this area will pave the way for the implementation of cutting-edge techniques in the production of healthier and safer fruit-based beverages.

CRediT authorship contribution statement

Boon-Seang Chu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **M. Adilia Lemos:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Slim Blidi:** Writing – original draft, Methodology, Formal analysis. **Branimir Kostic:** Formal analysis. **Rossella Di Monaco:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Nicoletta Antonella Miele:** Methodology, Formal analysis. **Elena Torrieri:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Moira Ledbetter:** Methodology, Formal analysis. **Óscar Rodríguez:** Methodology, Formal analysis. **Alberto Fiore:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data supporting the findings of this study are available within the article.

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