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A new drink rich in healthy bioactives combining lemon and pomegranate juices

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ABSTRACT

Nowadays, the interest in dietary antioxidants, mainly present in fruits and vegetables, has prompted research in the field of commercial polyphenol-rich beverages. The main objective of the present work was to produce new polyphenol-rich beverages using lemon and pomegranate juices in different proportions (at 25%, 50% and 75% for both juices). The bioactive composition (flavonoids and vitamin C) of the mixtures as well as its stability, antioxidant capacity and changes in colour over a 70 days storage period were studied. Our results suggest that the new drink made of 75% of pomegranate juice (PJ) and 25% of lemon juice (v:v), has potential for development of new healthy beverages or food products, emphasised by its high antioxidant capacity determined by its phenolic composition – punicalagin isomers, anthocyanins and vitamin C – and improved colour properties.

1. Introduction

Nowadays, the interest in the role of dietary antioxidants in human health has prompted research in the field of food science. Fruits are good sources of these bioactives, and there are a number of commercial polyphenol-rich beverages, which base their marketing strategies on antioxidant potency.

Pomegranate (*Punica granatum Linn.*) is a very rich source of anthocyanins (cyanidin 3,5-di and 3-*O*-glucoside, delphinidin 3,5-di and 3-*O*-glucoside, pelargonidin 3,5-di and 3-*O*-glucoside), ellagic acid, punicalagin isomers, different flavanols (catechins as catechins and epicatechin, and gallocatechins as gallocatechin and epigallocatechin) (Alighourchi, Barzegar, & Abbasi, 2007; García-Alonso, Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2004; Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000; Kulkarni, Mahal, Kapoor, & Aradhya, 2007; Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Pérez-Vicente, Serrano, Abellán, & García-Viguera, 2004). In addition, malic and citric acid have been described as the most abundant acids, whilst oxalic, succinic and fumaric are present in lower amounts (Mirdehghan et al., 2006). Moreover, pharmacological activities, anti-inflammatory, hepatoprotective, as well as preventive effects on cancer, cardiovascular and neurodegenerative diseases are associated with these compounds (Faria, Monteiro, Mateus, Azevedo, & Calhau, 2007; Sartippour et al., 2008).

On the other hand, lemon fruit (*Citrus limon* (L.) Burm. f.) is also a rich source of nutrients, including flavonoids, citric acid, vitamin C and minerals (e.g. potassium), which provide numerous health promoting properties (Del Río et al., 2004; González-Molina, Moreno, & García-Viguera, 2008). Among flavonoids, hesperidin and eriocitrin (flavanones), together with small amounts of diosmetin 6,8-di-*C*-glucoside (diosmetin 6,8-diglc), diosmin and vicenin-2 (flavones) are the main compounds present (Gil-Izquierdo, Riquelme, Porras, & Ferreres, 2004; González-Molina et al., 2008; Peterson et al., 2006). Moreover, additional minor flavonoids, such as quercetin and myricetin (Hertog, Hollman, & Van de Putte, 1993), as well as other hydroxycinnamic acids (Gil-Izquierdo et al., 2004) are also known to be present in very low concentrations.

Following our research focused on providing an alternative use for these typical Mediterranean crops, including the second qualities and over-ripe pomegranates (Martí, Pérez-Vicente, & García-Viguera, 2001; Pérez-Vicente et al., 2004) other than fresh consumption, the design of new polyphenol-rich beverages combining lemon juice plus pomegranate juice was carried out in this work.

Lemon juice is widely used as an antioxidant natural substitute for the synthetic ascorbic or citric acids (E300 and E330, respectively) (Martí et al., 2001). Moreover, lemon juice could prevent browning reactions and colour deterioration of pomegranate juice (Özkan, 2002). The aim of the present work was to study the antioxidant activity, organoleptic properties and stability effects that can take place during storage due to the possible synergism/antagonism between the compounds present in these juices in order to design a new beverage with high nutritive value.

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2. Materials and methods

2.1. Reagents and standards

Sweet over-ripe pomegranate juice (PJ) from cv. 'Mollar' was purchased from Cítricos de Murcia, S.A. (Ceutí, Murcia, Spain). The juice was obtained by pressure with a laboratory pilot scale press (Zumonat C-40). This juice was stored frozen (−20 °C) until analysed.

Lemon juice (LJ) was obtained using a domestic squeezer ('Citromatic', Braun Española S.A., Barcelona, Spain) and lemons were collected from the CEBAS-CSIC's Experimental Farm ('La Matanza', Santomera, Murcia, SE Spain) of 'Fino' clones. The juice was stored frozen (−20 °C) until analysed.

Standards. Phenolic compounds were obtained commercially: cyanidin 3-glucoside (Polyphenols, Norway); hesperidin (Merck, Darmstadt, Germany); diosmin (Genay, France); 2,2-diphenyl-1-picryl-hydrazyl (DPPH·) (Sigma, Steinheim, Germany); gallic acid (Doesder. Chem. Co., Barcelona, Spain) and ellagic acid (EA) (Sigma St. Louis, USA). Other reagents were, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Fluka Chemika, Neu-Ulm, Switzerland); Folin–Ciocalteau's reagent (Sigma, Steinheim, Germany); sodium carbonate anhydrous (Panreac Quimica S.A., Barcelona, Spain); potassium dihydrogen phosphate (Panreac Quimica S.A., Barcelona, Spain); citric acid (Sigma, Steinheim, Germany); benzoic acid (Sigma, St. Louis, USA); dimethylsufoxide, formic acid, and methanol were all of analytical grade (Merck, Darmstadt, Germany); ascorbic acid (AA) and dehydroascorbic acid (DHAA), both from Sigma–Aldrich (Steinheim, Germany); 1,2-phenylenediamine dihydrochloride (OPDA) (Fluka Chemika, Neu-Ulm, Switzerland). Ultrapure water used was produced using a Millipore water purification system (Molsheim, France).

2.2. Experimental design

Lemon and pomegranate juices were thawed at room temperature and mixed in different proportions: 75%PJ + 25%LJ, 50%PJ + 50%LJ and 25%PJ + 75%LJ, keeping also two control solutions of 100% pomegranate juice (PJ100) and 100% lemon juice (LJ100). Homogenised mixtures and pure juices were centrifuged (10 min at 2894*g*), and benzoic acid (100 mg l^{-1}) was added in order to prevent spoilage (Martí et al., 2001). Juices and mixtures (8 ml) were placed in screw capped class tubes (10 ml) and, after removal of air under N_2 atmosphere, were stored in the dark at room temperature for 70 days. All analyses were done in triplicate, and the mean values were reported in each case. The analyses were carried out in triplicate every 7 days during the first 30 days, and every 15 days during the last 30 days for each mixture and juice.

2.3. pH, titratable acidity, and total soluble solids

pH, titratable acidity (TA), and total soluble solids (TSS) were evaluated as quality indexes. The pH values were measured using a pH-metre (GLP 21, Crison Ltd., Barcelona, Spain). The TA was determined by titrating 2 ml of the mixture (rising 60 ml final volume with Milli-Q water) with 0.1 N NaOH (pH 8.1). Results were expressed as g citric acid per 100 ml of sample, in accordance with AOAC (1984). The TSS contents were recorded in a refractometre (Abbe WYA-S, Optic Ivymen® System, Barcelona, Spain) at 20 °C with values being expressed as °Brix.

2.4. HPLC analysis, identification and quantification of anthocyanins and noncoloured phenolics

All samples were centrifuged during 5 min at 10,500*g* (model Sigma 1-13, B. Braun Biotech International, Osterode, Germany) at 4 °C. The supernatant (soluble fraction) was filtered through a 0.45 μm PVDF filter (Millex HV13, Millipore, Bedford, Mass, USA) before injection into the HPLC. For identification and quantification of anthocyanins the method previously reported by Pérez-Vicente et al. (2004) was followed. Each sample was analysed on a Merck-Hitachi L6200 liquid chromatograph (Tokyo, Japan), equipped with a Diode Array Detector UV–vis Shimadzu SPD-M6A (Kyoto, Japan) and an autoinjector (Gilson International, model 234, Barcelona, Spain). Chromatograms were recorded and processed on a LC Workstation Class M10A Shimadzu PC-based chromatography data system.

A 20 μl sample was analysed on a Lichrocart RP-18 reversed-phase column (250 × 4 mm, particle size 5 μm) with a precolumn C_{18} (Lichrocart® 4-4, Lichrospher® 100 RP-18 (5 μm)) from Merck (Darmstadt, Germany), using a mobile phase of 5% formic acid (v/v) (solvent A) and HPLC grade methanol (solvent B) (Merck, Darmstadt, Germany). Elution was performed at a flow rate of 1 ml min^{-1}. The linear gradient started with 1%B, keeping isocratic conditions during 5 min, reaching 20% B at 20 min, 40%B at 30 min, 95%B at 35 min and 1%B after 41 min. UV chromatograms were recorded at 280, 360 and 520 nm. The analyses were done in triplicate and results expressed as mean value. The different phenolics were characterised by chromatographic comparison with analytical standards, accordingly to previous reports (Gil-Izquierdo et al., 2004; Pérez-Vicente et al., 2004), and quantified by the absorbance of their corresponding peaks in the chromatograms. Anthocyanins were quantified as cyanidin 3-glucoside (detected at 520 nm); punicalagin isomers and ellagic acid as ellagic acid (at 360 and 280 nm, respectively); flavanones, as hesperidin (at 280 nm); and flavones, as diosmin (at 360 nm).

2.5. Extraction and analysis of vitamin C

Ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents were determined by HPLC-UV as described elsewhere and fully detailed in González-Molina et al. (2008). The vitamin C content was calculated by the addition of ascorbic acid and dehydroascorbic acid contents, and results were expressed as mg per 100 ml.

2.6. Colour measurement

Colour measurement was determined as in Pérez-Vicente et al. (2004). Briefly, solutions were measured in glass cells of 2 mm path length (CT-A22) at 520 nm using a Minolta CM-508i® tristimulus colour spectrophotometer (Osaka, Japan) coupled with a CM-A760 transmittance adaptor, illuminant D65 and 10° observer according to the CIELAB 76 convention. Data (CIEL^*, a^* and b^*), were recorded and processed using the Minolta Software Chromacontrol S, PC-based colorimetric data system. *Hue angle* (H^*) was calculated from $\tan^{-1}$ (b^*/a^*), *Chroma* (C^*) from $(a^{*2} + b^{*2})^{1/2}$ and colour differences (ΔE^*) from $[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.

2.7. Determination of total phenols by Folin–Ciocalteau's reagent

Total phenol content was determined with the Folin–Ciocalteau method, adapted to a microscale by Arnous, Makris, and Kefalas (2001). In a 1.5-ml Eppendorf microtube, 790 μl of Milli-Q water, 10 μl of sample appropriately diluted with MeOH, and 50 μl of Folin–Ciocalteau reagent were added and vortexed. After exactly 1 min, 150 μl of aqueous 20% sodium carbonate were added, vortexed again and allowed to stand at room temperature in the dark, for 120 min. The absorbance was recorded at 750 nm, and quantified using gallic acid as a standard. Results were expressed as mg per 100 ml of gallic acid equivalents (GAE).

2.8. DPPH· assay

All samples were centrifuged at 10,500*g* (Sigma 1-13, B. Braun Biotech International, Osterode, Germany) for 5 min at 4 °C. The free radical scavenging activity was determined using the free radical DPPH· according to González-Molina et al. (2008). It was evaluated by measuring the variation in absorbance at 515 nm after 50 min of reaction in parafilm-sealed glass cuvettes (to avoid methanol evaporation) at 25 °C. All reactions started by adding 5 μl of the corresponding diluted sample, and 45 μl of MeOH to the cuvette containing the diluted stock solution up to absorbance ~1, (950 μl, 0.094 mM of the free radical (DPPH·)). The final volume of the assay was 1 ml. Reaction was followed with a spectrophotometer (UV-1603 Shimadzu, Tokyo, Japan). Results were expressed as mM Trolox.

2.9. Statistical analyses

All data were subjected to analyses of variance (ANOVA) using SPSS 14.0 software (Chicago, Illinois, USA). The data shown are mean values ($n = 3$) and the significance of the differences was compared using a multiple range test (Least Significance Difference, L.S.D.) at $P < 0.05$ probability level (Duncan's test).

3. Results and discussion

3.1. Changes in pH, titratable acidity (TA) and total soluble solids (TSS)

There were not significant differences over the 70 days storage in the quality parameters (pH, TA and TSS) ($P < 0.001$) assayed in the mixtures and control juices, reason why the results are presented as average values for all of the experiments (Table 1). The pH values in the mixtures decreased with the percentage of LJ added, ranging from 2.45 for LJ100 to 3.60 for PJ100 (Table 1).

Regarding TSS, in case of LJ100 these were in accordance with reported contents for cv. 'Fino' lemon juices (~8.22°Brix) (González-Molina et al., 2008); whilst PJ100 presented much higher values (~17°Brix), also in accordance with those obtained by other authors for over-ripe fruits (Table 1) (Gil et al., 2000; Pérez-Vicente et al., 2004). The TSS contents of the new formulations were representative of the theoretical addition of the individual components of each mixture.

Finally, TA in PJ100 was always around 0.43 g citric acid/100 ml^{-1}, in agreement with the over-maturity stage of the pomegranates used (Kulkarni & Aradhya, 2005), and 5.40 g citric acid 100 ml^{-1}, for LJ100. Consequently, this acidity incresed in the mixtures proportionatelly to the percentage of LJ added.

Table 1
Average pH, titratable acidity (TA) and total soluble solids (TSS) through the storage period in the different mixtures.

Mixtures	pH	TA	TSS
LJ100	2.45 ± 0.06	5.40 ± 0.23	8.22 ± 0.16
LJ75-PJ25	2.54 ± 0.09	4.14 ± 0.27	10.51 ± 0.58
LJ50-PJ50	2.62 ± 0.69	2.82 ± 0.12	13.09 ± 0.31
LJ25-PJ75	2.80 ± 0.06	1.71 ± 0.20	15.25 ± 0.17
PJ100	3.60 ± 0.25	0.34 ± 0.09	16.99 ± 0.11

Values are mean ± standard deviation ($n = 21$).
TA (titratable acidity) is expressed as g citric acid per 100 ml juice.
TSS (total soluble solids) is expressed as ° Brix (20 °C).

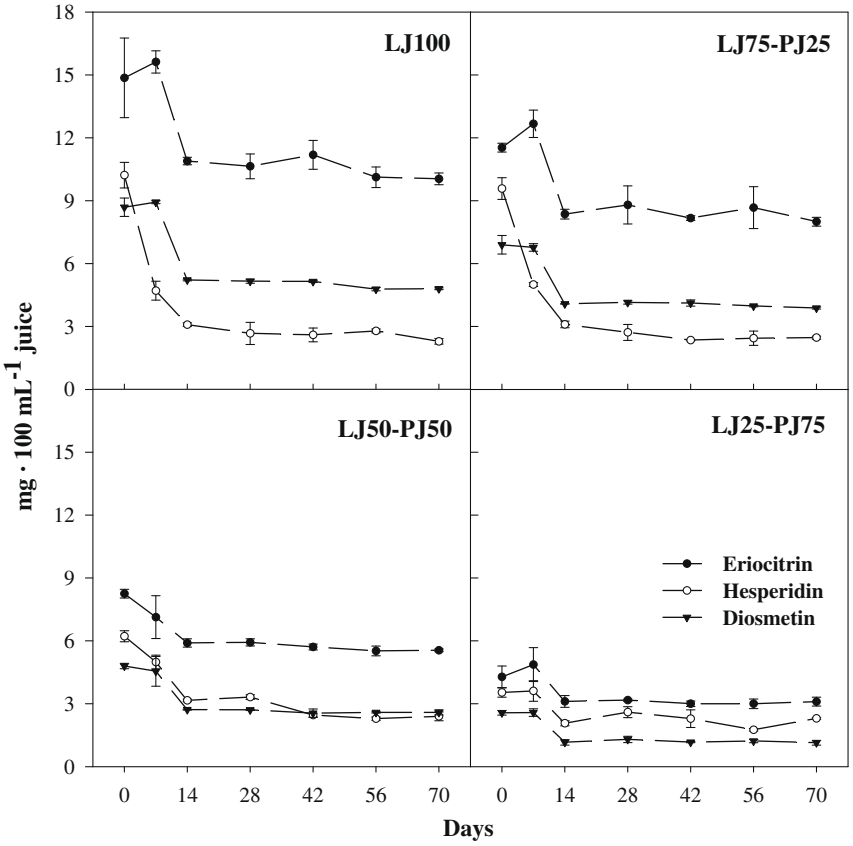

Fig. 1. Changes in hesperidin, eriocitrin and diosmetin contents (mg/100 ml) in the prepared mixtures through storage period. LJ100, 100% lemon juice; LJ75-PJ25, 75% lemon juice plus 25% pomegranate juice; LJ50-PJ50, 50% lemon juice plus 50% pomegranate juice; LJ25-PJ75, 25% lemon juice plus 75% pomegranate juice; PJ100, 100% pomegranate juice.

3.2. Flavanones and flavones stability during storage

LJ provided flavanones and flavones to the new mixtures. The initial content for LJ100 (Fig. 1) was 14.86, 10.22 and 8.69 mg/100 ml^{-1} for eriocitrin, hesperidin and diosmetin, respectively. In all mixtures, the flavanones, eriocitrin and hesperidin, characterised by a low solubility (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001; González-Molina et al., 2008), tended to precipitate (Fig. 1). In all cases the maximum decrease was found within the first 14 days of storage, with the percentage of loss being proportional to the concentration of LJ in the juice. In case of eriocitrin, the losses were less than 25% at the end of the storage period, in all cases. On the other hand, hesperidin was 50% reduced in only 7 days, reaching over 70% loss at the end of the experiment, for LJ100 and PJ25-LJ75. However, some degree of stabilisation could be observed for hesperidin in mixtures with 50% or 25% of LJ added, where losses did not exceed 50% at the end of the experiment (Fig. 1). After that, both flavanones remained rather constant, without significant differences until the end of the storage period ($P < 0.001$).

Regarding the diosmetin content, the losses were minor than 44–55% after the first two weeks, and remained invariable after that ($P < 0.001$) (Fig. 1).

3.3. Changes in the content of hydrolysable tannins and ellagic acid (EA)

PJ provided two isomers of punicalagin as hydrolysable tannins as well as ellagic acid. The initial content of the two punicalagin isomers in PJ100 were *ca* 4 mg/100 ml (punicalagin 1) and 9 mg/100 ml (punicalagin 2), decreasing during the storage. On the other hand, the content of free EA in PJ100 (initially, 1.52 mg/100 ml) increased up to 3.7 fold after 28-days of storage. The addition of LJ in the new mixtures stabilised the content of such compounds (Fig. 2).

Studies by Zafrilla, Ferreres, and Tomás-Barberán (2001), on pomegranate bioactive compounds, revealed an increase in free EA concomitant with the degradation of punicalagin isomers (Fig. 2). The release of hexahydroxydiphenic acid from such ellagitannins is transformed to ellagic acid (Fig. 3). For these reasons, the reported content for EA in the literature provides a big range depending on the cv. and maturity stage (Gil et al., 2000; Pérez-Vicente et al., 2004). Nevertheless, the presence of LJ reduced markedly this effect, since punicalagin hydrolysis was favoured at higher pH (pH ~ 7). These results could be of interest since, although ellagitannins are not absorbed *in vivo* (Hagerman et al., 1998) they could reach the colon and release EA which is metabolised by human microflora and presents health benefits (Larrosa, Tomás-Barberán, & Espín, 2006). Moreover, its bioavailability (Cerdá, Llorach, Cerón, Espín, & Tomás-Barberán, 2003a; Cerdá, Cerón, Tomás-Barberán, & Espín, 2003b; Heber et al., 2007; Larrosa et al., 2006) and non-toxic nature (Cerdá et al., 2003b; Heber et al., 2007; Kulkarni et al., 2007) render punicalagin as a promising multifunctional molecule.

3.4. Changes in vitamin C during storage

LJ provided an initial vitamin C content around 36.14 mg/100 ml (Fig. 4). On the other hand, PJ did not contain vitamin C (less than 6 mg DHAA/100 ml) in accordance with previous findings (Kulkarni & Aradhya, 2005; Martí et al., 2001).

Vitamin C in the mixtures varied according to the percentages of the LJ added to PJ. The AA form was the main contributor to vitamin C in LJ during the whole experiment (Fig. 4). Nevertheless, results revealed that after an initial general decrease (*ca* 50%) during

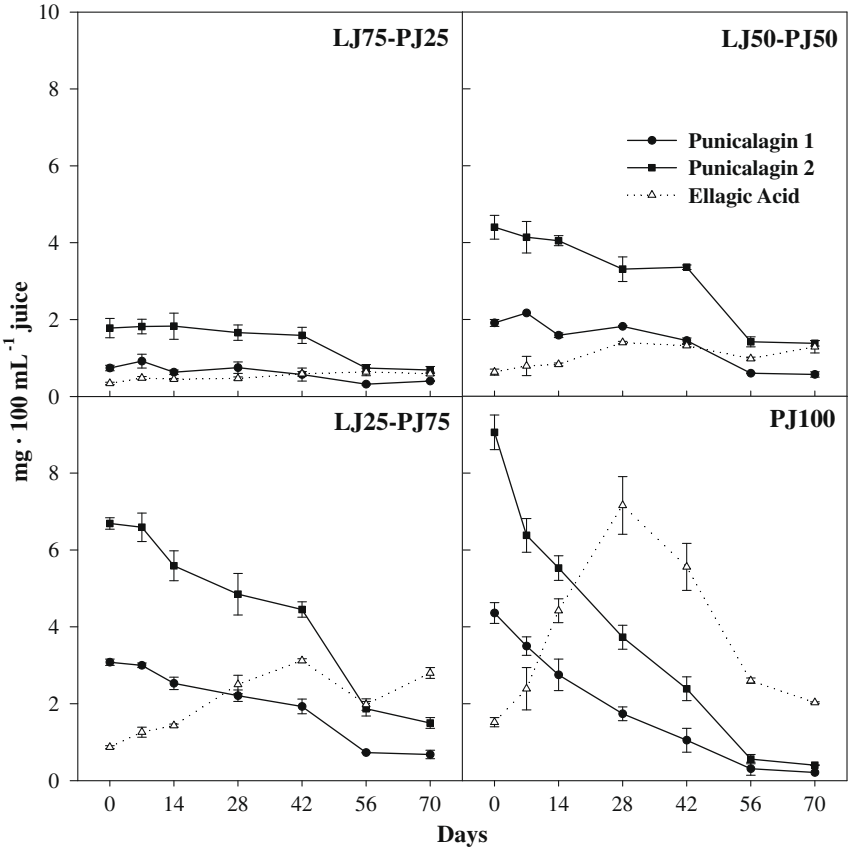

Fig. 2. Evolution in punicalagin isomers and ellagic acid contents (mg/100 ml) in the prepared mixtures through the storage period. Abbreviation as in Fig. 1.

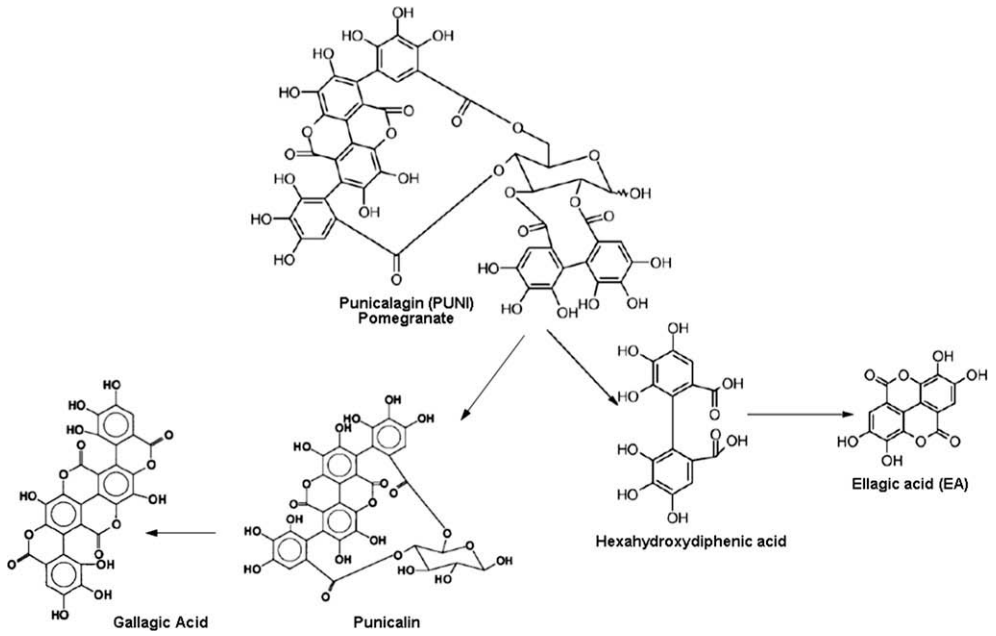

Fig. 3. Molecular structures of punicalagin and its derivatives, hydroxydiphenic acid, ellagic acid, and gallagic acid.

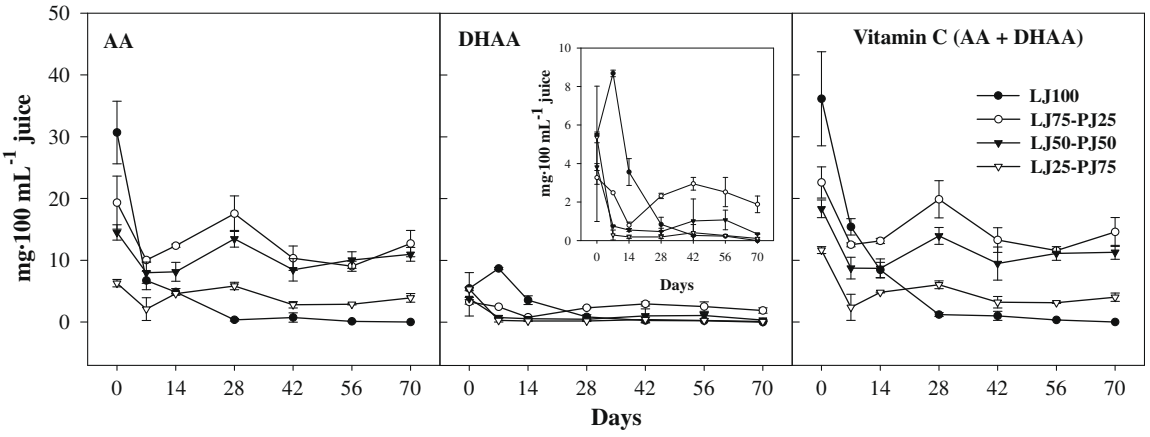

Fig. 4. AA, DHAA and vitamin C composition (mg/100 ml) of different juices through 70 days of storage. Abbreviation as in Fig. 1.

the first 7 days, a retention of vitamin C until the end of storage was registered ($P < 0.001$) (Fig. 4).

3.5. Stability of anthocyanins

PJ was characterised by six anthocyanins, namely, delphinidin 3,5-diglucoside (19%), cyanidin 3,5-diglucoside (42%), pelargonidin 3,5-diglucoside (4%), delphinidin 3-glucoside (7%), cyanidin 3-glucoside (23%), and pelargonidin 3-glucoside (5%), with the total anthocyanin concentration being 21.45 mg/100 ml (SD ± 1.08). The relative contents determined in the mixtures were in accordance with the percentage of PJ added (Fig. 5). The total anthocyanins showed a general decrease in all the samples, in accordance with previous reports (Pérez-Vicente et al., 2004). Nevertheless, the degradation rate was clearly influenced by the percentage of LJ added, being significantly higher for those with 75% LJ (100% loss), than for those with lower LJ added (70% loss for pure pomegranate juice, 80% loss for those with 25%LJ and 90% loss for 50%LJ added). Moreover, these losses were registered, mainly, at the beginning of the storage, due, probably to the vitamin C content of LJ (see below) (Fig. 4.) (Özkan, 2002).

In accordance with previous reports (García-Viguera & Bridle, 1999; Martí et al., 2001), the pomegranate anthocyanin diglucosides showed higher stabilities (~65% losses) than the mono-glucosides (~80% losses) (Fig. 6). Nevertheless, as mentioned above, the presence of LJ in the mixtures, affected significantly the anthocyanin stability, accelerating its degradation independently of its mono- or diglucoside structure (Fig. 6). This effect can be attributed to (1) the degradation products of ascorbic acid present in LJ (dehydroascorbic acid, furfurals and H_2O_2) (Özkan, 2002); or (2) to a direct condensation of anthocyanin pigment with AA Poei-Langston and Wrolstad, 1981.

3.6. Anthocyanins vs. vitamin C

It is well-known that anthocyanins and ascorbic acid resulted in the degradation of both compounds through a condensation reaction (Jurd, 1972). However, as we mentioned above, results showed a general decrease in anthocyanin content (Fig. 5 and 6), whilst vitamin C remained constant after the 7 first days (Fig. 4).

Studies carried out by Poei-Langston and Wrolstad (1981) and González-Paramás et al. (2006) reported that the model system

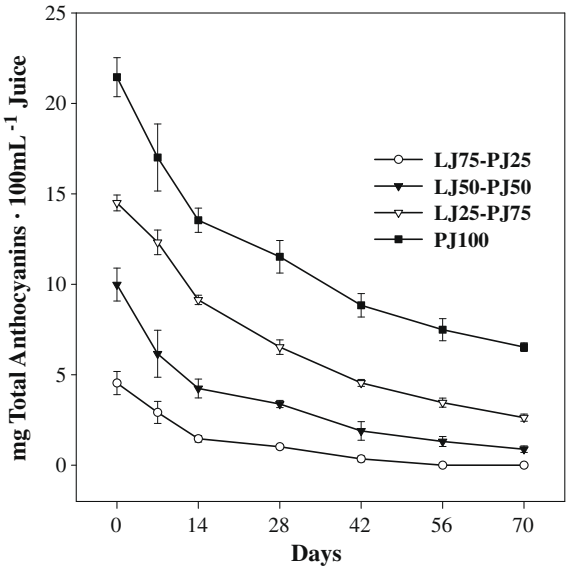

Fig. 5. Total anthocyanin content expressed as the sum of the individual anthocyanins (mg/100 ml) in the prepared mixtures through the storage period. Abbreviation as in Fig. 1.

'ascorbic acid–anthocyanin–flavanol' (e.g. catechin) under a nitrogen atmosphere could retarded the ascorbic acid degradation. Shrikhande and Francis (1974) also suggested the protective effect of flavonols (e.g. quercetin) on ascorbic acid in 'ascorbic acid–anthocyanin–flavonol' model systems. Iversen (1999) found that the degradation rate of anthocyanins in blackcurrant nectar was 3–4 times faster than the ascorbic acid, depending on the storage conditions (dark or daylight). All in all, flavanols and flavonols present in the pomegranate and lemon juices, respectively, could be acting in this way. Nevertheless, recent reports carried out by Martí et al. (2001) studying the influence of standard ascorbic acid on pomegranate juices, showed that no extra benefit was reached, as the ascorbic acid was totally degraded within 96 h and reduced the concentration of anthocyanins. Thus, LJ, as a natural source of vitamin C, provides something else which, in combination with the bioactives of the pomegranates, preserve the ascorbic acid content. Probably, similar reactions as we mention above are involved in the retention of the vitamin C.

3.7. Changes in colour during storage

During the storage period only slight alterations were observed in lightness (*CIEL** value), showing just small increases, due in some extent to the precipitation of different compounds as flavanones of the LJ (Gil-Izquierdo et al., 2001; González-Molina et al., 2008), different coloured polymers derived from the PJ (Boulton, 2001), or and other compounds, such as proteins. Nevertheless, considerable general decrease in redness (*CIEa**) was observed for all juices containing PJ, proportional to the amount of LJ added, ranging from 50% loss (pure PJ) to over 85% loss (LJ75-PJ25). On the other hand only little variations were observed in yellowness (*CIEb** value).

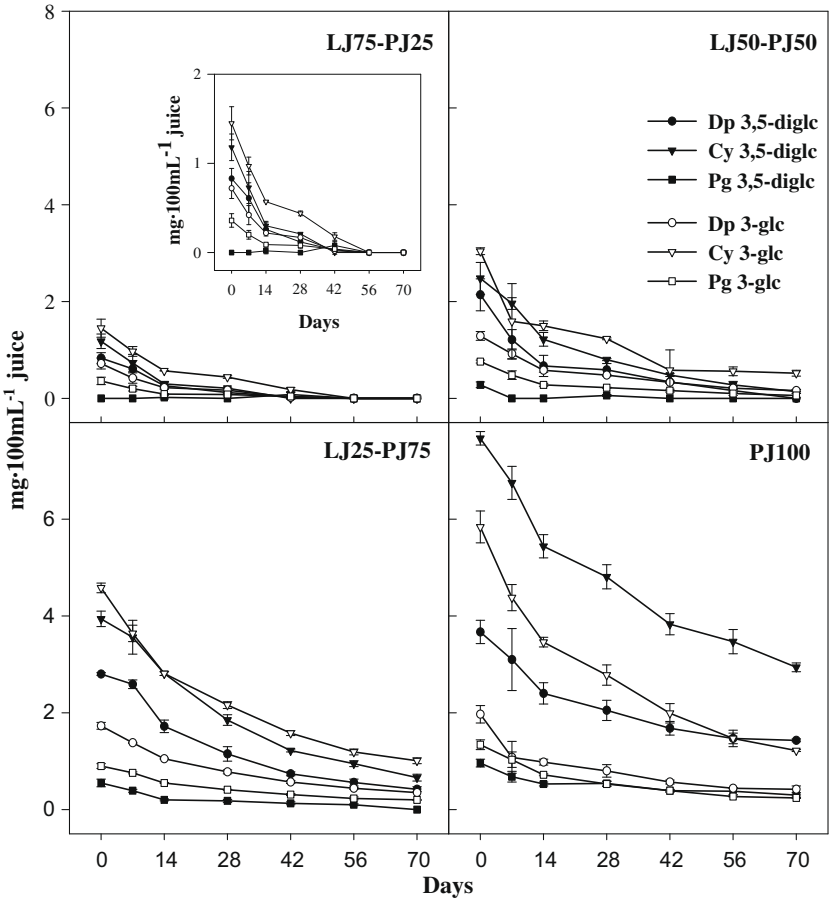

Fig. 6. Individual anthocyanin composition (mg/100 ml) of different mixtures through 70 days of storage. Dp 3,5-diglc, delphinidin 3,5-diglucoside; Cy 3,5-diglc, cyanidin 3,5-diglucoside; Pg 3,5-diglc, pelargonidin 3,5 diglucoside; Dp 3-glc, delphinidin 3-glucoside; Cy 3-glc, cyanidin 3-glucoside; Pg 3-glc, pelargonidin 3-glucoside. Abbreviation as in Fig. 1.

Table 2
Stability of *CIEL*a*b** values in the prepared juices during the storage period.

	Days	LJ100	LJ75-PJ25	LJ50-PJ50	LJ25-PJ75	PJ100
CIEL*	0	67.18e	59.56e	59.46e	60.04e	72.47cd
	7	73.89d	71.79d	69.72d	68.23cd	74.99acb
	14	79.24c	72.47cd	74.14c	67.23d	73.36cd
	28	84.74ab	76.17bc	74.68c	70.24bc	73.70bc
	42	88.47a	87.07a	81.39a	73.27a	76.51ab
	56	81.4bc2	87.00a	71.60d	70.49b	70.49d
	70	86.05a	78.23b	78.20b	73.13a	77.74a
	LSD	3.641***	3.801***	2.399***	2.040***	2.785**
CIEa*	0	3.46a	23.39a	37.68a	47.45a	27.59a
	7	2.97a	13.76b	25.50b	35.14c	20.67bc
	14	1.28b	11.95c	25.56b	36.52b	22.37b
	28	0.33cd	8.62d	20.26c	29.67d	17.81cd
	42	−0.01d	4.31e	15.97d	24.79e	14.99de
	56	0.67c	2.70f	13.51e	22.01f	12.18e
	70	−0.05d	3.49g	10.75f	19.41g	11.90e
	LSD	0.525***	0.703***	1.440***	0.629***	3.998***
CIEb*	0	21.68a	18.74a	13.23a	8.90bc	2.70f
	7	17.80b	15.42b	10.77ab	7.73cd	4.40e
	14	13.82c	14.08b	4.54cd	5.57e	5.55d
	28	9.25d	11.38c	6.84c	6.14de	7.20c
	42	6.73e	5.63d	3.90d	6.57de	9.28b
	56	11.88c	6.94d	12.28ab	11.22a	9.92ab
	70	9.05d	10.74c	10.36b	10.31ab	10.45a
	LSD	2.217***	1.948***	2.658***	1.515***	0.724***
Chroma	0	21.96a	29.97a	39.93a	48.16a	27.72a
	7	18.04b	20.67b	27.68b	36.00c	21.14bc
	14	13.87c	18.49c	26.04c	36.94b	23.08b
	28	9.25d	14.28d	21.44d	30.32d	19.25bcd
	42	6.73e	7.10f	16.45f	25.64e	17.64cd
	56	11.90c	7.47f	18.33e	24.71f	15.71d
	70	9.06d	11.30e	14.93f	21.97g	15.83d
	LSD	2.241***	1.785***	1.621***	0.646***	3.843***
Hue angle	0	80.93e	38.70c	19.35bc	10.65cd	5.59e
	7	80.55e	48.27b	22.90b	12.39bc	12.01d
	14	84.73d	49.36b	10.24d	8.67d	14.37d
	28	88.01bc	52.85b	18.57bc	11.69c	22.36c
	42	91.96a	52.39b	13.70cd	14.84b	31.79b
	56	87.08cd	68.57a	43.49a	27.00a	39.17a
	70	80.57ab	71.93a	43.88a	27.99a	41.29a
	LSD	2.739***	5.173***	5.873***	2.745***	3.634***

Values are mean ($n = 3$) ±standard deviation.
Means in the same column followed by different letters are significantly different at $P < 0.05$ according to Duncan's test.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

These changes render a consequently loss in *Chroma* and an increase in *Hue angle* (Table 2), changing the colour from more red/magenta to a more orange hue juices at the end of the experiment ($\Delta E^* = 28.43 \pm 2.01$ for LJ75-PJ25; $\Delta E^* = 32.94 \pm 2.360$ for LJ50-PJ50 and $\Delta E^* = 30.98 \pm 0.21$ for LJ25-PJ75, for mixtures analysed at day 0 and 70). Nevertheless, LJ25-PJ75 and LJ50-PJ50 colour remained very attractive during the storage period, even if visible differences were noticed between them at the end of the experiment ($\Delta E^* = 15.35 \pm 2.41$), but lower than at time 0 ($\Delta E^* = 36.27 \pm 0.10$).

3.8. Anthocyanins vs. colour

Red colour in the mixtures was due to the anthocyanin content of the PJ. Thus, the regression coefficients between individual or total anthocyanins and *CIEa** or *Croma* values ranged from 0.8 to 0.9 ($P < 0.001$) for mixtures containing LJ, and from 0.7 to 0.8 ($P < 0.001$) in the case of PJ100 (Table 3). The brighter colours in mixtures with LJ were due to the lower pH (Table 1), which favoured the coloured flavylium form of anthocyanins (Mazza & Miniati, 1993). Moreover, in case of mixtures with 25% or 50% of LJ added, the rate of colour loss was much slower than the rate of anthocyanin degradation, in agreement with previous studies for other fruit products (Martí et al., 2001). Probably, the attractive red colour in the juices could be due to other coloured polymers formed through the storage period (Boulton, 2001). Finally, the increase in CIEL* value in mixtures with LJ added mentioned above, could indicate a possible bleaching action of ascorbic acid on anthocyanins (Jurd, 1972).

3.9. Total phenolic compounds

When measuring the total phenol content by Folin–Ciocalteau's reagent in PJ100 and LJ100 (243.89 and 72.41 mg gallic acid/100 ml, respectively), an expected result was observed compared to other studies (Fig. 7) (Gil et al., 2000; González-Molina et al., 2008). Again, the values registered in the mixtures showed the sum of their components. On the other hand, the losses during storage were between 15% and 20% in all juices, except for LJ100, which reached over 35%, due, probably, to the precipitation of flavanones (Fig. 7) (Gil-Izquierdo et al., 2001).

3.10. Antioxidant activity

The antioxidant results obtained here are within the ranges previously published for pomegranate and lemon juices (~15.5 and ~3.7 mM Trolox, respectively) and the mixtures assayed provided between these values (Fig. 8) (Gil et al., 2000; González-Molina et al., 2008).

The antioxidant activity of LJ100 decreased rapidly during the first 14 days, reaching 70% loss by the end of the storage period. On the other hand, as previously reported for PJ100, the losses in antioxidant activity were lower than 20% (Gil et al., 2000; Pérez-Vicente et al., 2004), and did not exceed 30% loss through the storage period for any of the mixtures (Fig. 8).

Table 3
Analysis of correlations between individual and total anthocyanin content and colour parameters (*Croma* and *CIEa** values) in the juices during the storage period.

	LJ75-PJ25		LJ50-PJ50		LJ25-PJ75		PJ100	
	*CIEa**	*Croma*	*CIEa**	*Croma*	*CIEa**	*Croma*	*CIEa**	*Croma*
Dp diglc	0.867***	0.834***	0.895***	0.910***	0.867***	0.867***	0.717***	0.646**
Cy diglc	0.916***	0.882***	0.903***	0.895***	0.899***	0.889***	0.787***	0.697***
Pg diglc	–	–	0.584**	0.638***	0.843***	0.852***	0.752***	0.704***
Dp glc	0.933***	0.887***	0.925***	0.925***	0.937***	0.939***	0.789***	0.767***
Cy glc	0.955***	0.895***	0.869***	0.876***	0.943***	0.943***	0.841***	0.773***
Pg glc	0.893***	0.826***	0.901***	0.910***	0.918***	0.918***	0.780***	0.712***
Total anthocyanin	0.949***	0.897***	0.945***	0.951***	0.922***	0.918***	0.814***	0.741***

Correlations at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); ($n = 21$).
Dp diglc, delphinidin 3,5-diglucoside; Cy diglc, cyanidin 3,5-diglucoside; Pg diglc, pelargonidin 3,5 diglucoside; Dp glc, delphinidin 3-glucoside; Cy glc, cyanidin 3-glucoside; Pg glc, pelargonidin 3-glucoside.

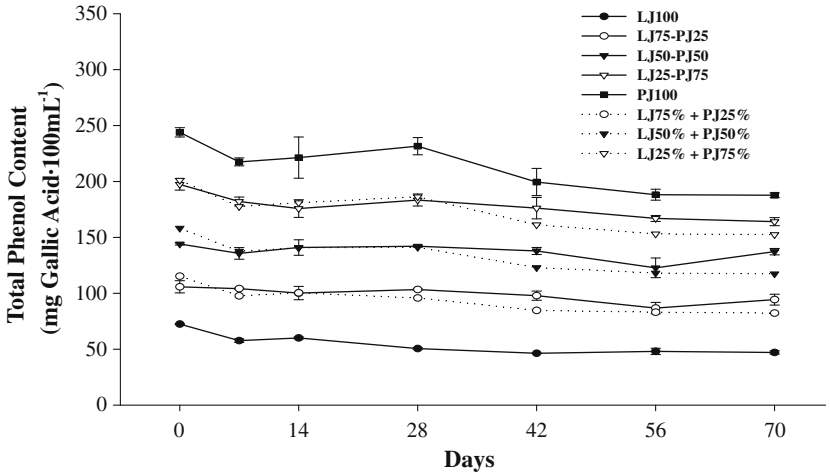

Fig. 7. Evolution of total phenolic content determined by Folin–Ciocalteau's reagent (mg gallic acid/100 ml) in the mixtures during 70 days of storage. Abbreviation as in Fig. 3. Dot lines indicate the theoretical addition of the individual components of each mixture.

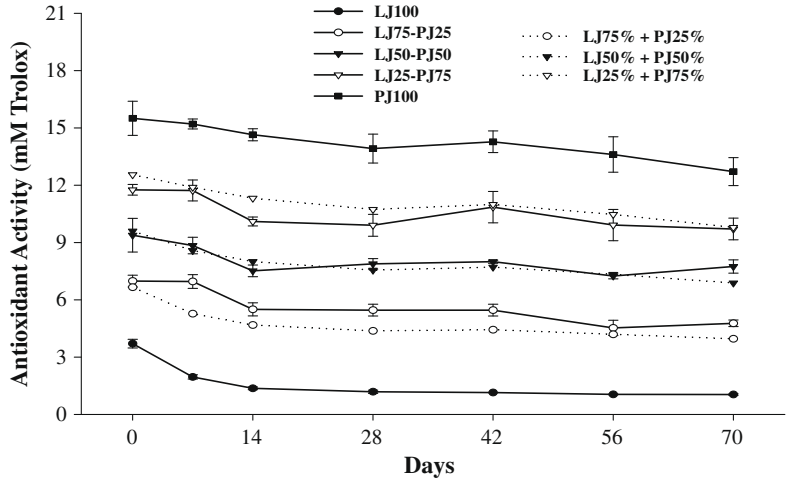

Fig. 8. Evolution of the antioxidant activity, expressed as mM Trolox, of the prepared mixtures by DPPH method through the storage period. Abbreviation as in Fig. 1. Dotted line indicate the theoretical addition of the individual components of each mixture.

The antioxidant activity in LJ was due, mainly, to its vitamin C ($R^2 = 0.951^{***}$) and total phenolic content (measured by FCR) ($R^2 = 0.830^{***}$), especially hesperidin ($R^2 = 0.953^{***}$). Thus, the great decrease observed in these bioactives at the beginning of the storage, resulted in the decrease of the antioxidant activity (Fig. 8).

On the other hand, PJ did not correlate with any of the compounds analysed, regarding the antioxidant activity of these compounds: (1) the anthocyanins, well-known by their ability to form complexes due to the hydroxyl functional groups linked to the (**B**) ring (Noda, Kaneyuki, Mori, & Packer, 2002; Sarma, Sreelakshmi, & Sharma, 1997); and (2) the hydrolysable tannins group, mainly punicalagin isomers, by the presence of 16 dissociable —OH groups in their structure (Fig. 3) acting not only as scavenger but also by forming metal chelates, which induced peroxidation (Gil et al., 2000; Kulkarni et al., 2007; Smyk, Pliszka, & Drabent, 2008). Nevertheless, although punicalagin isomers have been suggested as responsible for about half, or even >90%, of the total antioxidant activity of the juice in addition to ellagic acid, gallagic acid, and punicalin (Fig. 3) (Gil et al., 2000; Heber et al., 2007; Tzulker et al., 2007), we cannot exclude additional compounds as organic acids, proteins, etc. that might contribute to that antioxidant activity (Tzulker et al., 2007; Van Campenhout et al., 2006). Maybe, a possible synergism between them could explain the high antioxidant values in PJ. In accordance with previous reports, PJ was shown to posses a 3-fold higher antioxidant activity than that of red wine or green tea (Gil et al., 2000).

4. Conclusion

The new designed beverages, based on pomegranate and lemon juices, provided interesting results. The LJ75-PJ25 combination proved to be rich in vitamin C although it gave the lowest *in vitro* antioxidant capacity, high acidic content (4.14 g citric acid/100 ml), in the range of pure lemon juice, and a considerable decrease in redness during storage. On the other hand, the LJ50-PJ50 presented better organoleptic properties providing an attractive red colour, as well as an acceptable content of bioactive compounds and a moderate antioxidant activity. Finally, LJ25-PJ75, offered high antioxidant capacity due, at least in part, to its higher composition in phenolics (punicalagin isomers and anthocyanins) combined with vitamin C, together with stable and atracctive red colour, due to the small proportion of LJ added to the PJ that increased the CIEa^* values. Thus, LJ25-PJ75 provided a food product with good organoleptic (colour) properties, enhanced bioactive

composition and high antioxidant activity, interesting for the development of new healthy beverages or drinks.

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