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Changes in Vitamin C, Phenolic, and Carotenoid Profiles Throughout in Vitro Gastrointestinal Digestion of a Blended Fruit Juice

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ABSTRACT: The aim of this research was to evaluate the influence of an in vitro gastrointestinal digestion on the stability and bioaccessibility of vitamin C, phenolic compounds, and carotenoids, as well as the antioxidant activity in a blended fruit juice (BFJ) containing orange, pineapple, and kiwi. Vitamin C and most of the analyzed phenolic compounds were quite stable under gastric conditions (recovery > 75%), whereas carotenoids diminished significantly (to 64%). The concentration of all the evaluated compounds decreased during small intestinal digestion. The bioaccessibility of hydrophilic constituents was higher than that of lipophilic constituents. Flavonoids, vitamin C, and phenolic acids showed bioaccessibilities of 20.1, 15.0, and 12.7%, respectively. However, carotenes and xanthophylls were around 7.6 and 17.4% available for absorption. Despite the decrease in the concentration of these bioactive compounds after being subjected to an in vitro gastrointestinal digestion, results suggest that BFJ is an important source of bioaccessible constituents.

KEYWORDS: *blended fruit juice, bioactive compounds, in vitro gastrointestinal digestion, bioaccessibility, vitamin C, phenolic compounds, carotenoids, antioxidant activity*

INTRODUCTION

Fruit and vegetable consumption plays an important role in improving human health. The World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) recommend eating at least 400 g of fruits and vegetables per day (the "5 a day" program) for the prevention of chronic diseases such as heart disease, cancer, diabetes, and obesity.[1] However, fruit and vegetable intake per day in Spain, among other countries, is below the dietary recommendations at this time.[2]

Consumption of fruit juices has become a dietary concern worldwide. Fruit juices retain the physicochemical and organoleptical characteristics of fruits from which they are produced; therefore, their intake also should contribute to maintain health. Health benefits of fruit juices are attributed to a large number of compounds with biological activity present in these foods. These biological functions include radical scavenging activity, protecting proteins, lipids, and DNA from oxidative damage.[3] The major bioactive antioxidant compounds of fruit and vegetables juices are vitamin C and phenolic compounds,[4] as well as carotenoids. The intake of vitamin C reduces the risk of several cardiovascular and neurodegenerative diseases, among others.[5] The main biological functions of phenolic compounds are preventing some cancer types and cardiovascular and inflammatory diseases, and carotenoids avoid age-related macular degeneration.[6,7] For these reasons, the potential market of fruit juices is currently growing, and new fruit-derived products have been designed. Among the new products, blended fruit juices (BFJ) stand out to enhance the sensorial and nutritional characteristics of these products. Mixing different fruit juices provides increased concentrations of selected bioactive compounds, adds new nutrients, or improves flavor and appearance. Besides, it has been reported that absorption of bioactive compounds in fruit juices exceeds that after consumption of intact fruits.[4] Therefore, the bioavailability of these substances could be also enhanced through BFJ.

Many bioactive compounds must be released from the food matrix to exert their biological effects. In this sense, in vitro methodologies have been developed as a simple and fast approach to in vivo trials because the latter are expensive long-term studies with high variability between subjects.[8] In general, in vitro gastrointestinal digestion is useful in assessing the bioaccessibility of compounds with biological activity from food. This method followed by a dialysis has been mainly applied to study the bioaccessibility of food micronutrients (iron, calcium, zinc, copper, and manganese, among others).[9−12] The whole premise of dialyzability methods is that dialyzable compounds will be available for absorption.[10] In fact, iron dialyzability data have been reported to agree reasonably well with human absorption.[13] This could be one of the reasons why the application of gastric digestion with dialysis has been extended to other food constituents, including vitamin C, phenolic compounds, and isoflavones.[14−18]

It is known that not all of the quantity of food bioactive phytochemicals is able to be absorbed by the gastrointestinal tract. Information related to the concentration of substances with biological activity in individual fruit juices is available in the literature. However, studies in relation to the bioaccessibility of different bioactive compounds contained in BFJ are scarce.[19,20] Therefore, the aim of this research was to assess the changes in the concentration of vitamin C, phenolic compounds, and carotenoids, as well as the antioxidant activity,

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during an in vitro gastrointestinal digestion of a BFJ and to determine their bioaccessibility.

MATERIALS AND METHODS

Materials. A BFJ was manufactured by mixing three different fruits juices (orange, kiwi, and pineapple). Fruits were purchased at commercial maturity in a local supermarket. Each fruit was washed and peeled and the juice extracted. Afterward, every juice was filtered with a cheesecloth using a vacuum pump. Finally, the juices were combined in the following proportions: orange, 50%; kiwi, 36%; and pineapple, 14%. Fruit juice formulation was selected according to previous studies, in which the blend of these fruit juices showed high vitamin C and phenolic concentrations, as well as antioxidant activity.[21] The pH of BFJ was 3.40 ± 0.04 (Crison Instruments SA, Alella, Barcelona, Spain). The soluble solid content was measured in a Comecta refractometer (Abrera, Barcelona, Spain), being 10.75 ± 0.01 °Brix.

Pepsin from porcine stomach, pancreatin from porcine pancreas, bovine bile, DL-1,4-dithiothreitol (DTT), metaphosphoric acid, phenol standards (caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic acids; hesperidin, naringenin, rutin, quercetin, and (+)-catechin), carotenoid standards (*α*-carotene, *β*-carotene, zeaxanthin, lutein, *α*-cryptoxanthin, and *β*-cryptoxanthin), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and cellulose dialysis membrane (molecular weight cutoff of 12000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid, gallic acid, and Folin−Ciocalteu (F−C) reagent were acquired from Scharlau Chemie S.A. (Barcelona, Spain). Ultrafiltration devices (Amicon Ultra 3 K) and 0.2 μm membranes were bought from Millipore Iberica S.A. (Madrid, Spain).

In Vitro Gastrointestinal Digestion. The in vitro gastrointestinal digestion was carried out following the methodology described by Rodríguez-Roque et al.,[17] which consisted of two sequential phases: gastric and small intestinal digestion (including dialysis). In addition, a micellar fraction was prepared from digesta following the completion of the small intestinal phase.

Gastric Digestion. BFJ (200 mL) and pepsin (0.2 g) were mixed 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 and 90 rpm during 2 h (incubation chamber with orbital agitation Ovan, Badalona, Spain).

Small Intestinal Digestion with Dialysis Membrane. Segments of dialysis membrane were cut at 12 cm of length and filled with 25 mL of water/$NaHCO_3$ (0.5 N) mixture. The required amount of $NaHCO_3$ (0.5 N) to titrate the gastric digesta to pH 7.5 was that contained in the dialysis membrane. Every 20 mL of gastric digesta was placed into a polyethylene tube, and a dialysis membrane was completely immersed until a pH of 5.0 was reached. Later, 5 mL of pancreatin (4 g/L) and bile (25 g/L) mixture was added to each tube and incubated during 2 h at 37 °C and 90 rpm (incubation chamber with orbital agitation Ovan). Finally, the dialysis membrane was removed, rinsed with distilled water, and weighed. Bioactive compounds of small intestinal digesta corresponded to those obtained outside the dialysis membrane, and bioactive compounds that could be available for absorption were inside the dialysis membrane (bioaccessible fraction).

Micellar Fraction. Some lipophilic constituents, such as carotenoids, are usually micellarized before being absorbed, packed into chylomicrons, and secreted to the lymphatic system. To quantify the amount of carotenoids transferred to the aqueous-micellar fraction, a portion of small intestinal digesta (30 mL) was centrifuged at 5000 rpm during 20 min at room temperature.[22]

To monitor the changes in bioactive compound concentration during the in vitro gastrointestinal digestion of BFJ, aliquots were taken at the end of each digestive phase and immediately placed in a cold water bath during 10 min and frozen (−45 °C) until analysis.

Bioactive Compound Analysis. *Vitamin C.* Vitamin C extraction was performed through the method validated by Odriozola-Serrano et al.,[23] with some modifications. A sample of 5 mL of nondigested or digested BFJ was mixed with 5 mL of a solution containing 45 g/L metaphosphoric acid and 7.2 g/L DTT. Then, samples were filtered in 0.20 μm membrane. The filtrate was added into the ultrafiltration devices and centrifuged at 6000 rpm during 30 min at 4 °C to purify samples. The recovery of vitamin C after ultrafiltration was between 70 and 80%.

An aliquot of 20 μL was injected into the HPLC system consisting of a 600 controller, a 486 absorbance detector, and a reverse-phase C18 Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm × 250 cm) (Waters Corp., Milford, MA, USA). A 0.01% sulfuric acid solution adjusted to pH 2.6 was used as mobile phase, and the flow rate was fixed to 1 mL/min at room temperature. Detection was performed at 245 nm. Vitamin C identification was carried out by comparing the retention time and UV−visible absorption spectrum of samples with the standard (ascorbic acid). Results were expressed as milligrams of ascorbic acid per 100 mL of sample.

Phenolic Compounds. (a) Phenolic Compounds Determined by HPLC. The phenolic profile was analyzed using the methodology reported by Odriozola-Serrano et al.[24] Briefly, a portion of 5 mL of nondigested or digested BFJ was mixed with 5 mL of 62% aqueous methanol and 1 mL of 6 M HCl. The mixture was refluxed at 90 °C for 2 h, cooled, and diluted to 25 mL with methanol. Finally, the extracts were sonicated for 3 min, filtered (0.20 μm membrane), and frozen (−45 °C) until HPLC 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. Separations were performed on a reverse-phase C18 Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm × 250 mm) operating at room temperature with a flow rate of 1 mL/min. A gradient elution was employed with a mixture of two solvents: (A) 2.5% of acetic acid in water and (B) 2.5% of acetic acid in methanol as described by Odriozola-Serrano et al.[24] Individual phenols were identified by comparison of their retention times and spectra to those of the standards. Quantification of individual phenols was carried out by integrating the peak areas and using calibration curves. Results were expressed as milligrams of each phenolic compound per 100 mL of sample. Total phenolic compounds (TPC) were calculated as the sum of individuals.

(b) Total Phenolic Content Determined by Folin−Ciocalteu (F−C) Methodology. Total phenolic content was evaluated following the methodology reported by Singleton et al.,[25] with some modifications. A portion of 0.5 mL of nondigested or digested BFJ was mixed with 0.5 mL of F−C reagent and 10 mL of Na_2CO_3 (20%). The mixture was made up to 25 mL with distilled water and kept in the dark during 1 h at room temperature. Later, the samples were filtered using a 0.2 μm membrane, and the absorbance was measured at 725 nm (Cecil Instruments Ltd., Cambridge, UK). Concentrations were determined by comparing the sample's absorbance with a calibration curve built with gallic acid. Results were expressed as milligrams of gallic acid per 100 mL of sample.

Carotenoids. Carotenoids were extracted and quantified by HPLC following the procedure reported by Morales-de La Peña et al.,[26] with some modifications. Nondigested or digested BFJ (6 mL) was 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. All of 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

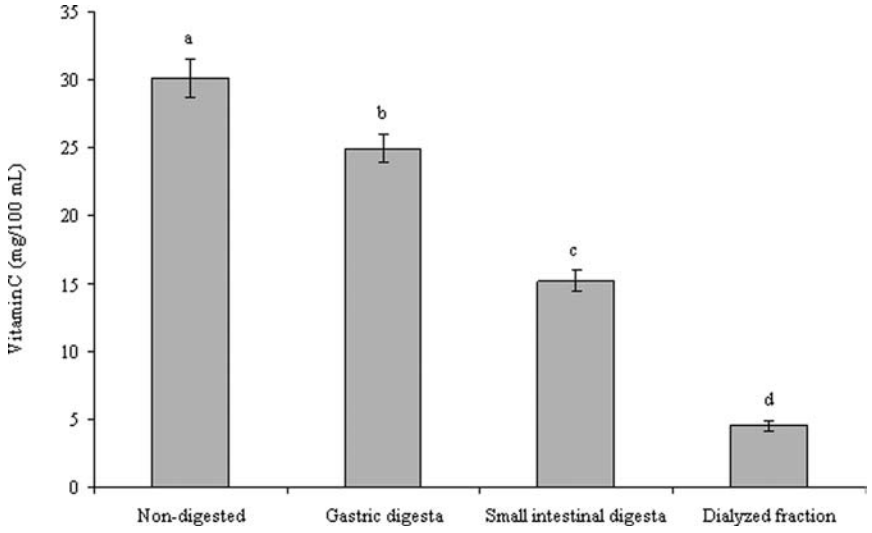

Figure 1. Vitamin C concentration during in vitro gastrointestinal digestion of a blended fruit juice. Different lower case letters indicate significant differences ($p < 0.05$).

amber glass vial. Finally, the solvent was evaporated under N_2 atmosphere and stored at (−45 °C) until analysis.

Carotenoids were determined in the same HPLC equipment used to assess phenolic compounds. The diode array detector 2996 (Waters Corp.) 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.[26] 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 according to the retention time and spectrum of standards, as well as by comparison of those reported in the literature. Carotenoid quantification was carried out by integrating the peak areas and using calibration curves. Results were expressed as micrograms of carotenoid compound per 100 mL of sample.

Hydrophilic and Lipophilic Antioxidant Activity. Extraction of hydrophilic and lipophilic fractions of nondigested or digested BFJ was performed using the colorimetric method described by Brand-Williams et al.,[27] as reported by Rodríguez-Roque et al.[17] Briefly, 5 mL of sample and 10 mL of methanol were mixed and centrifuged at 6000 rpm for 20 min at 4 °C. The supernatant was considered as the hydrophilic fraction, whereas the residue was mixed with 10 mL of tetrahydrofuran and centrifuged in the same conditions described above. The second supernatant was considered as the lipophilic fraction. The antioxidant activity was studied by evaluating the free radical scavenging effect of extracts on DPPH radical. Aliquots of 0.2 mL of hydrophilic or lipophilic extracts were mixed with 3.8 mL of DPPH methanolic solution (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 methanol. Results were expressed as percentage of DPPH inhibition, which can be related to the decrease in absorbance with respect to the control (methanolic solution of DPPH radical without extract).

Bioaccessibility Calculations. Bioaccessibility was expressed as percentage and determined using eq 1

$$\text{bioaccessibility}\,(\%) = \left(\frac{\text{BC}_{\text{dialyzed/micellar}}}{\text{BC}_{\text{nondigested}}}\right) \times 100 \quad (1)$$

where $\text{BC}_{\text{dialyzed/micellar}}$ corresponds to the bioactive compound concentration in the dialyzed (for vitamin C and phenolic compounds) or micellar fractions (for carotenoids) and $\text{BC}_{\text{nondigested}}$ is the concentration in nondigested BFJ.

Statistical Analysis. The in vitro gastrointestinal digestion of BFJ was carried out twice. Each studied parameter was analyzed three times in every in vitro gastrointestinal digestion ($n = 6$). Results were reported as the mean ± standard deviation. Analysis of variance (ANOVA) of the results was performed to determine significant differences ($p < 0.05$) between the concentration of bioactive compounds in nondigested BFJ and that obtained in each digestive phase (Statgraphics Plus v.5.1, Rockville, MD, USA).

RESULTS AND DISCUSSION

Vitamin C. Vitamin C concentration of nondigested BFJ was 30.1 mg ascorbic acid/100 mL. The recommended nutrient intake (RNI) for vitamin C ranges between 40 and 45 mg per day according to the FAO/WHO.[28] Therefore, the daily requirement of vitamin C is achieved by drinking 150 mL of the BFJ containing orange, pineapple, and kiwi.

The vitamin C concentration was reduced 17% in the gastric digesta, with regard to the content in nondigested BFJ (Figure 1). The acidic conditions of the gastric environment could protect the vitamin C against its chemical or enzymatic oxidation. This hypothesis is supported by Ball,[29] who reported that ascorbic acid was slowly attacked by oxygen when this molecule was fully protonated at low pH. Moreover, other authors demonstrated that gastric digestion had little effect on vitamin C stability, recovering 93% of this bioactive compound in broccoli inflorescences and 71% in pomegranate juice.[16,18]

On the other hand, the vitamin C concentration decreased 39% in the small intestinal digesta with regard to gastric digesta of BFJ. These results showed that vitamin C was unstable under intestinal conditions. The alkaline pH and some factors inherent to in vitro gastrointestinal digestion, such as temperature, oxygen, light, and the enzyme activity, could enhance the vitamin C oxidation or complex formation with other constituents. In this sense, Jeney-Nagymate and Fodor[30] observed that the concentration of ascorbic acid decayed when the pH was >4. In addition, Ball[29] reported that vitamin C oxidation in the gastrointestinal tract occurs due to its pro-oxidant behavior, maintaining the reduced state of other nutrients such as iron. This author also related the vitamin C degradation to the formation of metal−oxygen−ascorbate complexes.[29]

The vitamin C contained in the BFJ displayed a bioaccessibility of 15.0%. As far as we know, few papers have studied the in vitro bioaccessibility of vitamin C from food.

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Table 1. Concentration of Phenolic Compounds during in Vitro Gastrointestinal Digestion of a Blended Fruit Juice[a]

	concentration (mg/100 mL)				
phenolic compound	nondigested	gastric digesta	small intestinal digesta	dialyzed fraction	bioaccessibility (%)
phenolic acids					
hydroxycinnamic acids					
caffeic acid	0.332 ± 0.018a	0.337 ± 0.015a	0.267 ± 0.007b	nd	0.00A
chlorogenic acid	3.00 ± 0.10a	3.279 ± 0.016b	2.97 ± 0.08a	0.329 ± 0.009c	11.0 ± 0.5B
p-coumaric	1.06 ± 0.04a	1.204 ± 0.014b	1.007 ± 0.018c	0.176 ± 0.007d	16.7 ± 0.7C
ferulic	0.81 ± 0.05a	0.452 ± 0.006b	0.293 ± 0.013c	0.211 ± 0.009d	25.97 ± 1.6D
sinapic	1.30 ± 0.03a	0.470 ± 0.015b	0.411 ± 0.016c	0.229 ± 0.013d	17.7 ± 1.0E
total	6.49 ± 0.15a	5.74 ± 0.04b	4.95 ± 0.06c	0.83 ± 0.08d	12.7 ± 1.3F
flavonoids					
flavanones					
hesperidin	12.1 ± 0.4a	11.2 ± 0.3b	8.46 ± 0.06c	2.22 ± 0.10d	18.4 ± 0.7EG
naringenin	7.9 ± 0.3a	16.0 ± 1.0b	7.7 ± 0.6a	1.48 ± 0.03c	18.7 ± 0.7G
flavonols					
rutin	1.34 ± 0.06a	3.51 ± 0.08b	1.71 ± 0.08c	0.297 ± 0.010d	22.2 ± 0.8H
quercetin	0.916 ± 0.008a	0.68 ± 0.03b	0.288 ± 0.014c	0.264 ± 0.012d	28.9 ± 1.5I
flavan-3-ols					
(+)-catechin	7.04 ± 0.05a	6.13 ± 0.07b	3.95 ± 0.04c	1.631 ± 0.018d	23.16 ± 0.13J
total	29.3 ± 0.6a	37.5 ± 0.8b	22.1 ± 0.7c	5.90 ± 0.09d	20.1 ± 0.4K
total phenolic compounds					
sum of individuals	35.8 ± 0.6a	43.3 ± 0.8b	27.1 ± 0.8c	6.73 ± 0.13d	18.8 ± 0.4G
Folin−Ciocalteu method	73.4 ± 1.3a	78.9 ± 1.3b	64.1 ± 1.1c	8.4 ± 0.5d	11.5 ± 0.6B

[a]Values are expressed as the mean ± standard deviation. Lower case letters in the same row show significant differences ($p < 0.05$) among digestive phases. Capital letters within the same column indicate significant differences among the bioaccessibility of phenolic compounds. nd, not detected.

Cilla et al.[19] reported a similar bioaccessibility (12.58%) of this compound in a beverage made with soy milk and fruit juices (orange, pineapple, kiwi, and mango). However, they obtained higher vitamin C bioaccessibility (up to 70.19%) when the juice was blended with whole or skimmed milk. A high vitamin C bioaccessibility (around 44 and 83.7%) has been also reported by other authors in blended fruit juices made with grape, sweet orange, apricot, and peach after 135 days of storage.[20] Differences in these results and those found in the present research could be attributed to the food matrix composition, as well as to the fact that Cilla et al.[19,20] did not use a dialysis membrane. In contrast, the vitamin C bioaccessibility was around 2.5% and about 3.2% in pomegranate juice and broccoli inflorescences, respectively.[16,18] The vitamin C obtained in this research was between 4- and 6-fold more highly bioaccessible than that found in pomegranate juice and broccoli inflorescence when a dialysis membrane was also used. Probably, synergistic interactions between the vitamin C and other constituents were stronger in a BFJ than in food prepared with a single fruit or vegetable. Although the vitamin C concentration is significantly reduced during small intestinal digestion, human intervention studies demonstrated that the intake of orange juice and other vitamin C sources, such as potatoes and vegetables, increased the plasmatic concentration of this compound.[31−33]

Phenolic Profile. Changes in phenolic profile due to in vitro gastrointestinal digestion of BFJ are shown in Table 1, where two main groups (phenolic acids and flavonoids) were identified by HPLC. Total phenolic compounds (TPC) were calculated either as the sum of individuals or by F−C method.

The concentrations of phenolic acids and flavonoids in nondigested BFJ were 6.49 and 29.3 mg/100 mL, respectively. TPC determined by F−C were 2.0-fold higher than TPC calculated by HPLC. It is well-known that F−C reagent could be reduced by other nonphenolic substances, such as sugars, amines, organic acids, proteins, and ascorbic acid, leading to overestimation of the phenolic content in samples.[34]

Phenolic compounds showed different stabilities under gastric conditions. Chlorogenic and *p*-coumaric acids, as well as naringenin and rutin, increased their concentration, whereas ferulic and sinapic acids, hesperidin, quercetin, and (+)-catechin diminished. Caffeic acid remained unchanged in the gastric digesta. These results suggest that the phenolic stability might depend on some factors such as their physicochemical properties and the interaction with dietary or gastric constituents. Additionally, the low pH and the enzyme action of gastric digestion could hydrolyze some phenolic substances bound to proteins and carbohydrates from the food matrix, increasing the concentration of these bioactive compounds.[35] Other studies also support these findings. Rodríguez-Roque et al.[17] and Tagliazucchi et al.[36] showed that gastric digestion improved the phenolic release in soy milk and grapes, respectively. Bermúdez-Soto et al.[37] reported that the concentration of caffeic acid derivatives of chokeberry juice did not change in the gastric digesta, whereas the flavan-3-ols content was reduced a 15%. On the other hand, Laurent et al.[38] and Gil-Izquierdo et al.[39] reported that gastric digestion had no effect on the stability of flavonoids in grape seeds and orange juice, respectively. Differences between these results and those obtained in the present research could be explained by the fact that phenolic constituents may display antagonistic or synergistic interactions among themselves or with other substances, depending on the food matrix.[40]

During small intestinal digestion, the concentration of BFJ phenolic constituents significantly diminished. Flavonoid concentration was more reduced (41%) than that of phenolic acids (14%) in the small intestinal digesta with respect to gastric digesta. The phenolic instability under alkaline pH suggests that these compounds undergo several chemical

Table 2. Concentration of Carotenoids during in Vitro Gastrointestinal Digestion of a Blended Fruit Juice[a]

carotenoid	concentration (μg/100 mL)				bioaccessibility (%)
	nondigested	gastric digesta	small intestinal digesta	dialyzed fraction	
carotenes					
α-carotene	9.2 ± 0.3a	5.8 ± 0.4b	3.4 ± 0.3c	0.85 ± 0.06d	9.3 ± 0.8A
β-carotene	131 ± 4a	65.8 ± 0.7b	30.2 ± 1.4c	13.7 ± 1.6d	10.42 ± 1.04AB
total	140 ± 4a	71.6 ± 0.8b	33.6 ± 1.6c	14.5 ± 1.6d	10.3 ± 1.0AB
xanthophylls					
cis-violaxanthin + neoxanthin	81.8 ± 2.2a	29.8 ± 2.4b	9.17 ± 0.24c	6.20 ± 0.20d	7.6 ± 0.3C
cis-antheraxanthin	141 ± 11a	60 ± 3b	19.7 ± 1.5c	10.6 ± 1.6d	7.5 ± 0.9C
antheraxanthin	30.9 ± 1.6a	17.9 ± 1.1b	8.1 ± 0.3c	4.3 ± 0.5d	13.9 ± 1.6D
lutein	109 ± 3a	97.3 ± 1.5b	63 ± 3c	18.9 ± 0.9d	17.4 ± 1.0E
α-cryptoxanthin	12.2 ± 1.0a	6.45 ± 0.22b	4.45 ± 0.22c	1.75 ± 0.07d	14.4 ± 1.4D
β-cryptoxanthin	25.2 ± 1.5a	14.7 ± 1.2b	8.06 ± 0.24c	3.73 ± 0.23d	14.8 ± 1.4D
total	400 ± 16a	226.6 ± 7b	113 ± 3c	45.5 ± 2.1d	11.4 ± 0.4B
total carotenoids	540 ± 15a	298.2 ± 7b	146 ± 4c	60 ± 3d	11.1 ± 0.4B

[a]Values are expressed as the mean ± standard deviation. Lower case letters in the same row show significant differences ($p < 0.05$) among digestive phases. Capital letters within the same column indicate significant differences among the bioaccessibility of carotenoids.

reactions, mainly oxidation and polymerization, affording the formation of other phenolic derivatives, such as chalcones, which are not available for absorption because of their high molecular weight and low solubility. In fact, it has been reported that alkaline conditions transform 50−60% of flavanones into chalcones.[39] Furthermore, some dietary constituents such as fiber, proteins, and iron reduce the solubility and availability of phenolic compounds.[35,41]

As it can be seen in Table 1, the bioaccessibility of TPC determined as the sum of individuals was 18.8 and 11.5% by F−C assay. Phenolic acids and flavonoids were 12.7 and 20.1% bioaccessible, respectively. Ferulic acid was the most bioaccessible phenolic acid (26.0%) and quercetin the most bioaccessible flavonoid (28.9%). In contrast, caffeic acid did not show bioaccessibility under the conditions assayed in this research. These results demonstrate that phenolic bioaccessibility is widely influenced by pH changes and interactions with other constituents during the gastrointestinal digestion of food. Similar results were displayed by Gil-Izquierdo et al.,[39] who observed a flavanone bioaccessibility between 11 and 36% in orange juice. On the other hand, Vallejo et al.[18] showed that ferulic and sinapic acids derivatives had a low bioaccessibility (8.2 and 1.8%, respectively). In other studies, the bioaccessibility of phenolic compounds was >35%. For instance, Cilla et al.[42] reported that hydroxycinnamic acids, flavanones, flavones, and flavan-3-ols from a fruit beverage made with grape, orange, and apricot were between 36 and 63% bioaccessible. Cilla et al.[20] obseved a bioaccessibility of 90% in TPC determined by F−C in a fruit beverage (containing grape, sweet orange, apricot, and peach) after 135 days of storage. However, Cilla et al.[20,42] obtained the bioaccessible fraction by centrifugation instead of dialysis. This fact, as well the food matrix composition, could explain differences between the results obtained by these authors and that obtained in this research.

Carotenoid Profile. The in vitro gastrointestinal digestion influence on BFJ carotenoid profile is shown in Table 2. The concentrations of carotenes and xanthophylls in nondigested BFJ were 140 and 400 μg/100 mL, respectively.

Carotenes diminished 49% and xanthophylls 43% in the gastric digesta. Lutein was the carotenoid with the highest stability after gastric digestion (90%). However, the recoveries of α- and β-carotenes, α- and β-cryptoxanthines, *cis*-violaxanthin + neoxanthin, *cis*-antheraxanthin, and antheraxanthin ranged from 36 to 63%. Most of the BFJ carotenoids were unstable under gastric conditions perhaps due to oxidation reactions. Carotenoids are known to be unstable in acidic media because they are susceptible to oxidation owing to the numerous double bonds of their chemical structure.[43] Moreover, other factors, such as temperature and pH changes, can produce their oxidation.[44] In line with the results obtained in this research, Rich et al.[45] reported that trans-β-carotene concentration of raw spinach was reduced to 50% at low pH (2.5). Granado-Lorencio et al.[46] also showed that lutein was stable after gastric digestion of orange and kiwi fruits, recovering 80 and 100%, respectively.

The concentration of carotenoids decreased between 31 and 69% when the small intestinal digesta of BFJ was compared to gastric digesta. In this research, carotenoids were not detected in the dialyzed fraction. Likely, their concentration was below the limit of detection due to the fact that the digestion of BFJ was carried out without dietary fat, which enhances the absorption of carotenoids.[47] Similarly, Faulks and Southon[48] showed that when carotenoids were ingested with a meal, these compounds were almost completely absorbed. However, when carotenoids were consumed apart from a meal, their absorption was exceedingly smaller. In addition, intestinal epithelium contain other constituents (that were not present in the dialysis membrane) as membrane receptors (type B residual receptors, cluster of differentiation 36, and Niemann-PickC1-like 1 protein), enzymes (such as carboxyl-ester lipase), and proteins that facilitate the absorption of liposoluble compounds.[49] Carotenoids are highly hydrophobic compounds, which once released from the food matrix are dispersed in the gastrointestinal tract and solubilized in mixed micelles. Therefore, the formation of mixed micelles is one of the critical factors in carotenoid bioavailability.[50] With these findings taken into account, in the present study, the small intestinal digesta was centrifuged (5000 rpm/20 min at room temperature) to obtain the micellar fraction, which was considered to be the bioaccessible carotenoids.[22]

As can be seen in Table 2, the bioaccessibility of carotenoids ranged from 7.6% (*cis*-violaxanthin + neoxanthin and *cis*-anteraxanthin) to 17.4% (lutein). Similar results were reported by Granado-Lorencio et al.,[22] who observed 25% lutein

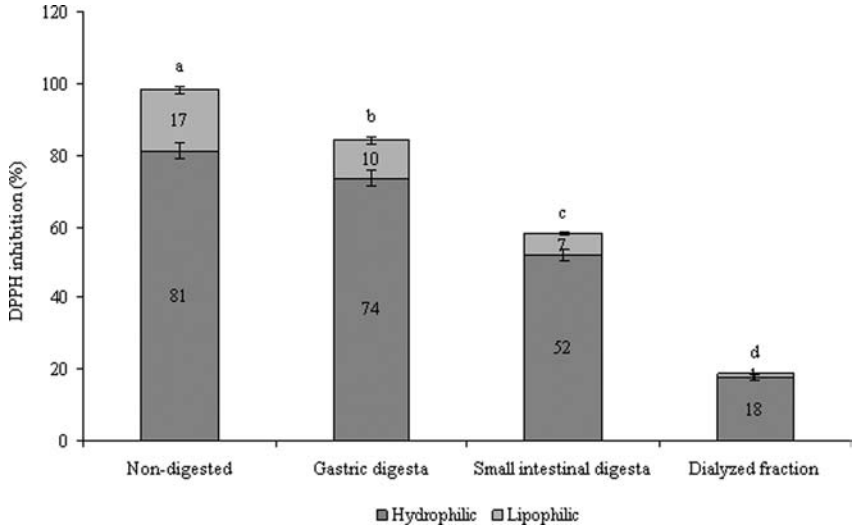

Figure 2. Changes in hydrophilic and lipophilic antioxidant activity during in vitro gastrointestinal digestion of a blended fruit juice. Different lower case letters indicate significant differences ($p < 0.05$) in the total antioxidant activity.

bioaccessibility in orange fruit. Dhuique-Mayer et al.[51] showed that β-cryptoxanthin was between 16 and 40% bioaccessible in different citrus juices (orange, mandarin, and lemon). Daly et al.[7] observed that β-carotene was up to 19% bioaccessible in different herb types, whereas β-cryptoxanthin and lutein + zeaxanthin were up to 27% bioaccessible. Hedrén et al.[43] showed that 3% of β-carotene from raw carrot pieces was bioaccessible. Hedrén et al.[43] also observed an increase of β-carotene bioaccessibility when the raw carrot pieces were made pure, cooked pulp or added oil, highlighting the importance of the particle size and oil presence on carotene bioaccessibility. Moreover, Cilla et al.[19] reported that the food matrix has a significant influence on the bioaccessibility of carotenoids, such as β-carotene, lutein, and β-cryptoxanthin, which displayed higher bioaccessibility when a blended juice was combined with whole milk (up to 148%) than with skimmed or soy milk (up to 63 and 38%, respectively).

In general, xanthophylls contained in the BFJ were more bioaccessible than carotenes. Changes in the molecular structure of carotenoids and a competitive inhibition between themselves could occur, affecting their incorporation into micelles, intestinal uptake, or lymphatic transport.[52] Granado-Lorencio et al.,[22] Nagao,[50] and Chitchumroonchokchai et al.[53] observed the same trend in in vitro carotenoid bioaccessibility: the xanthophylls zeaxanthin and β-cryptoxanthin were more available for absorption than carotenes. In addition, the results obtained in this research are in accordance with those reported in animal and human intervention studies, where the bioaccessibility and absorption rate of carotenoids is low because they interact with macromolecules within the food matrix, such as fiber, which decreases carotenoid absorption by entrapping them and increasing their fecal excretion.[54]

Antioxidant Activity. Total antioxidant activity (TAA) of BFJ was determined as the sum of hydrophilic and lipophilic antioxidant activity (HAA and LAA, respectively). The antioxidant activity in fruit juices depends on the composition and concentration of its antioxidants, such as vitamins, phenols, and carotenoids.[3] Nondigested BFJ displayed 98% DPPH inhibition, of which 81% corresponded to the hydrophilic fraction and 17% to the lipophilic (Figure 2). Ryan and Prescott[55] observed that the antioxidant activities of different fruit juices, including orange and pineapple juices, had a variation between 31 and 85% DPPH inhibition.

The HAA decreased 9% and LAA 38% in the gastric digesta, demonstrating that hydrophilic constituents were less affected by gastric digestion than those lipophilics. Phenolic compounds are among the most important BFJ bioactive compounds with hydrophilic antioxidant activity. These compounds exhibited a high stability under gastric conditions, which could explain the results obtained in this research. Furthermore, a significant correlation between the HAA and the TPC determined by HPLC was observed ($r^2 = 0.8198$, $p = 0.0458$).

The antioxidant activity of both hydrophilic and lipophilic compounds diminished significantly ($p < 0.05$) during the small intestinal digestion of BFJ, for which HAA and LAA were reduced 30 and 37%, respectively, compared to that obtained in the gastric digesta. The greatest losses of antioxidant activity were observed in the dialyzed fraction, where the DPPH inhibition for HAA was 18 and 1% for LAA. These results suggest that most BFJ bioactive compounds with antioxidant activity are unstable under intestinal conditions. The alkaline pH, as well as the digestive enzymes action, could transform these bioactive compounds in other substances with different chemical and physical properties, such as the conversion of flavanones into chalcones or complex formation among the bioactive compounds and other dietary constituents. Moreover, HAA displayed a good correlation with the vitamin C ($r^2 = 0.9819$, $p = 0.0001$) and the phenolic compounds determined as the sum of individuals ($r^2 = 0.9502$, $p = 0.0002$) during the in vitro gastrointestinal digestion. These data related the decrease in the HAA to the loss of both vitamin C and phenolic compounds during digestion of BFJ. A significant correlation was also found between LAA and total carotenoids ($r^2 = 0.9770$, $p = 0.0001$). This could explain the similar behavior between the LAA and the carotenoids during the in vitro gastrointestinal digestion of BFJ, although the contribution of other lipophilic compounds, such as fatty acids and some liposoluble vitamins, is not discarded.

The bioaccessibilities of hydrophilic and lipophilic compounds contained in BFJ were 21.6 and 7.89%, respectively. A similar pattern was reported by Rodríguez-Roque et al.,[17] who observed greater bioaccessibility of hydrophilic than of

lipophilic soy milk constituents. Changes in the food environment, such as heat, pH, additives, and modification of particle size, may alter the functional and structural properties of vitamins,[29] phenolic compounds, and carotenoids. As a result, the bioaccessibility of these compounds could be also influenced by these factors.

Results obtained in this research reveal the amount of bioactive compounds from a blended juice containing orange, pineapple, and kiwi that could be released from the food matrix and could be available for absorption in vivo. Therefore, in vitro methodologies, such as gastrointestinal digestion, allow rapid progress in understanding physicochemical changes, interactions, and bioaccessibility of bioactive compounds.

As can be seen in previous sections, the quantity of ingested compound differs widely from the bioaccessible fraction using this in vitro methodology. If these results could be corroborated by in vivo studies, it would mean that small quantities of bioaccessible compounds could influence cellular activities that modify the risk of several diseases and could be potentially beneficial in improving health.

On the other hand, the assessment of bioaccessibility and bioavailability of compounds with biological activity contained in food might provide more specific information concerning dietary requirements of these constituents to achieve health benefits beyond recommended dietary patterns reported at this time. Consequently, in vivo experimental designs are necessary to understand the biological effects and mechanisms of action of bioactive constituents of food.

In conclusion, most BFJ bioactive compounds were quite stable in the gastric digesta. Only small changes in the concentration of phenolic compounds and vitamin C were observed after acidic conditions. However, the concentration of all the analyzed compounds, as well as the antioxidant activity, diminished significantly during small intestinal digestion. The bioaccessibility of hydrophilic bioactive compounds was higher (vitamin C, 15%; phenolic acids, 13%; and flavonoids, 20%) than that of lipophilics (carotenes, 10%; and xanthophylls, 11%). Results suggest that, despite the significant decrease in the concentration of these bioactive compounds after being subjected to in vitro gastrointestinal digestion, the bioaccessibility of BFJ constituents could be high enough to be absorbed and utilized. The results obtained in this research should be compared with additional in vivo studies to correlate the bioaccessibility of BFJ bioactive compounds between in vivo and in vitro methodologies.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BFJ, blended fruit juice; BHT, butylhydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTT, DL-1,4-dithiothreitol; FAO, Food Agriculture Organization of the United Nations; F−C, Folin−Ciocalteu; HAA, hydrophilic antioxidant activity; HPLC, high-performance liquid chromatography; LAA, lipophilic antioxidant activity; RNI, recommended nutrient intake; TAA, total antioxidant activity; TPC, total phenolic compounds; WHO, World Health Organization

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