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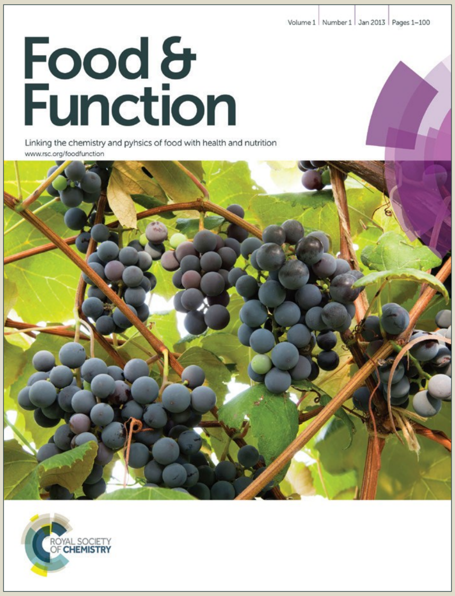

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Food matrix and processing influence on carotenoid bioaccessibility and lipophilic antioxidant activity of fruit juice-based beverages

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Abstract

The biological activity of carotenoids depends on its bioaccessibility and solubilization in the gastrointestinal tract. These compounds are poorly dispersed in the aqueous media of the digestive tract due to their lipophilic nature. Thus, it is important to analyze the extent to which some factors, such as the food matrix and food processing, may improve their bioaccessibility. Beverages formulated with a blend of fruit juices and water (WB), milk (MB) or soymilk (SB) were treated by high-intensity pulsed electric fields (HIPEF) (35 kV/cm with 4 μs bipolar pulses at 200 Hz during 1800 μs), high-pressure processing (HPP) (400 MPa at 40 ºC for 5 min) or thermal treatment (TT) (90 ºC during 1 min) in order to evaluate the influence of food matrix and processing on the bioaccessibility of carotenoids and on the lipophilic antioxidant activity (LAA). The bioaccessibility of these compounds diminished after applying any treatment (HIPEF, HPP and TT), with the exception of cis-violaxanthin+neoxanthin, which increased by 79% in HIPEF and HPP beverages. The lowest carotenoid bioaccessibility was always obtained in TT beverages (losses up to 63%). MB was the best food matrix for improving the bioaccessibility of carotenoids, as well as the LAA. Results demonstrate that treatment and food matrix modulated the bioaccessibility of carotenoids as well as the lipophilic antioxidant potential of beverages. Additionally, HIPEF and HPP could be considered as promising technologies to obtain highly nutritional and functional beverages.

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Introduction

Functional beverages are becoming more and more popular because they help maintaining well-being and health.[1] These beverages are generally made from fruits in combination or not with dairy and/or soy-derived products, which naturally provide great amounts of health-promoting compounds.[2,3] Fruit juices retain the physicochemical and organoleptical features of fruits from which they are produced. As a result, fruit juices represent an easy and convenient way for increasing the consumption of bioactive compounds. In addition, mixing different fruit juices allow increasing the concentration of selected bioactive compounds, adding new nutrients or improving the flavour and appearance of these beverages. For this reason, a variety of functional beverages are available in the market to suit different lifestyles of consumers, as well as to satisfy their preferences for tasty, nutritious, healthy and convenient products. [4]

Carotenoids are a widespread family of fat-soluble plant pigments. They have shown to play an important role in human health by their powerful antioxidant potential and because some of them possess provitamin A activity. These compounds have been associated with immune system enhancement, antiaging, antiinflammation, antiulcer and anticancer properties.[5] The main food sources of carotenoids are yellow and orange fruits, dark green vegetables and dairy products.[6] Among the most utilized ingredients for producing beverages with functional properties stand out fruit juices and milk, which are considered as wholesome and nutrient-rich foods. Therefore, functional beverages based on these food stuffs could also contribute to carotenoids intake. In many cases, soymilk is utilized as surrogate of milk for consumers who experience lactose intolerance, protein milk allergy or galactosemia.[7] Although soymilk does not

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contain carotenoids, it is an important source of other nutrients, such as phenolic compounds and isoflavones.[8,9]

It has been stated that the beneficial effect of foods on human health comes from the antioxidant activity of bioactive compounds contained in these products.[10] Particularly, carotenoids have potential antioxidant properties due to they quench singlet oxygen.[11] For this purpose, it is also interesting to evaluate the lipophilic antioxidant activity of this kind of products.

Thermal treatment (TT) has widely been used to preserve foods and beverages because of their excellent performance against microorganisms. Nevertheless, nutritional and sensorial features of food are affected by the high temperatures reached during this treatment.[12] In order to satisfy the increased demand of consumers for nutritious, healthy and tasty products, the food industry and food researches are looking for processing methods that do not compromise all these important characteristics. Non-thermal food processing technologies, such as high-intensity pulsed electric fields (HIPEF) or high-pressure processing (HPP), have widely been researched during the last decade due to they are alternatives to heat treatments.[13–16]

Bioaccesibility is defined as the portion of nutritients or bioactive compounds that is released from the food matrix into the gastrointestinal tract and thus become available for intestinal absorption.[17] Therefore, although functional beverages contain important amounts of nutrients, it does not mean that all these compounds can be absorbed. In particular, the availability of lipophilic constituents is limited because the hydrophobic nature of these compounds avoids their dispersion in the aqueous media of the digestive tract.[18] Carotenoids must be first released from the food matrix, dispersed in the digestive tract and solubilised into mixed micelles to be available for absorption. Thus, the formation of micelles is one of the most important factors that affect the absorption

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of carotenoids.[5] Bioaccessibility of nutrients is usually evaluated by *in vitro* gastrointestinal digestion[19] and represents a useful and fast approach previous to *in vivo* trials.

Processing involves changes on the microstructure of food (i.e. the disruption of cell walls and membranes), as well as on the release of carotenoids from carotenoid-protein complexes, and on their solubilisation (free and ester forms).[6] All these changes may modify the bioaccessibility of these nutrients. In addition to food processing, the surrounding environment in which carotenoids are contained also impacts on their bioaccessibility because interactions between carotenoid-carotenoid and/or carotenoid-food constituents (i.e. fiber and fat) could occur.[20] As a result, it is important to know the concentration of bioactive compound that is accessible for absorption after digestion and the extent to which food processing and food matrix may change their bioaccessibility. Recently, the bioaccessibility of carotenoids from single food matrices (i.e. mango, carrot, sweet potato, tomato, pungent peppers, papaya and orange juice) has been reviewed by Lemmens et al.[21] There is also some information available about the influence of food processing on the bioaccessibility of carotenoids. [22–26] However, to the best of our knowledge this is the first study focused on evaluating the influence of both factors (food matrix and food processing) on the bioaccessibility of carotenoids from complex matrices. For this reason, this research aimed to analyze the influence of food matrix (milk, soymilk and water) and food processing (HIPEF, HPP and TT) on the *in vitro* bioaccessibility of carotenoids and on the lipophilic antioxidant activity (LAA) of blended fruit juice-based beverages.

Material and methods

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Materials and reagents. Pepsin from porcine stomach (≥250 units/mg solid, P7000), pancreatin from porcine pancreas (P7545), bovine bile (B3883), carotenoid standards (α-carotene 50887 purity ≥98.0%, β-carotene C4582 purity ≥95.0%, zeaxanthin 14681 purity ≥95.0%, lutein 07168 purity ≥97.0% and β-cryptoxanthin C6368 purity ≥97.0%) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) radical were purchased from Sigma-Aldrich (St. Louis, MO, USA). The radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and the cellulose dialysis membrane (molecular weight cutoff of 12,000 Da) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Fruit juice-based beverages. Three beverages were prepared by mixing 75% of a blended fruit juice (orange (Valencia variety), kiwi (Hayward variety), pineapple (Extra sweet variety) and mango (Palmer variety)); 17.5% of milk (milk-fruit juice beverage, MB), or soymilk (soymilk-fruit juice beverage, SB), or distilled water (water-fruit juice beverage, WB); and 7.5% of sugar. The pH of the beverages was adjusted to 3.30 ± 0.20 (Crison Instruments S.A., Alella, Barcelona, Spain) with citric acid. The soluble solid content was analyzed in a refractometer Comecta S.A., Abrera (Barcelona, Spain), obtaining 18.0 ± 0.2, 18.5 ± 0.2, 19.3 ± 0.3 ºBrix for WB, SB and MB, respectively. Beverages formulations were selected based on a previous study, where similar concentration of these fruit juices resulted in a high bioaccessibility of bioactive compounds.[28]

Fruits (orange, kiwi, pineapple and mango) were purchased at commercial maturity in a local supermarket (Lleida, Spain). These fruits were washed, peeled and juice extracted. Each fresh-squeezed juice was filtered with a cheesecloth using a vacuum pump. A blended fruit juice was obtained by mixing 40% of orange, 33% of kiwi, 13.5% of pineapple and 13.5% of mango juices.

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Whole milk (Hacendado, Córdoba, Spain) and soymilk (Yosoy, Girona, Spain) were purchased at local supermarket. According to manufacturers, milk contained 3.6% of fat, 3.0% of protein and 4.5% of carbohydrates; while 1.8% of fat, 3.6% of protein, 0.7% of carbohydrates and 1% of fiber were reported in soymilk.

Food processing technologies

High-Intensity Pulsed Electric Fields (HIPEF). HIPEF treatment was carried out in a continuous-flow bench scale system (OSU-4F, The Ohio State University, Colombus, OH, USA), using square-wave pulses. Eight collinear chambers serially connected were used as treatment system. Each chamber consisted of two stainless steel electrodes separated by a gap of 0.29 cm. The flow rate was adjusted to 60 mL/min and controlled by a variable speed pump (model 752210-25, Cole Palmer Instrument Company, Vermon Hills, IL, USA). HIPEF treatment consisted in the application of 35 kV/cm field strength in bipolar mode, 4-μs pulse width, 200 Hz pulse frequency and 1800 μs total treatment time. Temperature was always kept below 35 ºC through a cooling coil connected before and after each pair of chambers and submerged in an ice-water shaking bath. These conditions were selected based on previous studies performed in our laboratory, where the nutritional and microbiological stability of similar beverages was achieved.[29,30]

High-Pressure Processing (HPP). HPP was performed in a hydrostatic pressure unit with a vessel of 2925 mL capacity, a maximum pressure of 900 MPa, and a maximum temperature of 100 ºC (High Pressure Iso-Lab System, Model FPG7100:9/2C, Stansted Fluid Power LTD., Essex, UK). Beverages (300 mL) were vacuum packed in flexible Doypack® bags (Polyskin XL, Flexibles Hispania, S.L.) and introduced in the pressure

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unit filled with pressure medium (water). Samples were HPP processed at 400 MPa with a holding time of 5 min. The rates of compression and decompression were both 3 MPa/s. Because of adiabatic compression, the maximum temperature in the vessel was 40 ºC at 400 MPa. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the process. HPP conditions were selected based on previous studies, where the nutritional and microbiological stability of fruit juices and similar beverages were obtained.[31,32]

Thermal Treatment (TT). Beverages were thermally processed at 90 ºC during 1min in a tubular stainless-steel heat exchanger coil immersed in a hot water shaking bath (University of Lleida, Spain). The flow rate of beverages was maintained through a gear pump. After thermal treatment, the beverages were immediately cooled down to 5 ± 1 ºC in an ice-water bath.

***In vitro* gastrointestinal digestion.** Once beverages were prepared and processed, they were digested through the *in vitro* methodology described by Rodríguez-Roque et al.[33] This procedure consisted of two digestive stages: gastric (pH 2, containing pepsin) and small intestinal digestions (pH 7, containing a pancreatine-bile mixture).

Briefly, each beverage (200 mL) was mixed with pepsin (0.2 g) in a beaker. Afterward, the pH was immediately adjusted to 2 by addition of 12 M HCl, and the mixture was incubated at 37 °C, 90 rpm during 2 h (incubation chamber with orbital agitation Ovan, Badalona, Spain). A portion of 20 mL of gastric digesta was placed into a baker and 5 mL of pancreatin (4 g/L) and bile (25 g/L) mixture was added. This mixture was incubated during 2 h at 37 °C and 90 rpm (incubation chamber with orbital agitation Ovan). Samples were immediately placed in a cold water bath during 10 min once

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digested. To quantify the amount of carotenoids transferred to the aqueous-micellar fraction, a portion of small intestinal digesta (30 mL) was centrifuged (5000 rpm during 20 min at 4 ºC)[34] and filtered (membrane of 0.22 µm). All samples from the micellar fraction were frozen (-45 ºC) until analysis.

Bioactive compounds analyses

Carotenoids. Carotenoids of non-digested or digested samples were extracted, separated, identified and quantified by HPLC following the methodology described by Morales de la Peña et al,[29] with some modifications.

Non-digested or digested beverages (6 mL) were mixed with 0.01 g of magnesium hydroxide carbonate, 0.01 g of butylhydroxytoluene (BHT), and 15 mL of ethanol/hexane solution (4:3 v/v) in an amber round-bottom flask under N_2 atmosphere and continuous agitation during 45 min. Afterward, the mixture was filtered using a low-ash filter paper 70 mm (Albert-Hahnemuehle, S.L.U., Barcelona, Spain), and the residue was washed and again filtered once with 10 mL of ethanol/hexane solution (4:3 v/v), twice with 5 mL of ethanol, and once with 5 mL of hexane. The filtrates were combined and washed with 10 mL of distilled water and 10 mL of 10% NaCl solution in an amber decanting funnel, discarding the aqueous phase each time. The organic phase was rotoevaporated at 40 °C until dryness. Then, the residue was saponified with 5 mL of methanolic KOH 0.5 M + 0.1% of BHT (v/w) and 5 mL of diethyl ether, under N_2 atmosphere during 30 min. Later, 5 mL of diethyl ether was added, and the solution was washed with 10 mL of distilled water and 10 mL of 10% NaCl solution. The organic phase was mixed with 5 mL of ethanol and rotoevaporated at 45 °C until dryness. The residue was dissolved with 4 mL of diethyl ether and placed in an amber

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glass vial. Finally, the solvent was evaporated under N_2 atmosphere and stored at -45 ºC until analysis.

The HPLC system was equipped with a 600 controller and a 2996 diode array detector (Waters Corp.), which was set to scan from 200 to 600 nm. Carotenoids were separated using a reverse-phase C18 Spherisorb ODS2 (5 µm) stainless steel column (4.6 mm × 250 mm) operating at 30 °C with a flow rate of 1 mL/min. A gradient elution was carried out to separate these compounds. Four eluents were employed as mobile phase: (1) methanol/ammonium acetate 0.1 M, (2) Milli-Q water, (3) methyl tert-butyl ether, and (4) methanol. Individual carotenoids were identified by comparing their retention time and spectrum with the standards and/or those reported in the literature. HPLC chromatograms of carotenoids in non-digested and untreated beverages are shown in Figure 1. Carotenoid quantification was carried out integrating the peak areas and using calibration curves (R^2 in the range of 0.9961 to 0.9995; concentration between 0.1 and 50 mg/L). Results were expressed as µg of carotenoid/100 mL of sample.

Lipophilic antioxidant activity (LAA). Extraction of lipophilic fraction of non-digested or digested beverages, as well as the determination of the antioxidant activity were performed according to the procedure of Rodríguez-Roque et al.[9]

Briefly, 5 mL of sample and 10 mL of tetrahydrofuran were mixed and centrifuged at 6000 rpm for 20 min at 4 °C. The supernatant was separated, whereas the residue was again mixed with 10 mL of tetrahydrofuran and centrifuged (6000 rpm for 20 min at 4 °C). Both supernatants were combined in order to analyze the LAA. The antioxidant activity was evaluated using the colorimetric method reported by Brand-Williams et al.[35], which is based on the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) assay. Aliquots of 0.2 mL of lipophilic extracts were mixed with 3.8 mL of DPPH methanolic solution

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(0.025 g/L). The homogenate was shaken vigorously and kept in the dark for 30 min. Afterward, the absorbance was measured at 515 nm against a blank of metanol. Results were expressed as percentage of DPPH$^\bullet$ inhibition.

Bioaccessibility calculations

Bioaccessibility was determined as the ratio of carotenoid concentration in the digested beverage ($BC_{digested}$) with respect to non-digested beverage ($BC_{non\text{-}digested}$) (Eq. 1). Results were expressed as percentage.

$$Bioaccessibility(\%) = 100x\left(\frac{BC_{digested}}{BC_{non-digested}}\right) \quad \text{Eq. 1}$$

Statistical analysis

The food processing technologies and the *in vitro* gastrointestinal digestion were conducted in duplicated. Each bioactive compound was extracted and analyzed two times (n=8). Analysis of variance (ANOVA) of the results followed by the least significant difference test (LSD) was carried out to determine significant differences ($p < 0.05$) in the concentration and bioaccessibility of bioactive compounds from beverages in relation to the factors studied in this research (food matrix and food processing). Multifactorial analysis of variance (ANOVA) was performed to study separately the main effects (food matrix and treatment) and the interaction effect (food matrix × treatment). As a significant interaction effect was observed in most of the variables, ANOVA, comparing the means within the same food matrix for different treatments and within the same treatment for different food matrix, was performed. All statistical analyses were performed with the program Statgraphics Plus 5.1 (Statistical

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Graphics Corporation, Inc., Rockville, MD, USA). Results were reported as the mean ± standard deviation.

Results and discussion

Carotenoids

Carotenoid profile in untreated, HIPEF, HPP and TT fruit juice-based beverages is presented in Tables 1 and 2. The concentration of total carotenoids (determined as the sum of individuals) was in the range of 322 to 426 μg/100 mL in untreated beverages, being xanthophylls up to 3.3 times higher than carotenes (Table 2). A similar concentration of carotenoids (between 223 and 540 μg/100 mL) was reported in mixed fruit juices and beverages, where xanthophylls were also the predominant forms.[28,29,33,36]

Processing exerted a significant influence on the concentration of carotenoids contained in the three beverages analyzed in this study ($p < 0.05$). The concentration of some carotenoids increased after applying HIPEF treatment with respect to untreated beverages, such as cis-violaxanthin+neoxanthin from both WB (9%) and MB (16%); cis-anteraxanthin from WB (8%); anteraxanthin (10%), lutein (23%) and zeaxanthin (28%) from MB. In the same way, HPP improved the concentration of cis-violaxanthin, anteraxanthin, lutein and zeaxanthin from MB (between 12 and 37%) as compared with untreated ones. An explanation of this trend could be attributed to greater stability of these products due to food processing, the inactivation of both hydrolytic and oxidative enzymes, as well as the disruption of cell membranes and proteins, releasing some individual carotenoids.[6,12] Torregrosa et al.[27] also observed a rise (in the range of 111 to 160%) in the concentration of 9-*cis*-violaxanthin+neoxanthin, antheraxanthin, lutein,

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zeaxanthin, β-cryptoxanthin, when an orange-carrot juice was HIPEF-treated at 35 kV/cm for 150 µs. Similarly, Cilla et al.[37] reported that lutein, zeaxanthin, and neoxanthin + 9–cis–violaxanthin improved their concentration (between 53 and 99%) in beverages made with fruit juices and milk or soymilk treated by HPP (400 MPa/40ºC/5 min).

Other carotenoids did not change their concentration in HIPEF- (mainly β-cryptoxanthin of the three samples), HPP- (α- and β-cryptoxanthin of all samples) and TT-beverages (some xanthophylls) compared with untreated ones. However, losses of some of these compounds were observed in beverages treated by any of the three technologies (HIPEF, HPP and TT), being TT the processing in which the greatest reductions were obtained (between 8 and 48%). Carotenoid denaturalization depends on their chemical structure[38] and most of them are molecules that easily oxidized and isomerized due to the double bounds of their chemical structure.[39] Thus, carotenoids could undergo several changes during processing, resulting in the degradation of these constituents.[29] Zulueta et al.[40] reported that treatment may affect the carotenoids concentration and their isomeric features. In addition, similar results in orange juice, orange-carrot juice, and fruit juices and milk/soymilk beverages processed by these technologies were reported.[13,27,29,31,37,41]

On the other hand, it was observed that the food matrix exerted a significant influence ($p < 0.05$) on the concentration of carotenoids extracted from beverages. MB displayed the highest concentration of all individual carotenes and xanthophylls, indicating that this beverage contained higher total carotenoid concentration than WB and SB (Tables 1 and 2). The concentration of total carotenoids from WB and SB was very similar in untreated and HPP beverages and no statistically significant differences were found. However, SB displayed the lowest concentration of total carotenoids in HIPEF and TT

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samples. Therefore, these results indicated that the composition of the food matrix exerted an important effect on the stability and concentration of carotenoids extracted from blended fruit juice-based beverages. In fact, it has been reported that the presence of dietary fiber, as well as the amount and type of fat are among the main dietary factors that may affect the carotenoids extraction and in consequence, the carotenoid profile of food.[5,20]

Carotenoid bioaccessibility. Tables 3 and 4 show the bioaccessibility of carotenoids from the beverages considered in this study. The bioaccessibility of these compounds was in the range of 9.2 to 31.4% in untreated beverages. Similar results were reported in a blend of fruit juices and in a fruit juice-soymilk or -milk beverages, where carotenoid bioaccessibilities were between 6.5 and 26.8%.[28,33,36] β-cryptoxanthin and β-carotene displayed bioaccessibilities in the range of 16 to 33% in citrus juices.[42]

Both food matrix and food processing exerted a significant influence ($p < 0.05$) on the bioaccessibility of carotenoids. In overall, the bioaccessibility of individual carotenoids diminished after applying any type of treatment, mainly in TT beverages where the biaccessibility declined up to 63%. HIPEF treatment decreased the bioaccessibility of carotenoids in the range of 7.6 to 48.2%, compared to untreated beverages. In the same way, carotenoids were less bioaccessible in HPP beverages (between 8.2 and 45.1%) than in those untreated. The carotenoids that showed the lowest bioaccessibility after applying each processing technology analyzed herein were: β-cryptoxanthin from WB after HIPEF or HPP; and α-cryptoxanthin from SB after TT. As far as we know, very few reports have evaluated the influence of non-thermal (HIPEF and HPP) or thermal (TT) processing technologies on the bioaccessibility of carotenoids. In one such report, Cilla et al.[37] observed that some carotenoids were around 15 and 58% less bioaccessible

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in fruit juice-milk based beverages treated by HPP than in the untreated beverage. However, these authors observed greater reductions in the bioaccessibility of carotenoids from fruit juice-milk or soymilk-based beverages treated by heat (between 30 and 90%).[37] Stinco et al.[43] reported that pasteurization reduced the bioacessibility of α-carotene and β-cryptoxanthin in orange juice as compared with fresh industrially squeezed juice.

In some cases, HIPEF processing improved the bioaccessibility of carotenoids in comparison with their respective untreated beverages, such as cis-violaxanthin+neoxanthin from the three beverages (between 9 and 79%), cis-antheraxanthin from SB (10%), and lutein from both MB (32%) and SB (16%). The bioaccessibility of total xanthophylls and total carotenoids from MB also increased 24.5 and 15%, respectively, when HIPEF treatment was applied. A similar trend was observed in beverages treated by HPP, where cis-violaxanthin+neoxanthin from the three beverages; cis-antheraxanthin and lutein from SB, total xanthophylls from both MB and SB, and total carotenoids from SB were more bioaccessible in HPP beverages (in the range of 6.5 to 65%) than in untreated samples. On the contrary, significant reductions in the bioaccessibility of carotenoids were observed in TT beverages (between 22 and 63%). The improvement in the bioaccessibility of some carotenoids in HIPEF and HPP beverages could be justified by changes in the structure of the food matrix due to processing effect, such as the breakdown of cell walls and membranes in which carotenoids are embebed. Thus, carotenoids could be released from the food matrix enhancing their interactions with digestive enzymes and their solubilisation into micelles. This hypothesis is supported by Stinco et al.,[43] who reported that the food matrix structure is one of the most important factors that affect the bioaccessibility of carotenoids. Additionally, Maiani et al.[6] found that some types of food processing can

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improve the carotenoid bioavailability. Cilla et al.[37] reported increases between 39 and 264% in the bioaccessibility of neoxanthin+9-cis-violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene from milk- or soymilk-based beverages treated by HPP with respect to untreated products.

The food matrix exerted a significant influence ($p < 0.05$) on the bioaccessibility of carotenoids. Total carotenoids from MB displayed the highest bioaccessibility with average value of 23.5%, followed by SB (15.9%) and WB (12.9%). These results suggest that the greater fat content of milk (3.6%) compared with soymilk (1.6%) and water (0%) could favour the incorporation of carotenoids into micelles and thus, increase their bioaccessibility in MB. In accordance with this hypothesis, it has been reported that dietary fat enhance the bioaccessibility of carotenoids from food.[5,20] Granado-Lorencio et al.[44] also found that the addition of milk to blended fruit juices improve the bioaccessibility of carotenoids.

Fiber is other food constituent that could affect the bioaccessibility of carotenoids. Dietary fiber could increase the viscosity of the intestinal content[45] entrapping bioactive compounds and decreasing the activity of digestive enzymes. Thus, the micellization and bioaccessibility of carotenoids are reduced due to the fiber content of food. In this sense, it could be expected that SB beverages contain more amount of dietary fiber than MB, explaining why the bioaccessibility of carotenoids diminished in SB beverages. In contrast to these results, Cilla et al.[37] did not find significant differences on the bioaccessibility of carotenoids in fruit juice-based beverages containing milk or soymilk.

Considering the effect of both food matrix and processing, it was observed that a milk matrix (MB) in combination with HIPEF processing increased the bioaccessibility of total carotenoids (15%) compared to untreated beverages. Carotenoids from MB were

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equally bioaccesible in HPP and untreated beverages. In SB, the technology that improved the bioaccessibility of total carotenoids was HPP (10%), whereas HIPEF slightly decrease them (7%). Both non-thermal technologies (HIPEF and HPP) decreased the bioaccessibility of total carotenoids in WB (around 17%). The lowest bioaccessibility was achieved in the three beverages treated by TT (losses up to 37%), showing that TT was not adequate for improving the bioaccessibility of carotenoids contained in these beverages.

Lipophilic antioxidant activity (LAA)

The LAA from non-digested beverages is displayed in Figure 2A, ranging between 5.3 and 16.7% of DPPH$^{\bullet}$ inhibition in untreated products. Similar results were previously reported in blended fruit juices (between 15.2 and 17% of DPPH$^{\bullet}$ inhibition) and in beverages based on fruit juice and soymilk or milk (11.9 and 16.6% of DPPH$^{\bullet}$ inhibition, respectively).[28,33,36]

Thermal treatment (TT) exerted a significant influence ($p < 0.05$) on the LAA of MB and SB beverages, where the percentage of DPPH$^{\bullet}$ inhibition diminished between 7 and 27% when compared with untreated beverages. SB beverages treated by HIPEF and HPP also exhibited a decrease of 22 and 17%, respectively, in the LAA in comparison with untreated products. In contrast, the LAA from WB and MB treated by both non-themal technologies (HIPEF and HPP) remained unchanged with respect to untreated samples ($p > 0.05$). When the three treatments (HIPEF, HPP, TT) were compared, it was observed that the lowest LAA was obtained in thermally-treated beverages. On the contrary, the highest LAA was observed in products treated by HIPEF (for WB) and HPP (for MB). To our knowledge, this is the first study addressing the influence of non-thermal and thermal technologies on the lipophilic antioxidant activity of beverages.

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However, there is available information about the influence of HIPEF, HPP and TT on total antioxidant activity of liquid food. In this sense, Morales-de la Peña et al.[46] observed that HIPEF treatment (35 kV/cm, 4μs bipolar pulses at 200 Hz for 1400 μs) did not affect the total antioxidant activity of a blended fruit juice-soymilk beverage in comparison with untreated juice. Elez-Martínez et al.[47] did not find significant differences in the antioxidant activity of HIPEF (15 – 35 kV/cm, 20 – 10μs mono or bipolar pulses at 50 – 450 Hz for 100 – 1000 μs), TT (90 ºC/ 1 min) and untreated orange juice. Plaza et al.[48] also showed that the antioxidant activity of orange juice was not affected by HIPEF (35 kV/cm, 4μs bipolar pulses at 800 Hz for 750 μs) and thermal treatment (70 ºC during 30s) as compared with untreated juice. On the other hand, Patras et al.[49] reported that TT (70 ºC /2 min) and HPP (400 MPa/20 ºC/15 min) decrease the anti-radical power of strawberry pure (25 and 19%, respectively), but not in blackberry pure treated by these technologies. Significant reductions in the antioxidant activity (between 7.5 and 11.5%) of an orange juice-milk beverage thermally treated (90 or 98 ºC for 21s) were observed.[13] However, the antioxidant activity remained unchanged in HPP samples (400 MPa /5 min) as compared with that untreated.[13]

Considering the food matrix influence, it was observed that the LAA of all beverages were statistically different ($p < 0.05$), where SB displayed the lowest percentage of DPPH$^\bullet$ inhibition (4%) and MB the highest (17%). Likely, the higher fat content of milk with respect to SB and WB matrices could improve the antioxidant activity of lipophilic constituents. Additionally, these results were in accordance with those found in carotenoids, where the greatest concentration of these compounds was obtained in MB (see previous sections). On the other hand, some protein and fiber types could mask the antioxidant activity of food[50] and soymilk contains fiber and greater amounts of proteins

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(up to 20%), explaining why the lowest LAA was found in SB. In fact, a strong correlation between the LAA and total xanthophyll concentration ($r^2 = 0.8495$, $p = 0.0000$) from SB, as well as between LAA and total carotenoid concentration ($r^2 = 0.7257$, $p = 0.0015$) was observed.

Digested beverages. The lipophilic antioxidant activity (LAA) of digested beverages is presented in Figure 2B. The DPPH$^\bullet$ inhibition ranged from 3.3 to 12.67% in untreated beverages, where MB showed the highest LAA.

All treatments (HIPEF, HPP and TT) increased between 7 and 17% the LAA of digested MB with respect to untreated beverages. Non-thermal technologies (HIPEF and HPP) also enhanced the LAA of digested WB (in the range of 47 to 53%), while non-significant differences were observed in the digested fraction of WB-TT. In contrast, the LAA of digested SB was reduced by any type of treatment, with losses between 21 and 30% as compared with untreated products. The LAA correlates well with the bioaccessibility of cis-violaxanthin+neoxanthin from MB ($r^2 = 0.7533$ $p = 0.0047$) and WB ($r^2 = 0.6487$, $p = 0.0225$), which was the carotenoid that increased its bioaccessibility after non-thermal processing. Therefore, the increment in the LAA of non-thermally treated beverages could be linked to the improvement in the solubilisation, digestibility and bioaccessibility of some lipophilic compounds with antioxidant activity, such as carotenoids.

The food matrix exerted a significant influence on the LAA of digested beverages. The lowest LAA was observed in digested SB, with around 2.30 and 3.3% of DPPH$^\bullet$ inhibition. On the other hand, digested MB displayed the highest LAA (between 12.67 and 15.6%). An explanation of these results could be attributed to the fact that the bioaccessibility of carotenoids was improved in matrices containing certain amount of

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fat (such as milk), as well as in beverages treated by non-thermal technologies (in the case of certain carotenoids). Therefore, the antioxidant potential and the bioaccessibility of these compounds could be modulated by both food matrix and food processing.

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Conclusion

Food matrix and food processing exerted a significant influence on the bioaccessibility of carotenoids, as well as on the lipophilic antioxidant activity (LAA) of beverages. Non-thermal technologies (HIPEF and HPP) were more effective than TT to preserve the concentration and bioaccessibility of carotenoids and other lipophilic compounds with antioxidant activity from beverages based on a blend of fruit juices (orange, pineapple, kiwi and mango) and water, milk or soymilk. The beverage with the highest bioaccessibility of total carotenoids (determined as the sum of individual compounds) was that containing milk (MB), followed by that made with soymilk (SB) and finally that of water (WB). A milk matrix (MB) in combination with HIPEF processing increased 15% the bioaccessibility of carotenoids as compared with the untreated product. In SB beverages, HPP increased 10% the bioaccessibility of these compounds, while all technologies (HIPEF, HPP and TT) diminished it in WB. Results demonstrate that both, food matrix and food processing, are able to modulate the bioaccessibility of carotenoids as well as the antioxidant potential of beverages, therefore these issues should be taken in consideration when developing functional food and beverages. In addition, HIPEF and HPP could be considered as promising technologies to obtain highly nutritional and functional beverages. Further studies should be carried out in order to evaluate the influence of food matrix and processing on the *in vivo* bioavailability of carotenoids.

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Table 1. Concentration of carotenoids in fruit juice-based beverages[a]

Beverages	Treatments	Carotenoid concentration (μg/100 mL)								
		Cis-violaxanthin +neoxanthin	**Cis-antheraxanthin**	**Antheraxanthin**	**Lutein**	**Zeaxanthin**	**α-cryptoxanthin**	**β-cryptoxanthin**	**α-carotene**	**β-carotene**
WB	Untreated	57.0 ± 2.2aA	82 ± 4aA	12.6 ± 0.5cA	43 ± 3aA	25.9 ± 1.3dA	8.2 ± 0.3cC	12.1 ± 0.8bA	4.7 ± 0.3cA	77 ± 5cA
	HIPEF	62 ± 4bB	89 ± 3bB	11.1 ± 0.4bA	37.4 ± 1.5bB	20.5 ± 1.0bA	7.3 ± 0.5baA	11.9 ± 0.7bA	3.59 ± 0.12bA	67.5 ± 1.6bA
	HPP	63 ± 3abB	85 ± 5abB	11.5 ± 0.4bA	40.8 ± 0.7aB	24.1 ± 1.2cA	7.9 ± 0.4bcA	12.3 ± 0.5bA	3.8 ± 0.3bA	66.5 ± 2.1bA
	TT	58.6 ± 1.8abB	81.3 ± 2.5aB	9.8 ± 0.4aA	35.5 ± 1.4bB	17.4 ± 1.2aA	6.7 ± 0.3aA	10.9 ± 0.6aA	3.20 ± 0.10aA	60 ± 3aA
MB	Untreated	66 ± 4aB	122 ± 3cB	18.3 ± 1.1aB	57 ± 4aB	34.3 ± 1.8aB	9.2 ± 0.3aB	15.3 ± 0.7aB	7.5 ± 0.3bcB	96 ± 4aB
	HIPEF	76.7 ± 2.2bC	109.2 ± 1.8bC	20.2 ± 1.0bB	70 ± 4bC	44 ± 3bB	8.7 ± 0.5aB	16.0 ± 0.6abC	7.1 ± 0.4abC	89 ± 4aC
	HPP	80 ± 4bC	110 ± 7bC	20.5 ± 1.4bB	75 ± 3bC	47 ± 3bB	9.2 ± 0.4aB	16.3 ± 0.5abC	7.9 ± 0.4cC	102 ± 4bC
	TT	70 ± 4aC	99 ± 4aC	18.9 ± 0.6abC	57.4 ± 2.4aC	32.3 ± 2.1aB	7.7 ± 0.3bB	15.7 ± 0.6abC	6.88 ± 0.12aC	85 ± 5aB
SB	Untreated	58 ± 3bA	87 ± 5cA	13.3 ± 0.9cA	48 ± 3dC	28.3 ± 1.9cA	7.2 ± 0.3bcA	14.2 ± 0.6bcB	5.1 ± 0.3abA	72 ± 2aA
	HIPEF	53 ± 3bA	71 ± 3bA	11.3 ± 0.5abA	29.7 ± 1.0bA	20.8 ± 1.4bA	6.69 ± 0.21abA	14.0 ± 0.7bB	4.8 ± 0.3aB	76 ± 3aB
	HPP	56 ± 4bA	75 ± 4bA	11.9 ± 0.3bA	33.9 ± 1.4cA	21.4 ± 1.1bA	7.3 ± 0.5cA	14.9 ± 0.6cB	5.5 ± 0.4abB	78 ± 5aB
	TT	43 ± 3aA	57.5 ± 1.7aA	10.8 ± 0.4aB	25.2 ± 1.2aA	15.3 ± 0.7aA	6.5 ± 0.3aA	12.4 ± 0.5aB	5.30 ± 0.23abB	61 ± 4aA

[a] Values are expressed as the mean ± standard deviation (n=8). Different lower case letters in the same column and beverage indicate significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high-intensity pulsed electric fields; HPP, high-pressure procesing; TT, thermal treatment.

Table 2. Concentration of total carotenoids in fruit juice-based beverages[a]

Beverages	Treatments	Carotenoid concentration (µg/100 mL)		
		Total xanthophylls	Total carotenes	Total carotenoids
WB	Untreated	240 ± 6bA	81 ± 4cA	322 ± 4dA
	HIPEF	238 ± 5bB	71.1 ± 1.7bA	309 ± 3bB
	HPP	244.4 ± 1.7bB	70.4 ± 2.2bA	315 ± 3cA
	TT	220 ± 4aB	63 ± 3aA	283 ± 3aB
MB	Untreated	322 ± 10bC	104 ± 4bB	426 ± 12bB
	HIPEF	345 ± 4cC	97 ± 4aC	441.8 ± 1.3cC
	HPP	358 ± 7dC	110 ± 4bC	467 ± 7dB
	TT	302 ± 5aC	92 ± 5aB	393 ± 10aC
SB	Untreated	256 ± 11dB	77.5 ± 2.1cA	334 ± 10dA
	HIPEF	206.7 ± 1.7bA	80 ± 3acB	287 ± 4bA
	HPP	220 ± 6cA	83 ± 5bB	303 ± 11cA
	TT	170 ± 4aA	66 ± 4aA	237 ± 6Aa

[a] Values are expressed as the mean ± standard deviation (n=8). Different lower case letters in the same column and beverage indicate significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high-intensity pulsed electric fields; HPP, high-pressure procesing; TT, thermal treatment. Total xanthophylls and total carotenes were determined as the sum of individual carotenoids of each family quantified by HPLC (see Table 1). Total carotenoids corresponded to the sum of total xanthophylls and total carotenes determined by HPLC.

Table 3. Bioaccessibility of carotenoids in fruit juice-based beverages[a]

Beverages	**Treatments**	**Bioaccessibility of carotenoids (%)**								
		Cis-violaxanthin + neoxanthin	**Cis-antheraxanthin**	**Antheraxanthin**	**Lutein**	**Zeaxanthin**	**α-cryptoxanthin**	**β-cryptoxanthin**	**α-carotene**	**β-carotene**
WB	Untreated	15.8 ± 0.8bB	14.0 ± 0.6bB	9.2 ± 0.6cA	16.0 ± 0.9dA	17.5 ± 1.2dA	17.5 ± 1.1bB	17.8 ± 1.1cA	17.7 ± 0.9cA	16.9 ± 0.7cA
	HIPEF	17.2 ± 0.5cA	10.4 ± 0.7aA	7.05 ± 0.17bA	14.8 ± 0.6cA	13.5 ± 0.6cA	12.1 ± 0.7bB	9.2 ± 0.4bA	13.2 ± 0.8bA	12.2 ± 0.8bA
	HPP	19.0 ± 1.2dA	10.0 ± 0.4aA	7.5 ± 0.3bA	13.8 ± 0.4bA	12.3 ± 0.4bcA	13.4 ± 0.8bB	9.8 ± 0.5bA	12.9 ± 0.5bA	13.1 ± 0.7bA
	TT	8.9 ± 0.5aA	9.8 ± 0.3aB	6.5 ± 0.4aA	12.1 ± 0.5aA	11.8 ± 0.7aA	10.4 ± 0.7aB	7.8 ± 0.3cA	8.5 ± 0.3aA	10.2 ± 0.3aA
MB	Untreated	21.6 ± 1.4bC	17.5 ± 0.8cC	14.6 ± 1.0cC	28.9 ± 1.4cC	30.0 ± 0.8cC	29,8 ± 1.3cC	20.0 ± 1.1cB	31.2 ± 1.4cC	31.4 ± 2.2cC
	HIPEF	38.7 ± 2.5dB	15.5 ± 0.6bC	13.0 ± 0.6bC	38.1 ± 1.7dC	29.1 ± 1.6bcC	30.1 ± 1.2cC	19.8 ± 1.0cC	28.5 ± 1.8bC	29.6 ± 2.0bC
	HPP	33.8 ± 1.8cC	15.8 ± 1.1bC	13.9 ± 0.9bcC	25.2 ± 1.0bB	27.6 ± 1.7bC	26.8 ± 1.8bC	13.7 ± 0.6bB	30.4 ± 1.6bcC	29.0 ± 1.3bC
	TT	15.9 ± 0.8aB	12.2 ± 0.5aC	10.6 ± 0.7aB	19.8 ± 0.9aB	23.3 ± 1.6aC	14.3 ± 1.0aC	12.8 ± 0.7aC	23.1 ± 0.7aC	22.9 ± 1.2aC
SB	Untreated	13.9 ± 0.4bA	12.2 ± 0.8cA	11.1 ± 0.6dB	22.7 ± 1.4aB	24.1 ± 1.6dB	15.1 ± 1.0cA	18.6 ± 0.8dAB	22.0 ± 0.7dB	20.1 ± 1.3cB
	HIPEF	17.1 ± 1.2cA	13.5 ± 0.6bB	9.4 ± 0.4bB	26.3 ± 1.4bB	17.3 ± 1.1bB	7.84 ± 0.22bA	11.9 ± 0.6bB	16.5 ± 1.1cB	15.6 ± 1.1bB
	HPP	21.5 ± 0.9dA	14.0 ± 0.8bB	9.66 ± 0.07cB	37.6 ± 1.5cC	20.7 ± 0.7cB	8.5 ± 0.6bA	15.9 ± 0.9cC	15.3 ± 0.5bB	16.1 ± 0.5bB
	TT	9.2 ± 0.5aA	7.8 ± 0.4aA	7.2 ± 0.4aA	23.6 ± 1.0aC	14.5 ± 0.5aB	5.6 ± 0.4aA	10.5 ± 0.6aB	11.8 ± 0.6aB	12.9 ± 0.9aB

[a] Values are expressed as the mean ± standard deviation (n=8). Different lower case letters in the same column for each beverage show significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high-intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment. The bioaccessibility of each carotenoid was determined as the ratio between the concentration of individual compound in the digested beverage (micellar fraction) and that of non-digested products (see Table 1).

Table 4. Bioaccessibility of total carotenoids in fruit juice-based beverages[a]

Beverages	Treatments	Bioaccessibility of carotenoids (%)		
		Total xanthophylls	Total carotenes	Total carotenoids
WB	Untreated	15.19 ± 0.12cA	17.0 ± 0.7cA	15.63 ± 0.17dA
	HIPEF	12.93 ± 0.19bA	12.3 ± 0.8bA	12.8 ± 0.3bA
	HPP	13.12 ± 0.19bA	13.1 ± 0.7bA	13.12 ± 0.21cA
	TT	9.85 ± 0.21aA	10.1 ± 0.3aA	9.91 ± 0.12aA
MB	Untreated	22.0 ± 0.7bC	31.4 ± 2.0bC	24.3 ± 0.6bC
	HIPEF	27.4 ± 0.5dC	29.5 ± 1.8bC	27.8 ± 0.3cC
	HPP	23.4 ± 1.1cC	29.1 ± 1.1bC	24.8 ± 0.7bC
	TT	15.68 ± 0.17aC	22.6 ± 1.1aC	17.3 ± 0.3aC
SB	Untreated	16.3 ± 0.6bB	20.2 ± 1.3cB	17.2 ± 0.7cB
	HIPEF	16.0 ± 0.4bB	15.7 ± 1.1bB	15.9 ± 0.3bB
	HPP	19.89 ± 0.20cB	16.1 ± 0.5bB	18.84 ± 0.22dB
	TT	11.21 ± 0.23aB	12.8 ± 0.8aB	11.65 ± 0.06aB

[a] Values are expressed as the mean ± standard deviation (n=8). Different lower case letters in the same column for each beverage show significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high-intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment. The bioaccessibility of total xanthophylls and total carotenes was determined as the ratio between the sum of the concentrations of individual compounds of each family quantified by HPLC in the digested beverage (micellar fraction) and that of non-digested products (see Table 2). The bioaccessibility of total carotenoids was determined as the ratio between the sum of the concentrations of total xanthophylls and total carotenes in the digested beverage (micellar fraction) and that of non-digested products.

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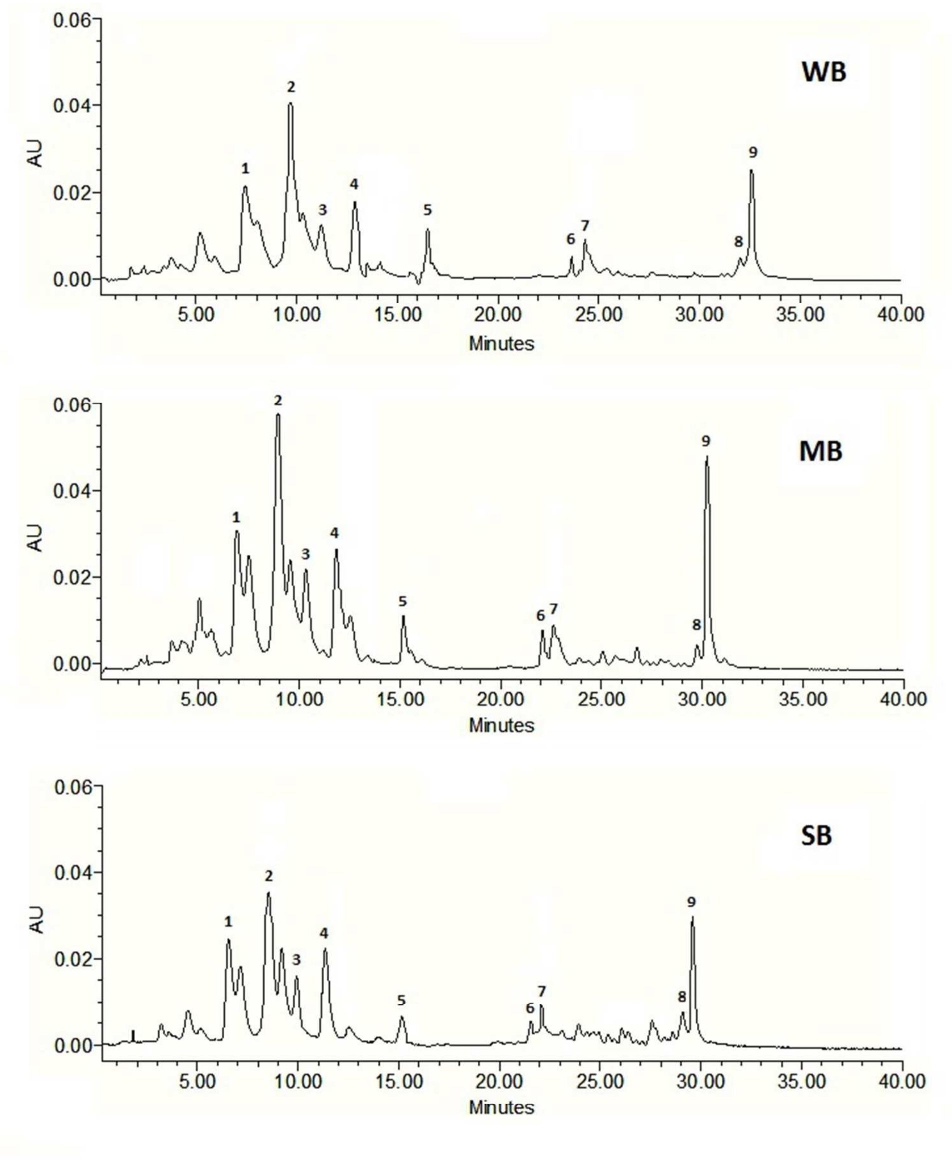

Figure 1. HPLC chromatograms of carotenoids in non-digested and untreated beverages at 450 nm. WB: water-fruit juice beverage; MB: milk-fruit juice beverage; and SB: soymilk-fruit juice beverage. Peaks: 1. Cis-violaxanthin+neoxanthin; 2. Cis-antheraxanthin; 3. Antheraxanthin; 4. Lutein; 5. Zeaxanthin; 6. α-cryptoxanthin; 7. β-cryptoxanthin; 8. α-carotene; and 9. β-carotene.
201x241mm (96 x 96 DPI)

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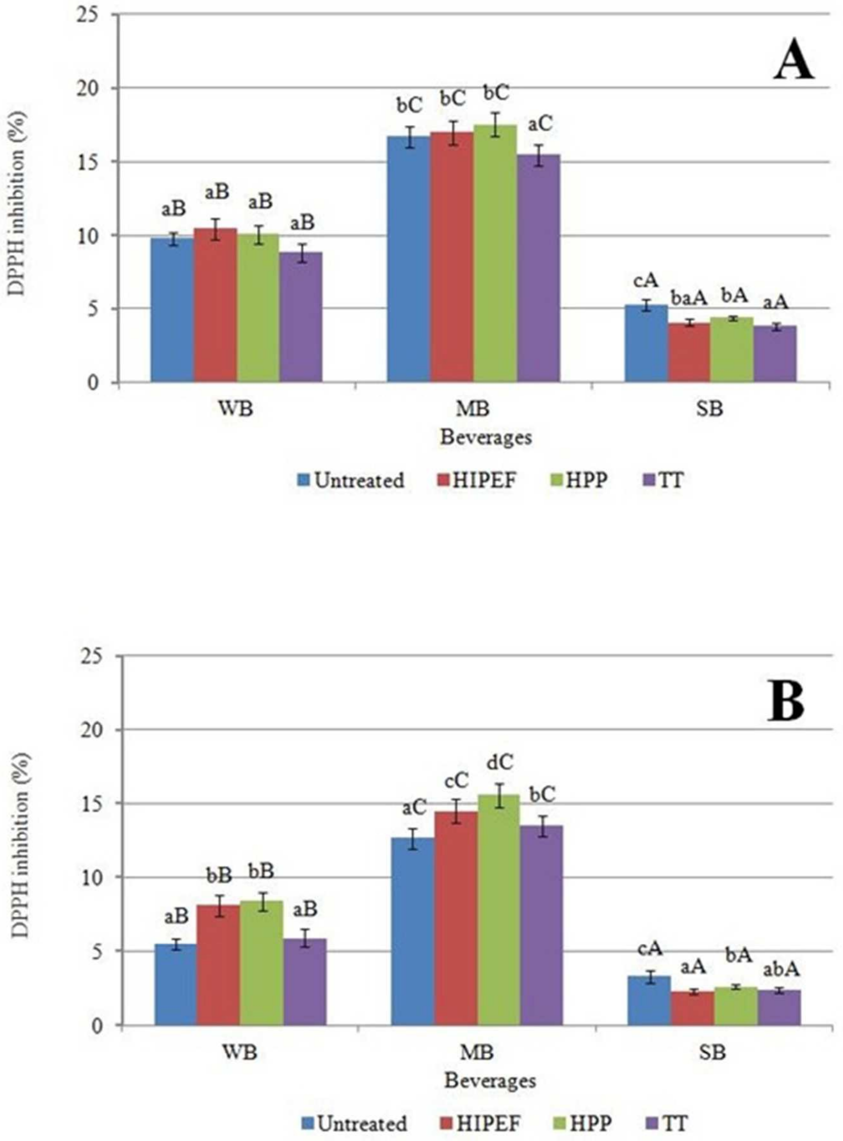

Figure 2. Lipophilic antioxidant activity (LAA) from fruit juice-based beverages. (A) LAA of non-digested beverages. (B) LAA of digested beverages Different lower case letters in the same beverage indicate significant differences ($p < 0.05$) within treatments. Different capital letters in the same treatment for WB, MB and SB beverages show significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high-intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.
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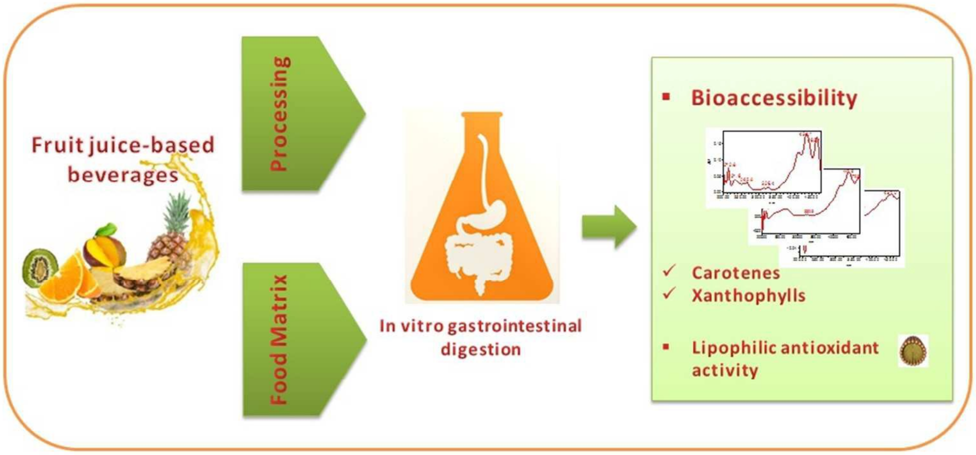

Understanding the extent to which food matrix and food processing modify the bioaccessibility of carotenoids is important for designing food and beverages with high nutritional and functional properties.

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