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Bioaccessibility of Tocopherols, Carotenoids, and Ascorbic Acid from Milk- and Soy-Based Fruit Beverages: Influence of Food Matrix and Processing

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Supporting Information

ABSTRACT: A study was made of the effect of high-pressure processing (HPP) and thermal treatment (TT) on plant bioactive compounds (tocopherols, carotenoids, and ascorbic acid) in 12 fruit juice−milk beverages and of how the food matrix [whole milk (JW), skimmed milk (JS), and soy milk (JSy)] modulates their bioaccessibility (%). HPP (400 MPa/40 °C/5 min) produced a significant decrease in carotenoid and ascorbic acid bioaccessibility in all three beverages and maintained the bioaccessibility of tocopherols in JW and JS while decreasing it in JSy. TT (90 °C/30 s) produced a significant decrease in tocopherol and carotenoid bioaccessibility in all three beverages and increased the bioaccessibility of ascorbic acid. With regard to the food matrix, α-tocopherol and ascorbic acid bioaccessibility was greatest in JW beverages and lowest in JSy beverages, whereas no significant differences were found among the three beverages in terms of carotenoid bioaccessibility. HPP-treated samples showed higher tocopherol and carotenoid bioaccessibility than TT-treated samples, thus indicating that HPP combined with a milk matrix positively modulates the bioaccessibility of certain types of bioactive components of food, mainly those of a lipophilic nature.

KEYWORDS: *bioaccessibility, tocopherols, carotenoids, ascorbic acid, milk- and soy-based fruit beverages, high-pressure processing, low pasteurization*

INTRODUCTION

Beverages containing fruit juices and milk provide calcium as well as bioactive compounds such as vitamin C, tocopherols, and carotenoids, which have been described as three of the main bioactive plant compounds affording health benefits and a reduction of the risk of suffering certain diseases (heart disease, cancers, macular degeneration).[1−3]

High-pressure processing (HPP) is a useful tool for obtaining safe and high-quality foods, because it inactivates and inhibits microorganisms and enzymes responsible for food quality loss, with minimal changes in its sensorial and nutritional characteristics.[4−7] HPP may also produce improvement of the extraction of potentially health-related compounds, due to the induction of many changes in plant food structure during food processing, which could favor their bioaccessibility and bioavailability.[6,8−15] Moreover, the combination of fruit juices with different matrixes such as whole milk, skimmed milk, and soy milk provide distinct environments for bioactive fruit components that can modify their stability and bioavailability.[16]

It is clear that data from human intervention studies constitute the reference standard and offer the highest scientific evidence referred to the bioavailability of a nutrient, whereas in vitro methods are used as surrogates for predictive purposes. Nevertheless, in vitro methods that apply human simulated digestion models are considered to be valuable and useful tools for the estimation of preabsorptive events (i.e., stability, bioaccessibility) of different nutrients such as ascorbic acid,[17,18] carotenoids and/or tocopherols[16,19−23] from different food sources including fruits, vegetables, and fruit juices and also for determining the effect that processing may have on nutrient bioavailability.

To our knowledge, there are no previous studies evaluating the effects of processing (thermal treatment and high-pressure processing) and the food matrix (whole, skimmed, and soy milk beverages) upon the bioaccessibility of bioactive compounds such as ascorbic acid, carotenoids, and tocopherols. Therefore, the aim of the present study was to evaluate ascorbic acid, carotenoid, and tocopherol bioaccessibility from different fruit juice beverages with milk or soy subjected to pasteurization or high-pressure processing.

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MATERIALS AND METHODS

Reagents. *Tocopherol Analysis.* (±)-α-Tocopherol (≥96% purity), (+)-γ-tocopherol (≥96% purity), and (+)-δ-tocopherol (90% purity) were obtained from Sigma (St. Louis, MO, USA). Chloroform Multisolvent, *n*-hexane 96% Multisolvent, and ethyl acetate Multisolvent were obtained from Scharlau (Barcelona, Spain). Methanol was obtained from J. T. Baker (Deventer, The Netherlands). Standard solutions of α-tocopherol (284 mg/L), γ-tocopherol (62.5 mg/L), and δ-tocopherol (263 mg/L) were prepared in *n*-hexane. These standard solutions were stable for at least 1 month at 4 °C in an N_2 atmosphere. Fresh working standard solutions were prepared daily by appropriate dilutions of standards in *n*- hexane.

Carotenoid Analysis. Butylated hydroxytoluene (BHT), β-carotene, β-cryptoxanthin, lutein, lycopene, Sudan I, and zeaxanthin were obtained from Sigma. Commercial standards, with purity >97% determined by HPLC as stated by Sigma, were employed. The concentration of stock solutions prepared in *n*-hexane was corrected according to this purity degree. Anhydrous sodium sulfate, hydrochloric acid 35%, citric acid monohydrate, magnesium hydroxide carbonate 5-hydrate, potassium hydroxide 85%, and sodium chloride were purchased from Panreac Química (Barcelona, Spain). Dichloromethane, diethyl ether, ethyl acetate, methanol, and tetrahydrofuran (THF) were obtained from Labscan Ltd. (Dublin, Ireland).

Vitamin C Analysis. Glacial acetic acid, formic acid, and L-(+) ascorbic acid (≥99% purity) were purchased from Panreac Química. DL-Dithiothreitol was obtained from Sigma.

In Vitro Digestion. Enzymes and bile salts were purchased from Sigma Chemical Co.: pepsin (porcine, 975 units/mg protein), pancreatin (porcine, activity equivalent to 4 × USP specifications), and bile extract (porcine). Working solutions of these enzymes were prepared immediately before use.

Samples. Three formulations of fruit beverages with different types of processing (nontreated, pasteurized, or high pressure) have been studied. The fruit juice−milk-based beverages were prepared by mixing 75% (v/v) of fruit juice (J) and 16.5% (v/v) of milk [whole milk (W), skimmed milk (S)] or 50% (v/v) of fruit juice (J) and 41.5% (v/v) of soy milk (Sy). Sugar (7.5%) and citric acid (1%) were added as sweetener and preservative, respectively. The formulation was designed to simulate the composition of commercial beverages. Immediately after preparation, the beverages were processed. The treatments received were high-pressure processing (HPP) and no treatment as control (C_a) and thermal treatment (TT) and no treatment as control (C_b). Two different controls were analyzed as two different batches of beverages were employed, one for each treatment. The 12 different samples studied were named as follows: JW-C_a, JW-HPP, JW-C_b, JW-TT, JS-C_a, JS-HPP, JS-C_b, JS-TT, JSy-C_a, JSy-HPP, JSy-C_b, and JSy-TT.

Fresh-squeezed juices were obtained from oranges (cv. Valencia Late, Valencia, Spain), kiwis (cv. Hayward, New Zealand), pineapples (cv. MD2 or SuperSweet, Costa Rica), and mangos (cv. Keitt, Ecuador) purchased in a local supermarket in December 2009. Orange juices were obtained using a domestic squeezer (Lomi model 4, Madrid, Spain) and filtered through 2 mm steel sieves. Kiwi, mango. and pineapple juices were obtained using a domestic liquidizer (Braun MP80 Multipress, Barcelona, Spain). After that, the source of milk [(whole milk and skimmed milk, commercial pasteurized and refrigerated with a mild thermal treatment of 72 °C/15 s (La Priegola) or soy milk (Soja Yosoy)], citric acid, and sugar were added to them and mixed. Briefly, the nutritional composition of the commercial milks employed was as follows: fat, 3.5% (W), 0.3% (S), and 1.8% (Sy); protein, 3.1% (W), 3.2% (S), and 3.6% (Sy); carbohydrates, 4.8% (W), 5% (S), and 1.3% (Sy); calcium, 119 mg (W), 123 mg (S), and 120 mg (Sy). Four 250 mL aliquots of each fruit beverage were vacuum packed in flexible Doypack plastic bags (Polyskin XL, Amcor Flexibles Hispania, SL, Granollers, Spain) and then processed or not. Finally, samples were kept frozen at −20 °C until analysis.

High-Pressure Processing. Fruit beverages vacuum packed in flexible Doypack bags were introduced in the pressure unit filled with pressure medium (water). HPP at 400 MPa was performed in a hydrostatic pressure unit with 2350 mL capacity, a maximum pressure of 500 MPa, and a potential maximum temperature of 95 °C (GEC Alsthom ACB 900 HP, type ACIP no. 665, Nantes, France). Before pressurization, the pressure chamber was heated/cooled to a desired level by means of a thermostat jacket connected to a water bath. Compression and decompression rates were 2.5 MPa/s. Samples were processed at 36 °C with a holding time of 5 min at 400 MPa. Because of adiabatic compression, the maximum temperature in the vessel was 40 °C at 400 MPa. Pressure, time, and temperature were controlled by a computer program, being constantly monitored and recorded during the process. On the basis of preliminary results of our group, these processing conditions were selected due to their effect on enzymatic inactivation[24] and microbial reduction.[25]

Thermal Treatment. Fruit beverages were heated at 90 °C for 30 s in the same packages used for HPP, quickly achieving uniform heat. Then the samples were cooled to room temperature. Treatment was carried out in an autoclave (Autotester-G, Selecta, Barcelona, Spain). The optimum TT conditions were selected according to the results of a previous study.[26]

In Vitro Simulated Gastrointestinal Digestion. An in vitro gastrointestinal digestion procedure mimicking the physiological situation in the upper digestive tract (stomach and small intestine) was used according to the method of Cilla et al.,[27] with 80 g of each sample.

Briefly, 80 g of fruit beverages was adjusted to pH 2.0 with 6 M HCl (GLP 21 pH-meter, Crison, Barcelona, Spain). The pH was checked after 15 min and, if necessary, readjusted to 2.0. Then an amount of freshly prepared demineralized pepsin solution sufficient to yield 0.02 g pepsin/g sample was added. The samples were made up to 100 g with cell culture grade water (Aqua B Braun, Braun Medical, Barcelona, Spain) and incubated in a shaking water bath at 37 °C/120 strokes per minute for 2 h (SS40-2, Gran Instruments, Cambridge, U.K.). The gastric digests were maintained in ice for 10 min to stop pepsin digestion. For the intestinal digestion stage, the pH of the gastric digests was raised to pH 6.5 by dropwise addition of 1 M $NaHCO_3$. Then an amount of freshly prepared and previously demineralized pancreatin−bile salt solution sufficient to provide 0.005 g of pancreatin and 0.03 g of bile salt per gram of sample was added, and incubation was continued for an additional 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. The pH was then adjusted to 7.2 by dropwise addition of 0.5 M NaOH.

Aliquots of 25 g of digested sample were transferred to polypropylene centrifuge tubes (50 mL, Costar, New York, NY, USA) and centrifuged at 3300*g* for 1 h at 4 °C (GT422 centrifuge, Jouan, Saint Nazaire, France). Supernatants obtained after in vitro digestion were used to determine the ascorbic acid, carotenoid, and tocopherol contents (bioaccessible fraction). Bioaccessibility (%) refers to the percentage of tested compound remaining in the bioaccessible fraction related to the original nondigested sample. This parameter can be calculated as follows: 100 × (bioaccessible content/total content).

Determination of Tocopherols. Tocopherol extraction and evaluation was carried out according to the method previously described by Rodrigo et al.[28] using 1 mL of sample (fruit beverage or bioaccessible fraction), with determination being performed by HPLC.

Quantification was made using external standard calibration curves containing α-, γ-, and δ-tocopherols. The α-tocopherol calibration curves for all food matrixes were in the range of 0.5−6 μg/mL. On the other hand, γ- and δ-tocopherols needed different calibration curves according to the food matrix involved. For soy milk-based samples, calibration curves ranged from 0.5 to 5 μg/mL and from 0.1 to 0.8 μg/mL for γ- and δ-tocopherols, respectively. In the case of cow's milk-based samples (whole and skimmed), calibration curves ranged between 0.01 and 0.1 μg/mL and from 0.005 to 0.05 μg/mL for γ- and δ-tocopherols, respectively. Results were expressed as micrograms of the corresponding tocopherol per 100 mL of fruit juice−milk beverage (or bioaccessible fraction).

Determination of Carotenoids. Extraction, separation, identification, and quantification of carotenoids were carried out according

Table 1. α-, γ-, and δ-Tocopherol Contents of Milk- and Soy-Based Fruit Beverages (Total and Bioaccessibility)[a]

	α-tocopherol			γ-tocopherol			δ-tocopherol		
	JW	JS	JSy	JW	JS	JSy	JW	JS	JSy
				Total (Micrograms per 100 mL of Fruit Beverage)					
C_a	399.3 ± 15.3 a	408.6 ± 7.6 a	259.9 ± 2.8 a	3.5 ± 0.6 a	2.7 ± 0.2 a	399.0 ± 2.2 a	nd	nd	24.0 ± 0.2 a
HPP	289.9 ± 1.1 b	356.3 ± 22.6 b	319.6 ± 13.0b	2.5 ± 0.2 b	2.7 ± 0.2 a	490.6 ± 20.7 b	nd	nd	28.6 ± 1.0 b
C_b	481.4 ± 8.3 c	446.0 ± 5.7 c	312.2 ± 9.7 c	4.1 ± 0.2 c	5.9 ± 0.04 c	537.8 ± 13.8 c	nd	nd	25.2 ± 0.5 c
TT	473.9 ± 4.7 c	485.0 ± 20.8 d	329.8 ± 12.8 c	3.9 ± 0.2 c	3.4 ± 0.3 d	553.0 ± 20.6 c	nd	nd	25.6 ± 0.8 c
				Bioaccessibility (Percent)					
C_a	71.7 ± 0.5 a	34.0 ± 1.5 a	50.5 ± 1.3 a	123.7 ± 29.1 a	nd	90.1 ± 2.5 a	nd	nd	102.0 ± 1.0 a
HPP	73.1 ± 7.0 a	34.6 ± 2.6 a	38.0 ± 1.7b	120.6 ± 10.2 a	nd	55.9 ± 3.6 b	nd	nd	81.6 ± 4.3 b
C_b	53.3 ± 1.00 c	32.4 ± 1.3 c	41.5 ± 4.5 c	98.3 ± 9.5 c	nd	70.7 ± 8.2 c	nd	nd	83.8 ± 8.9 c
TT	44.3 ± 3.1 d	20.3 ± 0.8 d	14.4 ± 0.4 d	89.6 ± 1.2 c	nd	20.3 ± 0.4 d	nd	nd	35.0 ± 3.1 d

[a]Data are expressed as the mean ± SD (n = 3). Bioaccessibility = 100 × (bioaccessible content/total content). Statistical analysis consisted of comparison (of each individual tocopherol isomer) of HPP samples and TT samples with their controls (C_a and C_b), respectively, using one-way ANOVA [(a, b) for C_a vs HPP, and (c, d) for C_b vs TT]. Different letters within columns indicate significant differences ($p < 0.05$). C_a, nontreated (control of HPP); HPP, high-pressure processing; C_b, nontreated (control of TT); TT, thermally treated; JW, whole milk−fruit beverages; JS, skimmed milk−fruit beverages; JSy, soy milk−fruit beverages; nd, not detected (below the detection limits of 0.16 and 0.06 μg/50 μL injection volume for γ- and δ-tocopherols, respectively).

to previous methods, with minor modifications,[29−32] from 50 mL of sample (fruit beverage or bioaccessible fraction). THF carotenoid extracts were saponified with 30% methanolic potassium hydroxide solution before being analyzed by HPLC-DAD. Separation and identification of carotenoids were performed by HPLC, comparing the retention time and UV−visible absorption spectrum obtained by an online diode array detector with those of the standards previously referred to and in accordance with the literature.[29−32] Carotenoids for which authentic standards are not available were tentatively identified by HPLC-DAD. Quantification was achieved using external standard calibration (β-carotene, β-cryptoxanthin, zeaxanthin, and lutein) in the range from 0.25 to 10 μg/mL, according to the procedure described by Hart and Scott.[33] A calibration curve of β-cryptoxanthin was used to quantify zeinoxanthin, and the rest of the xanthophylls were quantified against lutein. Results were expressed as micrograms of the corresponding carotenoid per 100 mL of fruit juice−milk beverage (or bioaccessible fraction).

Determination of Ascorbic Acid. Ascorbic acid was extracted and quantified by HPLC according to the procedure described by Sánchez-Moreno et al.[13] using 20 mL of sample (fruit beverage or bioaccessible fraction). Prior to the extraction of ascorbic acid, the bioaccessible fraction (pH 7.6) was acidified with hydrochloric acid to pH 4. Quantification was carried out using an ascorbic acid external standard calibration curve in the range of 10−100 μg/mL. Results were expressed as milligrams of ascorbic acid per 100 mL of fruit juice beverage (or bioaccessible fraction).

Statistical Analysis. Analysis of all components was conducted in triplicate, with values reported as the mean ± SD of two independent experiments. One-way (processing treatment) analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to the results obtained for comparison of HPP samples and TT samples with their controls (C_a and C_b), for each beverage (within columns in the tables).

In addition, two-way ANOVA (food matrix and processing treatment) followed by Tukey's post hoc test was performed for the comparison of HPP versus TT samples using relative increases after data transformation (available as Supporting Information), applying the following formulas for each value of tocopherol, carotenoid, and ascorbic acid concentration in HPP and TT samples:

$$C' = (C - C_a\text{m})/C_a\text{m} \text{ for HPP samples}$$

and

$$C'' = (C - C_b\text{m})/C_b\text{m} \text{ for TT samples}$$

where C = tocopherol, carotenoid, or ascorbic acid concentration value for each aliquot of HPP and TT samples, C_am = mean tocopherol, carotenoid, or ascorbic acid concentration value in C_a samples (control of HPP samples), and C_bm = mean tocopherol, carotenoid, or ascorbic acid concentration value in C_b samples (control of TT samples).

A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.1 (Rockville, MD, USA) was used.

RESULTS AND DISCUSSION

Tocopherol Content and Bioaccessibility. Results of α-, γ-, and δ- tocopherols [total and bioaccessibility (%)] in the 12 different milk- and soy-based fruit beverages analyzed are shown in Table 1.

Tocopherol Total Content and Processing Effects. The α-tocopherol contents found in the present study are in agreement with a previous study.[16] α-Tocopherol contents were found to be higher or equal when whole fat milk samples (JW) and skimmed milk samples (JS) were compared (Table 1), which is in agreement with values reported for commercialized dairy products in Spain.[34] In the case of γ-tocopherol isomer, soy milk beverage (JSy-C_a and JSy-C_b) had a significantly ($p < 0.05$) higher content than JW and JS (JSy ≫ JW = JS), and the δ-tocopherol isomer was detected only in soy milk-based fruit beverages (JSy).

On the other hand, when processing was considered, we found a general mean increase of 22% ($p < 0.05$) in the three tocopherols in JSy-HPP samples versus their controls (C_a), whereas in the case of α-tocopherol decreases of 27 and 13% versus the controls (C_a) ($p < 0.05$) were observed in JW-HPP and JS-HPP samples, respectively. Lastly, in the case of γ-tocopherol, a decrease of 28% ($p < 0.05$) was observed in JW-HPP versus its control (JW-C_a). In turn, for TT samples we observed a general trend with no changes versus controls (C_b), except a slight 9% increase ($p < 0.05$) in α-tocopherol for JS.

TT samples had higher α-tocopherol contents than HPP samples in fruit juices with cow's milk (whole and skimmed), but not in the case of JSy samples (Figure 1A, Supporting Information). In addition, TT yielded increased ($p < 0.05$) γ-tocopherol compared with HPP for whole milk beverage (JW) samples. Only in JSy were the contents of the three tocopherols higher ($p < 0.05$) in HPP beverages than in TT beverages (Figure 1A, Supporting Information).

Little information is available on the stability of tocopherols during pasteurization and even less during high-pressure processing. In line with our results obtained for fruit juices

with cow's milk (JW and JS), it has been reported in mature human milk that Holder pasteurization (62.5 °C/30 min) exerts no changes in α-, γ-, and δ-tocopherols versus control (nontreated) samples, whereas lower levels (though without reaching statistical significance) of the three tocopherols were found after HPP (400 MPa/5 min/22 °C) versus control samples.[35]

Tocopherol Bioaccessibility and Processing Effects. In relation to processing, JSy fruit beverages showed the highest decrease ($p < 0.05$) in α-tocopherol bioaccessibility after the applied treatments, with 25, 65, and 62% decreases for HPP versus C_a, TT versus C_b, and HPP versus TT, respectively. The same behavior was observed in the cases of γ-tocopherol and δ-tocopherol, with decreases ($p < 0.05$) of 64 and 57% for JSy samples for HPP versus TT, respectively. It is noteworthy that the bioaccessibility of α-tocopherol in JW-C_a and JS-C_a remained practically unchanged after HPP but decreased 17 and 37% after thermal treatment, respectively (Table 1).

The interactions of the two-way ANOVA (Figure 1B, Supporting Information) showed that when samples were compared according to food matrix factor, whole milk containing fruit beverages showed the highest bioaccessibility values for α-tocopherol ($p < 0.05$). This same trend was observed in the case of γ-tocopherol, but due to the low content of this vitamin E isomer in whole milk-containing fruit beverages, soy milk-based fruit beverages still constitute a better dietary source of γ-tocopherol despite their lower bioaccessibility values compared with whole milk-based beverages (Figure 1A,B, Supporting Information).

In addition, HPP conferred higher α-tocopherol bioaccessibility than TT in all samples (JW, JS, and JSy) and higher ($p < 0.05$) γ- and δ-tocopherol bioaccessibility for JSy samples (Figure 1B, Supporting Information). These results indicate that the thermal treatment applied (mild pasteurization at 90 °C for 30 s) strongly affects α-tocopherol bioaccessibility in comparison to HPP. It is possible that HPP can affect the food matrix, modifying the location of tocopherols in the food, changing their physicochemical states, or altering the amounts of absorption effectors (i.e., fibers, fats, and phytosterols),[20] thereby making these fat-soluble compounds more available for incorporation to the micelles after in vitro gastrointestinal digestion.

On comparing our results with those of other studies, we observe a decrease in α-tocopherol bioaccessibility in our JS-TT sample (20%) (Table 1) versus that of a pasteurized vitamin C-fortified juice made of orange, grape, and apricot and supplemented with skimmed milk 11% v/v (110%).[16] This discrepancy can be explained by slight differences in the food matrix as well as by differences in the in vitro digestion model used. The above authors employed more digestive enzymes than in our study (i.e., α-amylase, colipase, cholesterol esterase, taurocholate salts, and phospholipase A2) and, in particular, made use of a different supernatant isolation procedure. Indeed, these same authors found a 2-fold greater recovery of α-tocopherol in loquat and orange fruits after overnight sedimentation of the digests than that obtained with a low-speed centrifugation procedure.[21] However, our study is in agreement with other publications that use a similar digestion procedure for determining vitamin E bioaccessibility in different foods.[20,23] In this sense, Reboul et al.[20] reported α-tocopherol bioaccessibility values ranging from 0.47 to 98.8%, and γ-tocopherol bioaccessibility values ranging from 6.54 to 6.88% in apples and bananas, respectively. Thus, our values ranging from 14 to 73% and from 20 to 123% for α- and γ-tocopherol isomers, respectively, are similar to those previously noted. Likewise, O'Callaghan et al.[23] reported α-tocopherol bioaccessibility values ranging from 11% in apple sauce to 86% in beef, confirming that vitamin E bioaccessibility is highly variable among dietary sources.

Carotenoid Content and Bioaccessibility. The main xanthophylls (neoxanthin + 9-*cis*-violaxanthin, zeaxanthin, lutein, zeinoxanthin, and β-cryptoxanthin) and β-carotene from the 12 milk-based fruit juice beverages were studied in the saponified extracts. Individual carotenoids and total carotenoid contents (as the sum of the individual carotenoids studied) of the 12 beverages are shown in Tables 2, 3, and 4.

Table 2. Total Carotenoid Contents of Milk-Based Fruit Beverages (Total and Bioaccessibility)[a]

	total carotenoid content		
	JW	JS	JSy
	Total (Micrograms per 100 mL of Fruit Beverage)		
C_a	137.27 ± 3.5 a	121.55 ± 17 a	48.45 ± 8.1 a
HPP	219.53 ± 11 b	134.17 ± 13 a	32.30 ± 3.5 b
C_b	140.05 ± 7.8 c	126.46 ± 16 c	58.07 ± 6.9 c
TT	196.16 ± 0.5 d	157.81 ± 19 d	23.80 ± 0.8 d
	Bioaccessibility (Percent)		
C_a	91.70 ± 1.7 a	47.47 ± 8.1 a	39.84 ± 3.7 a
HPP	38.54 ± 1.5 b	37.75 ± 1.1 b	73.34 ± 6.4 b
C_b	99.36 ± 1.5 c	45.42 ± 5.0 c	36.94 ± 3.9 c
TT	51.88 ± 4.7 d	12.94 ± 0.9 d	18.35 ± 1.4 d

[a]Data are expressed as the mean ± SD ($n = 3$). Bioaccessibility = 100 × (bioaccessible content/total content). Statistical analysis consisted of comparison of total carotenoid content of HPP samples and TT samples with their controls (C_a and C_b), respectively, using one-way ANOVA [(a, b) for C_a vs HPP, and (c, d) for C_b vs TT]. Different letters within columns indicate significant differences ($p < 0.05$). C_a, nontreated (control of HPP); HPP, high-pressure processing; C_b, nontreated (control of TT); TT, thermally treated; JW, whole milk–fruit beverages; JS, skimmed milk–fruit beverages; JSy, soy milk–fruit beverages.

Carotenoid Total Content and Processing Effects. Results of total carotenoids (Table 2) were 3 times lower than those reported for similar milk-based fruit juice beverages,[36] but prepared with different fruit juice compositions. The carotenoid contents can vary in beverages depending on the type, variety, and quantity of fruits employed, the processing used, or the preparation method applied. JW and JS were prepared with the same fruit juice percentage (75%), so the total carotenoid content extracted from the beverage with whole fat milk (JW) was 11% higher ($p < 0.05$) than in the beverage with skimmed milk (JS). The higher fat content in whole milk beverage (3%) compared to skimmed milk (0.3%) could improve carotenoid extraction due to its lipophilic nature (Table 2).

Neoxanthin + 9-*cis*-violaxanthin and β-carotene were the predominant carotenoids, representing approximately 31–34 and 14–18% of total carotenoid content, respectively, in the beverages with cow's milk (JW and JS) (Tables 3 and 4). The rest of the carotenoids analyzed in the cow's milk–fruit beverages (JW and JS), zeinoxanthin, β-cryptoxanthin, zeaxanthin, and lutein, represented approximately 15, 12, 12, and 11%, respectively, of the total carotenoid content. In the beverages with soy (JSy), the predominant carotenoids were neoxanthin + 9-*cis*-violaxanthin and zeinoxanthin, representing

Table 3. Carotenoid Contents of Milk-Based Fruit Beverages (Total and Bioaccessibility)[a]

	neoxanthin + 9-*cis*-violaxanthin			zeaxanthin			lutein		
	JW	JS	JSy	JW	JS	JSy	JW	JS	JSy
	Total (Micrograms per 100 mL of Fruit Beverage)								
C_a	47.34 ± 0.3 a	46.89 ± 7.6 a	18.46 ± 3.4 a	14.68 ± 1.6 a	10.71 ± 0.57 a	4.41 ± 0.9 a	13.93 ± 2.57 a	7.31 ± 1.4 a	3.68 ± 1.8 a
HPP	94.08 ± 6.7 b	41.38 ± 6.7 a	14.05 ± 1.9 a	22.53 ± 0.8 b	16.97 ± 2.8 b	2.87 ± 0.8 b	24.40 ± 2.0 b	11.59 ± 0.9 b	1.90 ± 0.5 a
C_b	45.13 ± 2.6 c	53.03 ± 7.7 c	21.46 ± 4.8 c	18.97 ± 0.9 c	11.63 ± 1.3 c	5.21 ± 0.1 c	18.85 ± 0.25 c	8.00 ± 2.9 c	7.12 ± 0.8 c
TT	68.92 ± 2.9 d	55.49 ± 7.5 c	8.85 ± 0.6 d	17.82 ± 1.0 c	18.70 ± 3.7 d	2.48 ± 0.2 d	24.08 ± 4.5 d	12.65 ± 3.2 c	1.60 ± 0.3 d
	Bioaccessibility (Percent)								
C_a	25.97 ± 2.1 a	50.08 ± 10 a	35.28 ± 1.8 a	59.62 ± 6.5 a	35.23 ± 6.2 a	71.4 ± 11 a	53.34 ± 3.9 a	44.27 ± 4.0 a	37.86 ± 9.5 a
HPP	94.56 ± 3.9 b	46.65 ± 2.9 a	56.34 ± 0.9 b	30.19 ± 2.4 b	18.63 ± 0.7 b	100.5 ± 3.7 b	22.37 ± 2.5 b	18.89 ± 1.0 b	54.77 ± 6.1 b
C_b	54.45 ± 3.9 c	44.10 ± 8.4 c	30.45 ± 0.8 c	71.18 ± 8.4 c	32.59 ± 7.4 c	62.31 ± 3.6 c	62.01 ± 3.27 c	42.64 ± 9.0 c	35.20 ± 2.5 c
TT	75.54 ± 8.2 d	9.15 ± 1.3 d	12.99 ± 1.7 d	50.28 ± 3.8 d	7.54 ± 1.3 d	29.90 ± 0.5 d	29.83 ± 3.5 d	3.53 ± 1.0 d	67.39 ± 12 d

[a]Data are expressed as the mean ± SD (n = 3). Bioaccessibility = 100 × (bioaccessible content/total content). Statistical analysis consisted of comparison (of each individual carotenoid) of HPP samples and TT samples with their controls (C_a and C_b), respectively, using one-way ANOVA [(a, b) for C_a vs HPP, and (c, d) for C_b vs TT]. Different letters within columns indicate significant differences ($p < 0.05$). C_a, nontreated (control of HPP); HPP, high-pressure processing; C_b, nontreated (control of TT); TT, thermally treated; JW, whole milk−fruit beverages; JS, skimmed milk−fruit beverages; JSy, soy milk−fruit beverages.

38 and 25%, respectively, of the total carotenoid content, followed in descending order by lutein (10%), β-cryptoxanthin (9%), zeaxanthin (9%), and β-carotene (6%) (Tables 3 and 4). Therefore, significant differences are observed in the facility of extraction of the same carotenoid, depending on the type of milk present in the beverage. In this sense, on excluding the main compound formed by the quantification of two xanthophylls (neoxanthin + 9-*cis*-violaxanthin) together, in cow's milk beverages (whole and skimmed milk), most of the extracted carotene was β-carotene, versus zeinoxanthin in soy milk.

HPP and TT applied to JW produced statistically significant increases ($p < 0.05$) of 60 and 40%, respectively, in the amount of total carotenoid extracted, compared with their respective control beverages (JW-C_a and JW-C_b) (Table 2). The same trend was observed in skimmed beverage (JS) with a nonsignificant increase of 10% and a significant ($p < 0.05$) increase of 25% in total carotenoid content after HPP and TT, respectively. We have also observed that HPP and TT in application to soy beverage (JSy) exert an effect opposite to that observed in the other two beverages (JW and JS), with significant ($p < 0.05$) decreases of 33 and 60%, respectively, in total carotenoid content extracted compared with their respective controls (JSy-C_a and JSy-C_b). The three beverages ordered from greatest to least facility of total carotenoid extraction by HPP and TT processing would be as follows: JW > JS ≫ JSy (Table 2).

Most of the individual carotenoids (neoxanthin + 9-*cis*-violaxanthin, zeaxanthin, lutein, and zeinoxanthin) in the three beverages studied (JW, JS, and JSy) showed behaviors similar to that of the total carotenoid content, except for β-carotene and β-cryptoxanthin (Tables 3 and 4). Initial β-carotene and β-cryptoxanthin concentrations in controls of the three beverages remained practically unchanged as a consequence of HPP and TT, respectively. The literature offers many results showing greater facility of carotenoid extraction as a consequence of processing (mechanical, TT, HPP) that produces cell membrane disruption of plant products (orange juice, tomato puree, persimmon puree, persimmon slices, vegetables soups, etc.) and a better release of these compounds.[6,10,13,15,29,37] Also well-known is the capacity of carotenoids to bind to proteins, forming carotenoid−protein complexes that reduce the facility of carotenoid extraction. The extraction facility of β-carotene and β-cryptoxanthin from JW, JS, and JSy beverages caused by HPP and TT could be minimized by the presence of milk and soy proteins, which could form protein−β-carotene and protein−β-cryptoxanthin complexes more easily than with the other more hydrophilic carotenoids.[38] On the other hand, the balance between improved extractions as a consequence of cell disruption and the formation of carotenoid−protein complexes can be compensated due to this latter process in the case of soy beverages, for which the amount of carotenoids extracted after HPP and TT processing decreases significantly.

Considering the processing factor, the results obtained show that the treatment applied (HPP and TT) exerted no significant influence on the majority of the individual carotenoids and on total carotenoid content (Figures 2A, 3A, and 4A, Supporting Information). On the other hand, taking into account the food matrix factor, significant differences ($p < 0.05$) were found between whole and skimmed milk beverages (JW and JS) versus soy beverage (JSy) for zeaxanthin, zeinoxanthin, and total carotenoid contents (Figures 2A, 3A, and 4A, Supporting Information).

Carotenoid Bioaccessibility and Processing Effects. Results referred to individual and total carotenoid bioaccessibility (percent) in the 12 different milk-based fruit beverages are shown in Tables 2, 3, and 4. In general, a significant decrease in total carotenoid bioaccessibility was observed in HPP and TT beverages compared to control beverages (C_a and C_b).

When samples were compared according to food matrix, whole milk−fruit beverages (JW) showed the highest bioaccessibility values (percent) ($p < 0.05$) for β-carotene, β-cryptoxanthin, zeinoxanthin, lutein, and total carotenoid content, with values of 130, 116, 99, 60, and 95%, respectively. The highest values for zeaxanthin (67%) and 9-*cis*-violaxanthin + neoxanthin (47%) bioaccessibility corresponded to soy (JSy) and skimmed milk (JS) beverages, respectively. Under the in vitro gastrointestinal conditions employed, the observed carotenoid bioaccessibility ranging from 17 to 148% is consistent with data found in similar in vitro studies involving different vegetable products.[16,21] Moreover, the bioavailability found for lutein (43%), zeaxanthin (33%), β-cryptoxanthin (54%), and β-carotene (58%) in skimmed milk beverages is comparable to that reported by Granado-Lorencio et al.[16] in similar beverages.

Table 4. Carotenoid Contents of Milk-Based Fruit Beverages (Total and Bioaccessibility)[a]

	zeinoxanthin			β-cryptoxanthin			β-carotene		
	JW	JS	JSy	JW	JS	JSy	JW	JS	JSy
Total (Micrograms per 100 mL Fruit Beverage)									
C_a	19.36 ± 1.5 a	18.70 ± 2.8 a	13.99 ± 0.9 a	18.82 ± 4.1 a	14.68 ± 1.8 a	5.92 ± 1.1 a	24.95 ± 2.9 a	26.64 ± 2.6 a	3.21 ± 0.01 a
HPP	28.62 ± 0.7 b	27.69 ± 1.9 b	5.75 ± 0.7 b	19.72 ± 3.6 a	17.46 ± 1.2 a	5.45 ± 0.3 a	26.49 ± 3.7 a	24.43 ± 1.6 a	3.23 ± 0.52 a
C_b	22.26 ± 1.7 c	18.64 ± 2.2 c	13.18 ± 0.2 c	14.31 ± 2.3 c	12.14 ± 0.7 c	5.67 ± 1.1 c	19.05 ± 0.9 c	28.88 ± 0.2 c	3.24 ± 0.07 c
TT	35.56 ± 0.9 d	23.38 ± 0.7 d	4.99 ± 0.8 d	15.73 ± 2.5 c	26.13 ± 6.8 d	4.83 ± 0.3 c	21.03 ± 0.9 c	26.64 ± 2.6 c	2.42 ± 0.06 d
Bioaccessibility (Percent)									
C_a	104.14 ± 6.7 a	27.91 ± 3.9 a	33.63 ± 1.9 a	105.75 ± 6.7 a	53.29 ± 7.7 a	36.99 ± 11.9 a	113.89 ± 6.5 a	52.94 ± 9.8 a	42.40 ± 4.12 a
HPP	43.79 ± 3.5 b	17.56 ± 1.2 b	49.47 ± 6.1 b	48.90 ± 7.2 b	42.16 ± 3.5 b	77.75 ± 0.3 b	96.23 ± 2.3 b	41.56 ± 4.5 b	86.39 ± 4.8 b
C_b	95.02 ± 3.7 c	17.32 ± 0.4 c	33.32 ± 2.2 c	128.18 ± 4.6 c	55.58 ± 7.7 c	28.97 ± 2.7 c	148.58 ± 8.1 c	63.48 ± 3.1 c	38.83 ± 0.5 c
TT	33.32 ± 5.7 d	10.15 ± 0.9 d	3.76 ± 0.1 d	89.12 ± 4.6 d	11.61 ± 0.9 d	3.54 ± 0.4 d	88.58 ± 8.1 d	29.73 ± 4.2 d	13.38 ± 1.3 d

[a]Data are expressed as the mean ± SD (n = 3). Bioaccessibility = 100 × (bioaccessible content/total content). Statistical analysis consisted of comparison (of each individual carotenoid) of HPP samples and TT samples with their controls (C_a and C_b), respectively, using one-way ANOVA [(a, b) for C_a vs HPP, and (c, d) for C_b vs TT]. Different letters within columns indicate significant differences (p < 0.05). C_a, nontreated (control of HPP); HPP, high-pressure processing; C_b, nontreated (control of TT); TT, thermally treated; JW, whole milk−fruit beverages; JS, skimmed milk−fruit beverages; JSy, soy milk−fruit beverages.

Considering food matrix and processing, JS-HPP and JW-TT showed the smallest decrease in total carotenoid bioaccessibility (p < 0.05) (20 and 48%, respectively) compared to control beverages (C_a and C_b) (Table 2). On the other hand, the largest decreases in total carotenoid bioaccessibility (p < 0.05) (58 and 71%) were found in JW-HPP and JS-TT, respectively. Generally the same trend was observed for each of the individual carotenoids. Thus, the bioaccessibility of zeaxanthin, lutein, zeinoxanthin, and β-cryptoxanthin in HPP and TT beverages suffered a significant decrease (p < 0.05), with average values of 50 and 35% in whole and skimmed milk beverages, respectively, compared to their respective control beverages (Tables 3 and 4). The lowest reductions in bioaccessibility values (16 and 20%) as a consequence of processing were found in β-carotene in JW-HPP and JS-HPP beverages, respectively. In addition, the only carotenoid compound showing increased bioaccessibility as a consequence of HPP was 9-*cis*-violaxanthin + neoxanthin in whole milk beverages (JW), with a significant increase from 26% in JW-C_a beverage to 94% in HPP-JW beverage.

Surprisingly, the bioaccessibility of total carotenoid in HPP soy beverage (JSy-HPP), with a value of 73%, was significantly higher than that found in the untreated JSy-Ca (40%) (Table 2). Furthermore, a significant increase (p < 0.05), with average values of 48% (neoxanthin + 9-*cis*-violaxanthin, zeaxanthin, lutein, and zeinoxanthin) and 107% (β-carotene and β-cryptoxanthin) was observed for the bioaccessibility of the individual carotenoids in HPP soy beverages (Tables 3 and 4). In TT beverages, we recorded average decreases in bioaccessibility of 42, 64, and 70% for individual carotenoids in whole, soy, and skimmed milk beverages, respectively. It is noteworthy that when compared with the controls, the samples that increased total carotenoid extracted as a consequence of processing, such as JW-HPP (60%) and JW-TT (40%), could also suffer a significant decrease in their bioaccessibility (58 and 48%, respectively). Thus, a higher amount of total carotenoid content in the fruit juice beverage as a consequence of processing does not ensure an increase in the bioaccessibility of these compounds.

Despite the decrease in bioaccessibility as a consequence of processing (HPP and TT) compared to untreated samples, the interactions of the two-way ANOVA (Figures 2B, 3B, and 4B, Supporting Information) indicated that HPP confers higher total carotenoid bioaccessibility (p < 0.05) than TT in JS and JSy and a very similar bioaccessibility for JW-HPP and JW-TT. In general, the same behavior was found in relation to the bioaccessibility of each of the individual carotenoids.

The significant decrease in carotenoid bioaccessibility in the HPP and TT milk beverages compared to the controls found in our study indicates that the food-processing effects on the bioaccessibility of carotenoids are more complex than the positive effects that might be expected in relation to food processing (mechanical or thermal processing), which induces plant cell disruption with a greater release of very lipophilic carotenoids (lycopene and β-carotene) than of more hydrophilic carotenoids such as lutein.[15]

Ascorbic Acid Content and Bioaccessibility. Results referred to the ascorbic acid (AA) [total and bioaccessibility (%)] contents in 12 different beverages are shown in Table 5.

Ascorbic Acid Total Content and Processing Effects. The ascorbic acid contents in control samples (C_a and C_b) are consistent with the percentage of fruit juice in the beverages.

Table 5. Ascorbic Acid Contents of Milk- and Soy-Based Fruit Beverages (Total and Bioaccessibility)[a]

	ascorbic acid		
	JW	JS	JSy
Total (Micrograms per 100 mL of Fruit Beverage)			
C_a	44.94 ± 0.46 a	40.54 ± 2.10 a	25.94 ± 0.33 a
HPP	44.05 ± 0.46 a	36.20 ± 1.74 b	22.92 ± 1.20 b
C_b	40.90 ± 1.22 c	41.05 ± 0.88 c	31.85 ± 0.67 c
TT	34.03 ± 1.78 d	36.46 ± 0.82 d	25.70 ± 0.57 d
Bioaccessibility (Percent)			
C_a	70.19 ± 1.19 a	62.41 ± 5.6 a	12.58 ± 1.0 a
HPP	61.07 ± 0.88 b	57.11 ± 2.5 a	7.20 ± 0.43 b
C_b	68.57 ± 3.00 c	52.98 ± 1.3 c	39.92 ± 0.55 c
TT	97.60 ± 7.40 d	59.93 ± 6.7 d	60.17 ± 1.10 d

[a]Data are expressed as the mean ± SD (n = 3). Bioaccessibility = 100 × (bioaccessible content/total content). Statistical analysis consisted of comparison of ascorbic acid content of HPP samples and TT samples with their controls (C_a and C_b), respectively, using one-way ANOVA [(a, b) for C_a vs HPP, and (c, d) for C_b vs TT]. Different letters within columns indicate significant differences ($p < 0.05$). C_a, nontreated (control of HPP); HPP, high-pressure processing; C_b, nontreated (control of TT); TT, thermally treated; JW, whole milk–fruit beverages; JS, skimmed milk–fruit beverages; JSy, soy milk–fruit beverages.

These values are similar to previous results reported for commercial milk-based fruit juice beverages.[36]

High-pressure treatment produced a similar statistically significant decrease ($p < 0.05$) of 11% in AA content extracted from JS and JSy beverages, whetrsd HPP did not modify the initial AA content in JW beverages (Table 5). Thermal treatment produced a significant reduction ($p < 0.05$) in AA concentration in the three food matrices assayed. These results are in agreement with those obtained previously by our group, whereby ascorbic acid is maintained in the pressurized orange juice at levels similar to those found in freshly squeezed juice, with better preservation in HPP products than in TT products.[10,30] The three food matrices ordered from least to most AA resistance to processing (HPP and TT) would be as follows: JSy < JS < JW.

Considering treatments, the AA content was higher ($p < 0.05$) in HPP samples versus TT samples only for JW (Figure 5A, Supporting Information). In the case of food matrix, significant differences ($p < 0.05$) were also found between JSy and JW and between JSy and JS, but no differences were observed between JW and JS (Table 5).

Ascorbic Acid Bioaccessibility and Processing Effects. Whole milk beverages (JW) showed the highest bioaccessibility values for ascorbic acid, followed by skimmed beverages (JS) and soy beverages (JSy) (Table 5). These percentage bioaccessibility values are a consequence of a statistically significant decrease ($p < 0.05$) in ascorbic acid content in the bioaccessible fraction with respect to the original beverages, which were within the range found for a similar bioaccessible fraction from a beverage of combined fruit juice, skimmed milk, zinc, and iron (16.3–56%), although the authors found no differences between the beverages with or without the addition of skimmed milk.[18] However, AA bioaccessibility was higher than for pomegranate juice (>80% decrease) employing a similar in vitro gastrointestinal digestion process, but applying dialysis instead of centrifugation, a fact that could explain the greater decrease in AA bioaccessibility compared with the present study.[17]

The significant decrease in AA content in the bioaccessible fraction compared to that found in the original beverage has been explained in the literature as a consequence of important ascorbic acid degradation during the in vitro digestion process, due to pH changes and the presence of oxygen.[17,18] Milk combined with fruit juice produces an emulsion in the aqueous phase that could reduce the amount of oxygen in contact with ascorbic acid and thus prevent its oxidation; this in turn could play a significant role in increasing ascorbic acid bioaccessibility in the whole milk–fruit juice beverage (JW).

With regard to the treatments assayed, a significant decrease ($p < 0.05$) was observed in the bioaccessibility of AA in HPP-treated samples with respect to their controls. On the other hand, TT beverages showed an increase ($p < 0.05$) in AA bioaccessibility compared with their controls (Table 5). Statistical analysis showed that the treatment applied and the food matrix exerted a significant influence ($p < 0.05$) upon the bioaccessibility of ascorbic acid (Figure 5B, Supporting Information). In general, AA bioaccessibility was higher in TT samples than in HPP JW and JSy beverages. In addition, the lowest AA bioaccessibility corresponded to JSy-HPP.

It is well-known that HPP and TT can affect the properties of whey proteins from milk, with the formation of aggregates.[39,40] However, in the present study we do not know which treatment affected the formation of such aggregates most. From the results obtained, it can be speculated that HPP treatment could better facilitate the formation of aggregates than TT, leading to an increase in viscosity and making the ascorbic acid molecules less accessible in HPP than in TT beverages in the in vitro gastrointestinal model.

In conclusion, the effect of processing upon the bioaccessibility of phytochemicals in a milk-based fruit juice beverage seems to be very dependent on the type of compounds (tocopherols, carotenoids, and ascorbic acid), the milk employed (whole, skimmed, and soy), and the processing involved (HPP and TT). With regard to the food matrix, we noted that α-tocopherol and ascorbic acid bioaccessibility were highest in fruit beverages containing whole milk and lowest in soy milk-based fruit beverages, whereas no significant differences ($p < 0.05$) were found among the three beverages (JW, JS, and JSy) in terms of carotenoid bioaccessibility. In addition, samples subjected to high-pressure processing showed higher tocopherol and carotenoid bioaccessibilities than pasteurized (TT) samples, thus indicating that this kind of processing technology combined with a milk matrix positively modulates the bioaccessibility of certain types of bioactive components in food, mainly those of a lipophilic nature. This fact can define this technique as an alternative to traditional heat processing in the development and manufacture of functional foods with implemented health-related benefits. The in vitro method used in our study measures only bioaccessibility (a prior and necessary step for further absorption). However, it is a useful tool that may allow comparisons among different foods according to their relative bioavailabilities. Further studies in humans are needed to confirm whether this model is able to predict the effect of food matrix and processing upon the bioavailability of ascorbic acid, carotenoids, and tocopherols from different food sources.

■ ASSOCIATED CONTENT

Supporting Information

Five figures representing the interaction of the two factors of two-way ANOVA (food matrix and processing) for tocopherols, carotenoids, and ascorbic acid (data transformed) (A) and bioaccessibility (data transformed) (B). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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