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Original article

Fortification of carrot juice with a high-pressure-obtained pomegranate peel extract: chemical, safety and sensorial aspects

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Summary High-pressure extraction was used to produce pomegranate peel extract, later incorporated in carrot juice. Chemical, microbiological and sensorial analyses were performed during storage to untreated, high-pressure and thermally processed juices incorporating pomegranate peel extract. Fortified juices showed lower counts for mesophiles and psychrophiles than the nonfortified ones ($P < 0.05$). Total phenolic and hydrolysable tannin contents, and antioxidant activity had superior values in supplemented juices during storage. The extract did not affect any sensorial parameter. On the 28th day, pressurised juices exhibited lower microbial loads in comparison with heated process, but phenolic compounds, antioxidant activity and several sensorial descriptors were identical between both processing technologies. The β- and α-carotene content decreased after processing, and pressurised juices exhibited higher residual activity for peroxidase ($P < 0.05$). These data suggest that the application of pomegranate peel extract in treated carrot juices (2.5 mg mL^{-1}) improves their microbial safety and antioxidant capacity, without impairing the sensorial aspects.

Keywords Beverage fortification, bioactive compounds, fruit residue, high-pressure technology, microbial safety.

Introduction

Each year, about 1.3 billion tons of the food generated globally is wasted. Among these losses, the fruit processing industry generates large amounts of by-products entailing economic and environmental concerns (FAO, 2011; Kowalska *et al*., 2017). Nonetheless, fruit by-products are rich in high value-added compounds with antioxidant and antimicrobial properties, and can be valorised through their incorporation – directly or via extracts – in food products. This sustainable approach could meet the consumer current demands for safer and health-promoting foods (Trigo *et al*., 2019).

Pomegranate juice is one of the most convenient ways to consume this fruit with numerous health benefits; however, its juice yield is about 332 L per ton of fruit, thus resulting in large amounts of by-products, mainly in the form of peels (Qu *et al*., 2009; Prospectiva 2020, 2015). The antimicrobial and antioxidant properties of pomegranate peel have been well reported in the literature, and this last one may prevent ailments such as cardiovascular diseases, cancer, diabetes and degenerative processes related to ageing (Hassan *et al*., 2017; Trigo *et al*., 2019).

The extraction of bioactive compounds from pomegranate peel is of paramount importance for its use in food applications. However, current extraction techniques display several limitations, such as (i) overheating of the by-product matrix; (ii) high energy and solvent consumption; (iii) long reaction times; and (iv) low yields. To overcome such problems, new pressure-assisted extraction technologies such as supercritical fluids and high pressure are being increasingly studied (Putnik *et al*., 2018; Alexandre *et al*., 2019). This latter technology relies on the ability of high pressure to cause physical damage to plant cells, thus enabling the extraction of internal components at room temperature. Despite the most well-studied application of high pressure being the preservation of food products, high-pressure-assisted extraction (HPE) has shown great efficiency in recovering bioactive compounds from fruit by-products (Alexandre *et al*., 2017, 2019; Ferrentino *et al*., 2017). To the best of our knowledge, no study had evaluated the chemical, safety and sensorial aspects of food products fortified with HPE-obtained extracts.

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Our work aimed to valorise an abundant by-product from the fruit juice industry and increase the value of carrot juice. This traditional food product was selected due to its (i) low antioxidant potential when compared to other vegetable juices and (ii) high pH, thus making it highly prone to microbial growth (Wootton-Beard *et al*., 2011).

In this work, pomegranate peel extract (PPE), obtained by HPE, was added to raw carrot juice in different concentrations (0, 1 and 2.5 mg extract per mL juice). Later, high-pressure and thermal preservation methods were employed to process the juices, which were stored for 42 days. Throughout storage, chemical, safety and sensorial tests were performed on these juices. Such tests allowed to compare the effect of (i) processing by high pressure with traditional thermal processing and (ii) increasing doses of PPE in carrot juices. With this work, we attempt to produce a fortified carrot juice with improved antioxidant and safety properties, without impairing the sensorial parameters.

Materials and methods

Preparation of pomegranate peel extract

About 70 kg of ripe pomegranates were collected in Évora, a region of south Portugal. The peels were manually separated from the fruit and dried at 40 °C. Dried samples were then frozen, grounded in a colloid mill and stored at −20 °C until used for HPE extraction.

High-pressure-assisted extraction

The extraction was performed in low-permeability polyamide–polyethylene bags (PA/PE-90) containing 8 g of ground pomegranate peel and 500 mL of deionised water (Alexandre *et al*., 2019). These bags were placed in a cylindrical loading vessel at room temperature and pressurised at 400 MPa for 30 min by a high-pressure equipment (Model 55; Hyperbaric, Burgos, Spain) with a pressure vessel of 55 L (Alexandre *et al*., 2019). After pressurisation at around 32 °C, the extract was centrifuged at 12 880 *g* for 10 min at 4 °C and the supernatant was filtered (Whatman®; Grade 2; 8 μm) and collected. The filtered extract was freeze-dried and stored in a desiccator protected from light. The total polyphenol content (TPC) of these extracts was compared by Alexandre *et al*. (2019), where TPC significantly improved from 201 ± 3.1 to 219 ± 2.6 mg gallic acid eq./g DW in unpressurised and high-pressure extraction, respectively.

Carrot juice preparation

Carrots (*Daucus carota* var. *Nantes*) were purchased from a local market (Porto, Portugal) and stored at 4 °C until further processing. Before juice preparation in a domestic high-speed juice extractor, the carrots were washed, and their edges were cut. Straight away, the juice was transferred to sterilised flasks where PPE was added in the following concentrations: 0, 1 and 2.5 mg PPE per mL juice. The extract was freeze-dried to not dilute the juice samples.

High-pressure and thermal processing

Juice aliquots (30 mL) were placed in PA/PE-90 bags. The bags were pressurised at 600 MPa for 10 min (Kim *et al*., 2001) in the same equipment used for HPE. For thermal processing (TP), the same volume of juice was heated at 80 ± 2 °C in a water bath for 7 min with a lag period of ~ 3.5 min (Dede *et al*., 2007). After TP, the samples were immediately chilled in a mixture of water/ice.

All samples were tested in duplicate and stored at 4 °C in the dark. The sampling time for untreated (UT) samples was on days 0 and 2, whereas for TP- and HPP-treated juices the selected days were 0, 7, 14, 28 and 42. The purpose of analysing day 2 in UT juices was solely to test whether the extract would have any antimicrobial effect *per se*. For microbiological analyses, samples were directly used, and for the remaining physicochemical parameters, all samples were first centrifuged at 18 659 *g* for 10 min and the supernatant was frozen at −80 °C until further evaluations.

Microbiological analysis

Total aerobic mesophiles and total aerobic psychrophiles were counted in plate count agar. The plates were incubated at 30 ± 1 °C and 7 ± 1 °C during 3 and 5 days, respectively (Berizi *et al*., 2016; Pintado *et al*., 2016). Yeasts and moulds were enumerated in Rose-Bengal Petri plates that were incubated at 25 ± 1 °C during 72 h (Ramos *et al*., 2012). Results were expressed as the decimal logarithm of colony-forming units (CFU) per mL of juice. The quantification limit associated with the method used was 2.78 log10 CFU mL^{-1}.

Total phenolics and hydrolysable tannins

Total phenolic content (TPC) was evaluated by the Folin–Ciocalteu method adapted by Oliveira *et al*. (2014). The total hydrolysable tannin content (HTC) was carried out according to Saffarzadeh-Matin & Khosrowshahi (2017). All measurements were carried out in triplicate.

Antioxidant capacity – ABTS and ORAC assays

The ABTS assay was based on the method described by Re *et al*. (1999), and the oxygen radical absorbance

capacity (ORAC) was performed according to the method developed by Dávalos *et al*. (2004).

HPLC quantification of individual carotenes

Carotenoids were extracted as described by Oliveira *et al*. (2016). The separation was performed in a reversed-phase C18 column (250 × 4.6 mm i.d., 5 μm particle size) using an HPLC-DAD system. Injection volume was 20 μL, and the detector was set at 454 nm. β- and α-carotene from Sigma-Aldrich were used as standards (concentration range of 0.98 - 62.50 and 0.49 - 31.25 mg L^{-1}, respectively). The results were expressed as mg L^{-1} juice, and three injections were performed in each of the duplicate samples.

Enzymatic analysis

Peroxidase (POD) activity assays were performed as reported by Rao *et al*. (1996) with minor modifications. Aliquots of 80 μL of sample were mixed with 800 μL of guaiacol (30 mM) and 400 μL of H_2O_2 (100 mM). Absorbance values were read at 470 nm during 10 min at room temperature *versus* a blank composed of 800 μL of guaiacol (30 mM) and 400 μL of H_2O_2 (100 mM). Guaiacol and H_2O_2 solutions were prepared in potassium phosphate buffer (100 mM, pH 6.0). Polyphenol oxidase (PPO) activity was tested according to the method described by Rocha & Morais (2001) with slight modifications. Aliquots of 150 μL of sample were mixed with 1120 μL of substrate solution containing catechol in sodium phosphate buffer (200 mM, pH 6.5), and the absorbance was measured at 420 nm during 5 min at room temperature.

The enzymatic activity was obtained from the linear portion of the absorbance–time curve. The quantification of POD activity was expressed as UI (quantity of enzyme that converts 1 μmol of subtract per min) and is given by Equation 1, being $\frac{\Delta Abs}{min}$, the absorbance of guaiacol in function of the time; V_{test}, the reaction volume (mL); ε, the extinction coefficient of guaiacol (2.52×10^{-2} M^{-1} cm^{-1}); and V_{enzyme}, the volume of enzyme added to the reaction solution (mL). All measurements were carried out in triplicate.

$$\frac{UI}{mL} = \frac{\frac{\Delta Abs}{min} \times V_{test} \times 1000}{\epsilon \times V_{enzyme}} \quad (1)$$

Sensorial analysis

The sensorial analysis used in this work was based on the quantitative descriptive analysis performed by Picouet *et al*. (2015). Sessions took place in the ISO 8589:2007 compliant sensory testing facilities of Escola Superior de Biotecnologia – Universidade Católica Portuguesa (ESB-UCP) and the trained panellist (n = 10) belonged to the ESB-UCP sensory evaluation panel. The sensorial descriptors evaluated included appearance, odour, taste and flavour, and mouth-feel. Regarding scoring scales, for appearance and odour attributes '0' meant orange colour and raw carrot odour, respectively, while '10' meant rust-brown colour and boiled carrot odour, respectively. The remaining attributes were evaluated using a nonstructured scoring scale, where '0' meant the absence of the descriptor and '10' meant the highest intensity of the descriptor.

Statistical analysis

The statistical analysis of the results was performed using SPSS Statistics v25.0.0.0 software. The normality of the distributions was evaluated using the Shapiro–Wilk test. Statistical significance of the effects was assessed by factorial analysis of variance (three-factor ANOVA) with Tukey's *post hoc* test to compare means at the $P < 0.05$. The differences were considered statistically significant at a 5% confidence degree level. The results were expressed as mean ± standard deviation.

Results and discussion

Microbiological analysis

The evolution of total aerobic mesophiles and psychrophiles, and yeasts and moulds counts (log_{10} CFU mL^{-1}) in carrot juices is depicted in Figure S1 of the Supplementary Material. During storage of unprocessed juices, the incorporation of PPE did not affect the microbial loads of the juices (Figure S1a-b). The initial pH value of nonfortified and fortified carrot juice was 6.42 ± 0.00 and 5.76 ± 0.01, respectively, suggesting that PPE had a significant impact on pH decreasing ($P < 0.05$). Pressure and heat treatment had a significant effect upon all microbial groups since microbial counts were reduced from 6.92 ± 0.06, 6.64 ± 0.19 and 5.08 ± 0.09 log_{10} CFU mL^{-1}, respectively, to undetectable levels. On days 7 and 14, total aerobic mesophiles and psychrophiles remained below the quantification limit (Figure S1a-b), whereas no yeasts and moulds were detected over storage (Figure S1c). On the 28th day of storage, pressurised samples exhibited lower microbial counts when compared to heated samples ($P < 0.01$); pressure treatment has been more effective in delaying microbial growth when compared to thermal processing (Picouet *et al*., 2015). Moreover, the supplementation of treated juices with 2.5 mg PPE per mL led to a significant microbial count reduction for both groups of microorganisms ($P < 0.05$); for

instance, in total aerobic mesophiles and psychrophiles, a count decrease ($P < 0.05$) of about 0.4 and 0.6 $\log_{10}$ CFU mL^{-1} was achieved for pressure- and heat-treated juices, respectively. Both PPE concentrations (1 and 2.5 mg PPE per mL) significantly reduced the total aerobic mesophile and psychrophile counts ($P < 0.01$), yet higher reductions were achieved for the 2highest concentration. In total aerobic mesophile group, the microbial reduction attained, on average, 0.4 and 0.8 $\log_{10}$ CFU mL^{-1} for 1 and 2.5 mg PPE per mL ($P < 0.01$), while for total aerobic psychrophiles the microbial reductions were about 0.2 and 0.6 $\log_{10}$ CFU mL^{-1}, respectively ($P < 0.01$). Despite the clear reduction of microbial loads in treated and fortified juices, further microbiological safety studies should be conducted, especially concerning the germination of spores of *Clostridium botulinum* type E (Segner *et al.*, 1966). As a low-acid beverage, carrot juice can pose a risk of botulism poisoning even after pasteurisation since lower processing temperatures could not be sufficient to eliminate spores of *Clostridium botulinum;* temperature abuse conditions during storage could also lead to toxin production in pasteurised carrot juices (FDA, 2007). Indeed, one limitation of HPP is that most of its products need transportation and storage under refrigeration since pressure treatment alone is not enough to inactivate spores of harmful pathogens (Huang *et al.*, 2017). On day 42, all juices presented signs of evident microbial spoilage due to its rotten odour, so the chemical and enzymatic parameters were not analysed in these samples. To the best of our knowledge, our work is the first reporting a significant antimicrobial effect of PPE in beverages.

Total phenolics and hydrolysable tannins

The evolution of TPC and HTC during the 28 days of storage is presented in Figure 1. No significant variations on TPC (Figure 1a) were found between days 0 and 28 of storage, and between HPP and TP samples ($P > 0.05$). As for the effect of PPE, fortified carrot juices showed higher TPC levels ($P < 0.05$). Indeed, this increase was almost proportional to PPE concentration; for instance, on day 0, TPC values, in supplemented juices with 1 and 2.5 mg PPE per mL, were improved 1.14 to 1.74 times and 1.99 to 2.50 times, respectively, when compared to the untreated juice without PPE (UT-0).

Hydrolysable tannin content was improved from undetectable levels in nonfortified juices to 0.166 ± 0.033, 0.182 ± 0.004, 0.244 ± 0.026 and 0.476 ± 0.042 mg tannic acid eq. per mL juice for HPP-1, HPP-2.5, TP-1 and TP-2.5, on day 0, respectively (Figure 1b). Pressure treatment did not have any impact on HTC ($P < 0.05$). However, heat treatment enhanced HTC in 51% when compared to the content

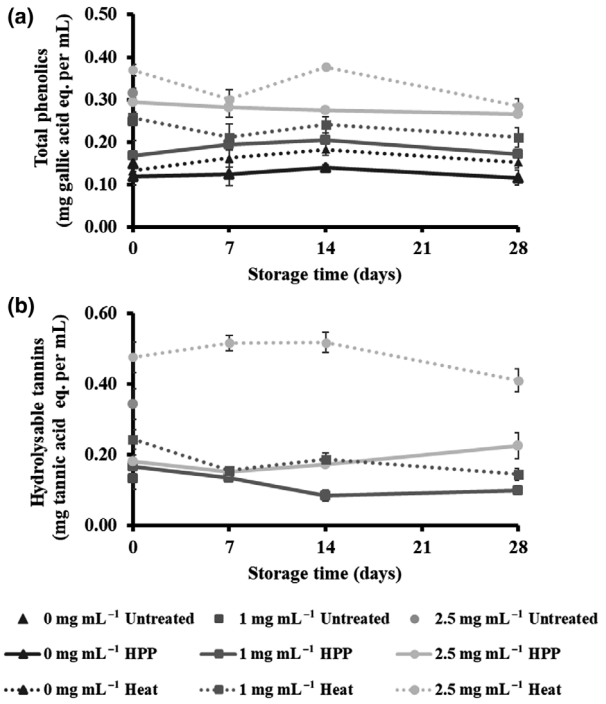

Figure 1 Evolution of (a) total phenolic content and (b) hydrolysable tannin content during 28 days of storage at 4 °C. In this figure can be seen the effect of pomegranate peel extract addition and the effect of processing type.

prior to processing ($P < 0.05$). Therefore, heat processing could induce a breakdown of hydrolysable tannins with the release of less complex tannins as well as ellagic acids and gallic acid (Colantuono *et al.*, 2018). In both treatments, the HTC was constant until day 14 ($P > 0.05$), and on day 28, no differences ($P > 0.05$) between HPP and TP were noticed, probably because the hydrolysable tannin degradation was greater in heat-treated samples.

Antioxidant capacity – ABTS and ORAC assays

Figure 2 summarises the ABTS results obtained during storage of carrot juices. No differences were detected between treatments on day 0 ($P > 0.05$). On the other hand, the fortification with PPE improved ABTS values up to 1.7, 2.8, 3.5, 1.7 and 4.0 times for UT-1, UT-2.5, HPP-2.5, TP-1 and TP-2.5, respectively, comparatively to UT-0 ($P < 0.01$). The antioxidant activity remained constant ($P > 0.05$) until day 14 and declined henceforth ($P < 0.05$). Between days 14 and 28, samples supplemented with 1 mg PPE per mL juice had similar results to the nonsupplemented ones ($P > 0.05$).

ORAC values were significantly influenced by the presence of PPE ($P < 0.01$). On day 0, antioxidant potential improved from 1.63 ± 0.04 to 2.28 ± 0.16,

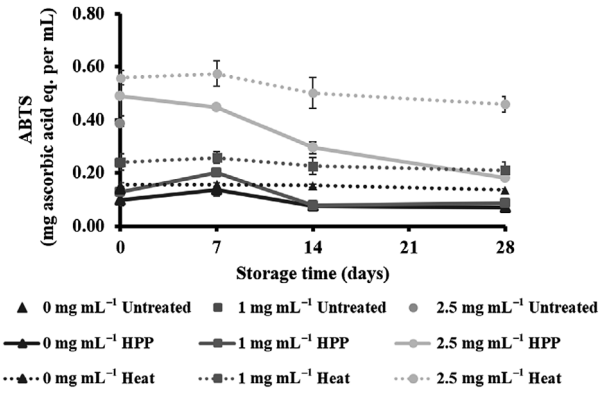

Figure 2 Evolution of ABTS during 28 days of storage at 4 °C. In this figure can be seen the effect of pomegranate peel extract addition and the effect of processing type.

2.17 ± 0.15 and 2.94 ± 0.30 mg Trolox eq. per mL for UT-2.5, HPP-2.5 and TP-2.5, respectively. In this way, TP-2.5 juice had the highest ORAC value possibly due to the thermal hydrolysis of HT; however, this difference was not significant when compared to HPP-2.5 and UT-2.5 juices ($P > 0.05$). During storage, a decline in the scavenging activity was noticeable ($P < 0.05$), particularly in TP samples. This could be explained by the instability of free gallic acid at relatively high pH value as is the case of carrot juice's pH (Friedman & Ju, 2000).

HPLC quantification of individual carotenes

The main carotenoids in carrot juice (i.e. β- and α-carotene) were quantified by HPLC, and their evolution throughout storage time is shown in Figure S2a-b of the Supplementary Material. For UT-0 juices, the β- and α-carotene contents were 11.3 and 2.7 mg L^{-1} for β- and α-carotene, respectively. Zhang *et al.* (2016) reported similar values for both carotenes (about 17 and 8 mg Kg^{-1} juice for β- and α-carotene, correspondingly). For UT and HPP samples, adding PPE decreases the β- and α-carotene content. This can be attributed to the probable formation of complexes between proteins of PPE and carotenoids, thus leading to less extractability of carotenoids (Natukunda *et al.*, 2016). On the other hand, the application of heat may prevent the formation of these complexes. The application of HPP and TP resulted in a decrease of β-carotene ($P < 0.01$) of 46-63% and 5-51%, respectively. For α-carotene, the percentage reductions were 40-47 and 3-43%, correspondingly. On days 14 and 28, HPP was significantly different from TP ($P < 0.01$). After TP, carotenoids may be entrapped within complexes from the cellular components formed after cell wall depolymerisation, hence protecting them from the oxidation reactions caused by oxygen/enzymes (Rodriguez-Amaya, 1997).

Enzymatic analysis

Peroxidase is involved in the loss of phenolic compounds, and its activity in UT-0 samples at day 0 was 6407 ± 759 UI mL^{-1}. According to Figure S3 of the Supplementary Material, heated samples had a negligible POD activity. Conversely, after pressurisation, POD residual activity decreased to 6.4-14.7%. Pomegranate peel extract reduced POD residual activity in all cases, yet this effect was only significant in HPP-2.5 samples from days 14 and 28 ($P < 0.01$). The

Table 1 Sensory scores for treated (by HPP and TP) carrot juice without and with PPE

	Thermal processing		High-pressure processing	
	0 mg mL^{-1}	2.5 mg mL^{-1}	0 mg mL^{-1}	2.5 mg mL^{-1}
Appearance				
Orange colour (0)	2.7 ± 0.5^{Aa}	5.3 ± 0.5^{Aa}	7.0 ± 0.8^{Ba}	9.0 ± 0.8^{Ba}
Rust-brown colour (10)				
Odour				
Raw carrot (0)	1.3 ± 0.5^{Aa}	3.7 ± 1.7^{Aa}	4.0 ± 2.2^{Aa}	6.3 ± 1.2^{Aa}
Boiled carrot (10)				
Taste and Flavour (0–10)				
Sweet taste	6.7 ± 0.9^{Aa}	7.8 ± 1.0^{Aa}	7.2 ± 1.2^{Aa}	8.2 ± 1.9^{Aa}
Acid taste	1.0 ± 0.0^{Aa}	1.0 ± 0.0^{Aa}	1.0 ± 0.0^{Aa}	1.0 ± 0.0^{Aa}
Fresh carrot	7.3 ± 0.9^{Aa}	6.2 ± 0.6^{Aa}	5.3 ± 1.2^{Ba}	4.5 ± 1.1^{Ba}
Boiled carrot	1.3 ± 0.5^{Aa}	1.8 ± 0.2^{Aa}	2.5 ± 0.7^{Ba}	3.3 ± 0.5^{Ba}
Persistency	6.0 ± 0.8^{Aa}	6.0 ± 0.8^{Aa}	6.0 ± 0.8^{Aa}	6.0 ± 0.8^{Aa}
Mouth-feel (0–10)				
Sliminess	1.7 ± 0.5^{Aa}	1.7 ± 0.5^{Aa}	2.0 ± 0.0^{Aa}	2.0 ± 0.0^{Aa}
Grittiness	1.7 ± 0.9^{Aa}	1.7 ± 0.9^{Aa}	1.7 ± 0.9^{Aa}	1.7 ± 0.9^{Aa}

All sensorial parameters were evaluated from 0 to 10 score. Within the same day, different upper case letters mean significant differences between treatments ($P < 0.05$); within the same day, different lower case letters mean significant differences between PPE concentrations ($P < 0.05$).

inhibitory action of phenolic compounds upon POD can occur through different mechanisms, such as the presence of oxidisable constituents capable of acting on the POD's prosthetic group causing enzyme inhibition and by chelation of metals like iron, which are essential to keep enzyme's activity (Malle *et al*., 2007). We also evaluated polyphenol oxidase (PPO) activity and found similar results to the latter ones, yet the extract did not inhibit PPO activity.

Sensorial analysis

Table 1 shows the panellist evaluation, after 1 day of storage, pertaining to sensorial descriptors such as appearance, odour, taste and flavour, and mouth-feel.

Juices enriched with PPE did not differ from the nonsupplemented ones in all sensory attributes tested ($P > 0.05$). Likewise, no major differences between HPP- and TP-treated juices were detected in the odour, sweet/acid taste, persistency and mouth-feel attributes ($P > 0.05$). However, heated samples had, in comparison with pressurised samples, a lower score for appearance, which translated into a higher orange colour intensity ($P < 0.01$). This is in agreement with the instrumental colour analysis since from day 0 onwards heated juices were more reddish (a*) and yellowish (b*) than pressurised juices (data not shown). The greater action of PPO detected in HPP juices was probably responsible for the decline of colour observed in these samples, while the high level of colour change (ΔE*) in TP juices can be related to the positive effect of heat on phenolic substances (anthocyanins and hydroxycinnamates) as suggested by Dede *et al*. (2007). Figure S4 of the Supplementary Material shows the visual aspect of the process juices.

Conclusion

Pomegranate peel extract (2.5 mg mL^{-1} juice) reduced the microbial counts, and the combination of PPE with HPP allowed higher microbial reductions than the one between TP and PPE. Total phenolic content improvement was almost proportional to the PPE dosage, and no significant variations were found during storage and between HPP and TP; a similar trend was found in hydrolysable tannin content. For antioxidant capacity (ABTS and ORAC assays), the results were similar between untreated and treated samples. However, throughout storage, antioxidant potential declined and only fortified juices (2.5 mg mL^{-1}) had higher ABTS values. On days 14 and 28 of storage, PPE did not influence β- and α-carotene contents of treated samples and peroxidase enzymatic activity was higher in pressurised juices. Lastly, all sensory descriptors were statistically similar between fortified and nonfortified juices. Incorporating a high-pressure-obtained PPE in HPP- and TP-treated carrot juice (2.5 mg mL^{-1}) reduces its microbial load and antioxidant capacity, without impairing the sensorial parameters.

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Conflicts of interest

The authors hereby declare there is no conflict of interests.

Data availability statement

Research data are not shared.

Ethical guidelines

Ethics approval was not required for this research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Evolution of (a) total aerobic mesophiles, (b) total aerobic psychrophiles, and (c) yeasts and moulds counts during 42 days of storage at 4 °C.

Figure S2. Evolution of (a) β-carotene and (b) α-carotene contents during 28 days of storage at 4 °C.

Figure S3. Evolution of PPO residual activity during 28 days of storage at 4 °C. In this figure can be seen the effect of pomegranate peel extract addition and the effect of processing type.

Figure S4. Visual aspect of the treated (by HPP and TP) carrot juices without and with increasing doses of pomegranate peel extract.