

Bioaccessibility and Caco-2 uptake of complex carotenoids profiles from Pitanga (*Eugenia uniflora*) and Buriti (*Mauritia flexuosa*) fruits

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Abstract

Carotenoids are compounds from secondary metabolism of plants which have many essential health benefits for humans. Tropical fruits contain considerable amounts of carotenoids that have antioxidant, anti-inflammatory, provitamin A and anticancer actions, such as β -carotene and lycopene. The fate of complex carotenoid profiles from several fruit matrix still unknown. Pitanga and Buriti are very tasty, colorful and productive fruits from Brazilian biodiversity with potential to increment the nutrition of several populations. These fruits were analyzed for their carotenoid profiles, stability, bioaccessibility and uptake by *in vitro* digestion coupled to Caco-2 cells culture model. Complete profile of carotenoids were obtained by HPLC-DAD allowing to access detailed behavior of 14 carotenoids during the *in vitro* studies. Pitanga showed more complex profile of carotenes and xanthophylls than buriti which had higher concentration of total carotenes. Pitanga carotenoids are statistically less stable than buriti, while xanthophylls were less stable than carotenes. Violaxanthin, lutein and zeaxanthin from pitanga were significantly more bioaccessible than other carotenes excepting *cis*- γ -carotene. Similarly, in buriti, xanthophylls and γ -carotene forms were significantly more bioaccessible than the other carotenoids. The Caco-2 cells experiment showed that an important fraction of xanthophylls are absorbed in their esterified forms, despite β -carotene had the highest uptake. Pitanga presented higher cellular absorption of carotenoids than buriti. This work evidence the essential need to better understand the bioaccessibility of carotenoids in diverse plant food matrix.

1. Introduction

Food and nutritional sciences highlight fruit bioactive compounds' health benefits, especially carotenoids. Moreover, food researchers are elucidating the health-related mechanisms of carotenoids and promoting new ways of its consumption, processing, and innovation [1]. Diverse antioxidant properties in carotenoids have been identified, highlighting the unique capacity to quench singlet oxygen [2]. Furthermore, epidemiological evidence suggests that consuming fruits and vegetables rich in lycopene, α -carotene and β -carotene reduces the risk of cancer, such as breast and prostate tumors [3]. In addition, low carotenoid concentrations were identified in older individuals and patients with cardiovascular disease, besides a widespread carotenoid deficiency in the general population [4]. However, not all carotenoids act the same way and are available for absorption in the human digestive system. Bioaccessibility is dependent on food matrix, intrinsic chemical structure, and carotenoid x carotenoid interactions [5]. Since, bioaccessibility data can help professionals, companies and policymakers to properly guide dietary changes to specific needs, it is essential to understand new sources' carotenoid composition and bioaccessibility.

Although the impressive biodiversity of native fruits of Brazil is recognized as the most diverse in the world [6], [7], information regarding their carotenoid composition and potential health benefits remains limited [8]. Among those native fruits, pitanga (*Eugenia uniflora*) and buriti (*Mauritia flexuosa*) are locally commercialized but can potentially scale up their production and establish a productive chain. For example, it is estimated that 1.3–1.7 tons/year of pitanga is produced in the Brazilian state of

Pernambuco, and 7.2 tons/year of this fruit is commercialized at the São Paulo Terminal Warehouse, the largest supply center for fruits from Latin America [9], [10]. On the other hand, the buriti constitutes the livelihood of many extractivist families from the Brazilian cerrado region, being used from the pulp to the seeds [11].

Pitanga is an edible fruit originating from the Brazilian *restinga* region and other coastal semi-deciduous forests. This fruit has high economic value due to its exotic flavor and high vitamin contents. It is processed into juice, frozen pulp, and nectar that are relatively rich in lycopene and xanthophylls [12]–[15]. Meanwhile, buriti is a palm fruit native from the Amazon forest, grown in the Northeast, Central-west, and Southern-east Brazilian regions. It contains high amounts of polyphenols and carotenoids, particularly all-*trans*- β -carotene [16]–[18]. Besides its bioactive compounds, the buriti also provides the oil with antibacterial activity, which promotes wound healing and makes cosmetics and therapeutic products [19], [20].

Carotenoids' health-promoting benefits primarily depend on carotenoid bioaccessibility, i.e., they must be released from the food matrix and partitioned into bile salt micelles during small intestinal digestion. Once incorporated into micelles, carotenoids are delivered to the brush border membrane of absorptive epithelial cells lining the gut lumen [21]. The extent of carotenoid liberation during the digestion of the plant food matrix is influenced by the compound's physical and chemical characteristics, as well as the chromoplast morphology and pectin methoxylation degree [22], [23]. Moreover, the presence and composition of fats can affect the formation of the mixed micelles and influence the intestinal uptake of carotenoids [24]. On the other hand, the fruits' naturally occurring sugars may also affect the carotenoid's bioaccessibility, as shown in the literature [25]. Accordingly, the micellarization and Caco-2 cells uptake are modulated by the proportion, concentration, and polarity of carotenoids, as well as their interactions with the medium [26], [27].

This research aimed to explore the nutritional potential of carotenoids from pitanga and buriti. Thus, we tracked the digestion profile of these fruits' carotenoids using *in vitro* digestion protocols and evaluating the uptake by the Caco-2 cells monolayers. For the best of our knowledge this is the first-time carotenoids bioaccessibility from these unique plants was assessed.

2. Materials And Methods

2.1 Fruit Samples

Pitanga (*Eugenia uniflora*) was harvested from the orchard at the Department of Agrifood Industry, Food and Nutrition of the Luiz de Queiroz College of Agriculture (LAN/ESALQ) Piracicaba, São Paulo, Brazil. Only red ripe fruits were collected. Afterward, selected fruits (integral and homogeneous) were washed, pulped using a juicer (Philips Walita, Brazil), frozen at -26°C, and stored until analysis (approx. two months). Buriti (*Mauritia flexuosa*) pulp was produced by the agricultural cooperative Sertão Veredas (Chapada Gaúcha, Brazil), which peeled the ripe fruits, soaked them in warm water, and crushed them to

separate seeds from the pulp. Aliquots were stored at -26°C (for a maximum of 2 months) in 50 mL tubes and put into sealed black bags. All the analytical procedures were carried out at the Food Analyses and Nutrition Laboratory.

2.2 Reagents

Commercial carotenoid standards, β -apo-8'-carotenal, and enzymes for *in vitro* digestion were purchased from Sigma-Aldrich (St Louis, USA). Organic solvents for extraction and HPLC analysis were chromatographic grade (Tedia®). Caco-2 cell cultures were purchased from American Type Culture Collection (ATCC®HTB37™, Manassas, USA). Dulbecco's Modified Minimum Essential Medium (DMEM), penicillin-streptomycin, fungizone, non-essential amino acids solution, and L-glutamine were purchased from Gibco (Waltham, USA). Heat-inactivated fetal bovine serum was purchased from Sigma-Aldrich (St Louis, USA).

2.3 Carotenoid extraction

Aliquots for extraction ranged from 100–200 mg of fresh fruit, 5 mL of digested food, i.e., chyme, 6–10 mL of micelle fraction, and cell pellets. The carotenoid extraction followed the method previously described in the literature [28]. Briefly, thawed samples were transferred to 50 mL centrifuge tubes along with 15 mL of a mixture of hexane, ethanol, and acetone (2:1:1, v/v/v) containing 0.1% BHT. The tubes were mixed using a vortex for 1 min and placed in an ultrasonic bath (Unique- Ultra Cleaner 1400, Frequency 40 kHz; 135 W/cm²; 80VA, Merse, Brazil) for 5 min. Then, saturated salt solution (3–5 mL) was added to the mixture, which was vortexed for 30 sec. Samples were centrifuged for 5 min at 800 x g, 4 °C, and the upper phase was collected and dried under a stream of nitrogen gas. To hydrolyze xanthophyll esters, replicate samples were extracted identically and added with an equal volume of methanolic KOH solution (10 %; 0.1 % HT). The mixtures were incubated overnight in the dark at room temperature. After centrifugation, the upper phase was collected and washed three times with distilled water to remove residual KOH before adding anhydrous sodium sulfate (~ 0.2 g) to remove residual water. The final extract was dried under a stream of nitrogen gas. The dried extract was dissolved in methanol and MTBE (1:1 v:v) with total volume (0.2–4 mL), depending on the carotenoid content, and the solution was filtered using a syringe filter unit (Millex PTFE, 0.22 μ m pore size, Sigma-Aldrich, St Louis, USA) into vials. β -apo-8'-carotenal was added randomly to samples before extraction (10 μ g/mL in methanol). The average recovery of β -apo-8'-carotenal was 98.6 % $\pm$ 17.2 SD from 18 spiked samples.

2.4 Carotenoid analysis by HPLC-DAD

The liquid chromatographic system was coupled to a diode array detector (HPLC-DAD, LC-20A Prominence, Shimadzu, Japan). The mobile phase consisted of methanol and MTBE gradient at 90:10 (Methanol:MTBE) from 0–40 min, changing to the linear gradient of 40:60 by 60 min before returning to initial conditions and washed for 15 minutes for re-equilibration. The flow was 0.8 mL/min, and injection volume ranged from 10 μ L to 40 μ L, depending on the carotenoid concentration. Separation was achieved with the polymeric Carotenoid C30 column (150 mm x 4.6 mm, 5 μ m particle size, YMC, Japan). The identification of carotenoids was carried out by combining commercial standards and using the elution

order described by Kimura et al.[29] compared to a certified reference material (CRM485) and the diode array absorption spectra. Furthermore, comparisons with publications were conducted to confirm the identity of carotenoids in HPLC-MS for the same fruits [17], [30]. Standard curves of violaxanthin, lutein, all-*trans*- α -carotene, all-*trans*- β -carotene, all-*trans*- γ -carotene, and all-*trans* lycopene were prepared and used to calculate the concentrations of carotenoids. The correlation coefficient (r^2) values were superior to 0.99 for the standard curves. Zeaxanthin, β -cryptoxanthin, *cis* and *trans* rubixanthin, and *cis* isomers of β -carotene quantities were estimated using the standard curve for all-*trans*- β -carotene standard curve as they share the same chromophore and similar wavelength maximums [31].

2.5 Carotenoid bioaccessibility

The three-phase static *in vitro* digestion model described in the literature[32], [33] was used with minor changes. Fruit samples (1–2 g) were homogenized with 10 mL of basal salts solution (BSS; NaCl, 120 mol/L; CaCl₂, 6 mmol/L; KCl, 5 mmol/L) by centrifugation. Following the homogenization, BSS was used for volume adjustment as needed. The pitanga pulp lipids content (0.4% wet weight) was determined previously using a literature method [34]. Hence, 2% (wt/wt) of soybean oil was added to promote the mixed micelles generation. On the other hand, the buriti pulp, which presented 19.2% of lipids in its composition, demanded no addition of oil during the *in vitro* digestion.

In the oral phase, 6 mL of previously prepared artificial saliva containing 106 units/mL of α -amylase (A3176, Sigma-Aldrich, St Louis, USA) were added to the sample and incubated at 37°C for 10 min in a rocker shaker (120 rpm). Subsequently, the gastric digestion phase was simulated by adjusting the pH to 2.5 with 1 M HCl and adding 2 ml of porcine pepsin (P7000, Sigma-Aldrich, St Louis, USA) solution (50000 units/ml in HCl 100 mM). The total volume was adjusted to 40 ml, and the tubes were incubated for 1 h at 37°C, 120 rpm. Then, the pH was increased to 6.0 with 1 M NaHCO₃ to terminate the gastric phase. Next, porcine bile extract solution (B8631; 3 ml, 40 mg/ml in 100 mM NaHCO₃, Sigma-Aldrich, St Louis, USA) and 2 ml solution containing pancreatin (P1750, Sigma-Aldrich, St Louis, USA) and lipase (L3126, Sigma-Aldrich, St Louis, USA) ~ 4000 units/ml and ~ 1000 units/ml in 100 mM NaHCO₃, respectively, were added. Before incubation for 2h at 37°C, pH and volume were adjusted to 6.5 and 50 ml, respectively. After the intestinal phase digestion, homogenized aliquots (12 ml) were centrifuged at 5000 x g for 60 min at 4 °C. The supernatant was collected and sterile filtered (Millipore®, 0,22 μ m pore size) to prepare the micellar fraction. The ratio between the carotenoids incorporated into the micelle fraction and the initial content of the fresh fruit pulp represents the nutrient bioaccessibility. Aliquots of digested fruits (chyme) and their bioaccessible fraction were stored under nitrogen gas at -26°C until carotenoid analysis by HPLC-DAD as described in the previous section.

2.6 Carotenoid uptake by Caco-2 cells culture

Caucasian colon adenocarcinoma (Caco-2) cells from American Type Culture Collection (ATCC®HTB37™, Manassas, USA) were cultured to evaluate the apical uptake of carotenoids. As described elsewhere [35], Caco-2 cells (passages 22–27) were seeded in T25 flasks (25cm² of area at 2–3 x 10⁵ cells), and the cultures were maintained at 37°C in a humidified atmosphere with 95% air and 5% CO₂. Dulbecco's

Modified Minimum Essential Medium (DMEM, Gibco, Waltham, USA) enriched with L-glutamine (1%), non-essentials amino acids (1%), penicillin-streptomycin (1%), fungizone (0.2%) and 15% heat-inactivated fetal bovine serum (FBS) were used to maintain the cells growing until the culture acquire confluence at which the FBS content was reduced to 10%. The medium was changed every two days. The integrity of the monolayer and the presence of domes were monitored regularly by phase-contrast microscopy.

Caco-2 cells were cultivated until generating monolayers of differentiated enterocytes (10–14 days post-confluency) and then were incubated with the micellar fraction diluted in DMEM medium (1:4 v/v) for 4 h. First, monolayers were washed with Minimum Essential Medium (MEM, Gibco, Waltham, USA). Next, fresh DMEM without FBS, pH 6.5, and containing 25% micellar fraction generated from *in vitro* digestions was added to the monolayers (final volume 5 mL) and incubated for 4 h. After that, the medium was removed, and monolayers were washed once with ice-cold phosphate-buffered saline (PBS) containing 2 g/L of albumin (pH 7.0) and twice with cold PBS (pH 7.0), collected after using a rubber scrapper, and centrifuged at 500 x g/10 min at 4°C. Finally, the protein content of cell samples was determined by a rapid assay (Quick start Bradford, Bio-Rad Laboratories, Hercules, USA) using bovine serum albumin as a standard.

2.7 Statistics

The *in vitro* digestion assays for assessing bioaccessibility were performed in triplicate with freshly prepared homogenized fruit pulp and simulated digestion fluids. Moreover, the carotenoid profile analyses were also conducted in triplicate. The ANOVA test was used to evaluate the significant differences between carotenoid species and fruit. In addition, means were compared using the Tukey test at the 95% significance level ($p < 0.05$). Statistical Assistance software Assistant 7.7 beta (UFCEG, Campo Grande, PB, Brazil) was used for the ANOVA and Tukey tests.

3. Results And Discussion

3.1 Carotenoid profile of pitanga and buriti

While chromatographic analyses identified 14 carotenoid species in the pitanga pulp, the buriti pulp had 9 species (Fig. 1, Table 1). Buriti pulp showed 8 carotenoids with provitamin A activity, i.e., that contained the β -ionone ring on its chemical structure [36] at high concentration, ranging from 19 to 434 $\mu\text{g/g}$ on a wet weight basis (Table 1). Therefore, almost 90% of the buriti carotenoids are the provitamin A species. It is worth mentioning that vitamin A is an essential nutrient, performing physiological roles related to human vision, immunity, growth, and reproduction [37]. Compared to 23 vegetables that are good sources of all-*trans*- β -carotene [38], the buriti pulp displayed 4.9 times more of this provitamin A carotenoid than any listed vegetables. These results agree with the literature, where similar levels of all-*trans*- β -carotene and α -carotene were found for buriti [39]. The concentrations of *cis*- β -carotene isomers in non-saponified extracts of buriti pulp are also similar to those previously reported [30]. It is worth observing that the formation of *cis*- β -carotene isomers may occur when this palm fruit is collected after falling to the forest ground, during extraction of edible pulp with warm water, or during lengthy transportations.

On the other hand, the pitanga pulp presented relatively lower provitamin A carotenoid levels than buriti, being β -cryptoxanthin the most abundant (Table 1). In contrast to buriti, pitanga's major carotenoid is the lycopene which is at least twice higher than the 6 xanthophylls it presented (Table 1), a unique characteristic among plant foods [40]. All carotenoids found in the pitanga pulp in our study were previously identified by HPLC-MS [17]. Furthermore, the carotenoid content determined for pitanga pulp samples evaluated here (Table 1) is in accordance with results reported for pitanga fruits grown in a different Brazilian region with the same climate and mesoregion [41]. It is important to observe that carotenoids contained in fruits are part of secondary plant metabolism, depending on plant variety, sunlight, soil, climate, and maturity [42].

Buriti presented higher amounts of xanthophylls, lutein, and β -cryptoxanthin compared to pitanga (Table 1). However, these compounds represent only 7.8% of the total buriti's carotenoids. It is essential to mention the nutritional relevance of buriti's lutein content because this is a crucial component for ocular health that may prevent macular degeneration [43]. After a saponification step of extracts obtained from buriti pulp, no significant differences in identified xanthophylls were observed. Nevertheless, trace amounts of mono and di-esters of xanthophylls may be present in buriti since typical small peaks disappeared after saponification. On the other hand, there were observed increases of 13-*cis* (28%), 9-*cis* (22%), and 15-*cis* isomers (10%) of β -carotene and *cis*- γ -carotene (27%) due to the aggressive solvent extraction and saponification steps for analysis that also can produce these compounds [31]. The saponification of pitanga pulp extracts resulted in the degradation of lycopene (23%) and γ -carotene (37%), as shown in Fig. 1. Thus, results for carotenes from pitanga and buriti pulp were calculated considering the non-saponified extracts.

Pitanga presents a higher proportion of xanthophylls (47%) than buriti, with relatively high amounts of mono- and di-esters of xanthophylls (Fig. 1A). This result was confirmed by the loss of these peaks with increases in the AUC of rubixanthin, β -cryptoxanthin, zeaxanthin, and violaxanthin after saponification (Fig. 1B). The following amounts of esterified xanthophylls were detected in the pitanga pulp: violaxanthin 0.35 $\mu\text{g/g}$, zeaxanthin 0.83 $\mu\text{g/g}$, β -cryptoxanthin 2.99 $\mu\text{g/g}$, rubixanthin 4.29 $\mu\text{g/g}$, and *cis*-rubixanthin 3.46 $\mu\text{g/g}$. Proportionally, the xanthophylls that were more esterified were violaxanthin > *cis*-rubixanthin > rubixanthin > β -cryptoxanthin > zeaxanthin. Moreover, there was no significant increase for lutein after saponification, while 63% of violaxanthin was in the esterified form. Analytical advances during the past decade have revealed that xanthophyll esters are common in fruits. Mono and di-esters of xanthophylls often occurs in plant cells that have no photosynthetically function where esterified xanthophylls are lipid-dissolved [37], [44]. The peculiar carotenoid profile and proportion of xanthophylls/carotenes from pitanga can be compared to other edible plant foods such as rose hips (*Rosa rubiginosa*) which presents all-*trans*-rubixanthin laurate as a major carotenoid and has 53.9% of total carotenoids as esterified xanthophylls and 43.7% of carotenes like lycopene [44].

Table 1
– Estimated content and bioaccessibility of carotenoids in pitanga and buriti pulps.

Carotenoid	Pitanga		Buriti	
	Pulp (µg/g)	Bioaccessible (µg/g)	Pulp (µg/g)	Bioaccessible (µg/g)
1. Violaxanthin	0.55 ± 0.12 ^h	0.19 ± 0.02 ^d	nd	nd
2. Lutein	2.33 ± 0.18 ^f	0.75 ± 0.08 ^c	37.8 ± 2.8 ^{de}	19.7 ± 0.32 ^b
3. Zeaxanthin	4.03 ± 0.74 ^e	1.11 ± 0.02 ^b	nd	nd
4. β-cryptoxanthin	11.4 ± 0.25 ^c	1.74 ± 0.07 ^a	21.5 ± 0.91 ^e	8.34 ± 0.14 ^d
5. 15-cis-β-carotene² + 13-cis-β-carotene²	2.01 ± 0.02 ^f	0.10 ± 0.02 ^d	56.8 ± 8.4 ^{bcd}	21.6 ± 1.03 ^b
			42.3 ± 2.65 ^{cd}	14.3 ± 0.30 ^c
6. α-carotene	2.58 ± 0.17 ^f	0.39 ± 0.01 ^d	65.0 ± 5.63 ^b	20.9 ± 1.38 ^b
7. all-trans-β-carotene	3.98 ± 0.26 ^e	0.77 ± 0.19 ^c	434 ± 16.3 ^a	115 ± 4.17 ^a
8. 9-cis-β-carotene	1.82 ± 0.08 ^{fg}	0.14 ± 0.07 ^d	60.8 ± 0.23 ^{bc}	21.2 ± 1.20 ^b
9. rubixanthin	14.2 ± 0.37 ^b	1.48 ± 0.15 ^a	nd	nd
10. cis-rubixanthin	6.23 ± 0.11 ^d	0.75 ± 0.21 ^c	nd	nd
11. γ-carotene	1.42 ± 0.17 ^{fgh}	0.31 ± 0.01 ^d	19.8 ± 4.15 ^e	8.86 ± 0.31 ^d
12. cis-γ-carotene	0.73 ± 0.05 ^{gh}	0.27 ± 0.01 ^d	20.9 ± 4.10 ^e	9.12 ± 0.19 ^d
13. lycopene	30.8 ± 1.03 ^a	0.35 ± 0.03 ^d	nd	nd

Carotenoid	Pitanga		Buriti	
	Pulp	Bioaccessible ($\mu\text{g/g}$)	Pulp	Bioaccessible
	($\mu\text{g/g}$)		($\mu\text{g/g}$)	($\mu\text{g/g}$)

¹Data are mean $\pm$ standard deviation of three replicates ($\mu\text{g/g}$ on wet weight basis). Analysis of variance ANOVA ($n = 3$) and Tukey test for means comparison were carried out. Different letters in the column correspond to significant difference ($p < 0.05$) between carotenoids levels of same fruit. ²15-*cis*- and 13-*cis*- β -carotene partially co-eluted and could only be adequately quantified as separate species for pulp and bioaccessible buriti.

3.2 Stability of carotenoids during simulated digestions

Figure 2 shows the carotenoid profile of pitanga *in natura* without saponification (2A), representing the *in vitro* digestion effect on its carotenoids stability (2B) and bioaccessibility (2C), i.e., carotenoids that were released from the food matrix and incorporated into mixed micelles, as well the carotenoids uptake by human intestine cells Caco-2 (2D). Comparing the first and second chromatograms (Fig. 2A and 2B), one may notice that the carotenoids remained relatively stable during the *in vitro* digestion. Thus, carotenoids from pitanga were statistically less stable than buriti ($p < 0.05$) since, generally, xanthophylls were less stable than carotenes. For instance, while only 52% of pitanga β -cryptoxanthin and zeaxanthin remained stable after, *in vitro* digestions, 82% of buriti β -cryptoxanthin made it (Fig. 3). Regarding carotenes, stability ranged from 62–104% for lycopene in pitanga and γ -carotene in buriti, respectively. Meanwhile, *cis* isomers of β -carotene increased slightly during simulated digestion – 113% stability for buriti 15-*cis* and 13-*cis* β -carotenes (Fig. 3). Although carotenoid stability during *in vitro* digestions usually exceeds 75% [45], Courraud et al. [46] reported all-*trans*- β -carotene stability of 31%, 100%, and 40%, respectively for commercial standard, carrot juice, and spinach. These authors also showed the high acidity of the gastric phase promotes the instability of all-*trans*- β -carotene. Therefore, the digestive environment and the food matrix can affect carotenoids' stability differently. The acidity may also explain why carotenoids obtained from pitanga are less stable than buriti's since the former's pH (2.8) was found to be more acid than the latter's (4.8). Moreover, carotenoids solubilized/emulsified in lipids, which is the case of those found in buriti pulp, are more likely to be protected against degradation [47], consequently more stable during the *in vitro* digestion simulations.

3.3 Bioaccessibility of carotenoids after in vitro digestions

Comparing the chromatograms in Fig. 2A and 2C, one may notice the efficiency of carotenoids' micellarization, representing the bioaccessibility of those compounds. Since xanthophylls are less hydrophobic than carotenes, they are easily incorporated into mixed micelles because of the oxygen in their chemical structure [44]. Hence, higher peaks displayed in Fig. 2C correspond to violaxanthin, lutein, zeaxanthin, and β -cryptoxanthin. Markedly, Fig. 2C also shows that lycopene was poorly incorporated into mixed micelles, which agreed with its low bioaccessibility of 1.1%, as represented in Fig. 4. Furthermore,

the behavior of free and esterified forms of xanthophylls (Fig. 2C) suggests that the esterification of the fat during digestion reduced the carotenoid incorporation into mixed micelles. Indeed, the pitanga pulp composition diverges from buriti's. While the former possesses a considerable amount of fibers, low oil content, high humidity, and acidity, the latter is an oily palm fruit.

Regarding the carotenes' bioaccessibility, Fig. 4 shows that pitanga pulp violaxanthin, lutein, and zeaxanthin were significantly more bioaccessible than other carotenes such as lycopene and *cis* forms of β -carotene. The same pattern was observed in buriti pulp samples, where xanthophylls and γ -carotene forms were also significantly more bioaccessible than the other carotenoids (Fig. 4). These results indicate that the various carotenoid forms may compete to get into micelles during intestinal digestion. The overall bioaccessibility of buriti carotenoids (26–52%, Fig. 4) was significantly higher than pitanga's (1–37%, Fig. 4). It is worth noting that dietary lipids, mainly those composed of unsaturated fatty acids, increase the micellarization and absorption of carotenes; on the other hand, saturated fat acid has minimal influence on the xanthophylls bioaccessibility [27]. Thus, one may imply that the buriti pulp fat content (19%), compared to pitanga pulp (2.4%, initial plus added oil for *in vitro* digestions), influenced the bioaccessibility results. The publication by Failla et al. [48], where the bioaccessibility of a salad meal containing 3% wt/wt of fat from different sources was evaluated, reported results that corroborate our findings. These authors found values ranging from 45–57% for lutein and zeaxanthin, 9–20% for all-*trans*- β -carotene, and 1.5–5.3% for lycopene. Therefore, it was observed that xanthophylls might act as an inhibitor of lycopene and β -carotene micellarization through a competition mechanism

Pitanga, when consumed *in natura*, contains lycopene, which may prevent prostate cancer, but β -cryptoxanthin had 5 times higher bioaccessible concentrations (Table 2). Micellar phase of digested pitanga would first provide higher amounts of xanthophylls than the desirable carotenes – β -cryptoxanthin > rubixanthin > zeaxanthin > lutein > *cis*-rubixanthin. There is a marked relation between carotenoid bioaccessibility and antioxidant activity. It has demonstrated that hydroxylation and cyclization of carotenoid terminal end groups are not only responsible for augmented bioaccessibility but also for higher antioxidant capacity against oxygen reactive species [37], [46], [49]. [50] reported antioxidant capacity of carotenoid rich micellar fraction by Trolox equivalent antioxidant capacity (TEAC) assay being rubixanthin > lycopene > β -cryptoxanthin > γ -carotene > β -carotene. They associated rubixanthin higher TEAC values with the twofold enhancing effect of one ψ -end and one hydroxyl group on the β -ionone ring, thus being closely connected to the micelle's surface and, in addition, possessing a reactive open-chain end. Rubixanthin's health effects have been neglected because it is uncommon in foods. In pitanga, it is possibly the most important functional carotenoid because of its high amount in the micellar phase (Table 2). β -carotene and lycopene have been shown to be inversely related to the risk of cardiovascular diseases and certain cancers whereas lutein and zeaxanthin to the disorders related to the eye [51]. β -cryptoxanthin have anti-inflammatory effects, enhance the immune system and may reduce the risk of osteoporosis[52], [53]. Thereby pitanga may have much more potential as a functional fruit due to β -cryptoxanthin, zeaxanthin and lutein than lycopene bioaccessible amounts.

The all-*trans*- β -carotene is the most abundant compound found in the micellar phase of the buriti pulp samples evaluations. It is important to mention that all-*trans*- β -carotene is converted to retinol by BCO1 and BCO2 enzymes in enterocytes twice more than other carotenes [54]. All carotenes from buriti, except lutein, have vitamin A activity, which suggests that buriti is an interesting source of vitamin A, which might be used in programs dedicated to combating the deficiency of this nutrient. Considering that the FAO[55] established a mean requirement ingestion of retinol equivalent (RE) for adult women of 300 μ g RE/day, the buriti pulp offers 96.7 μ g RE/g, calculated according to FAO (25% of bioaccessibility). Noteworthy, as we showed here, buriti provitamin A carotenoids bioaccessibility ranged from 26–46%. Then, a single small fruit of buriti (> 5 g portion) is enough to provide FAO's requirement of vitamin A, which is incredibly helpful for peoples that live in rural areas, forests, an insulated regions. It is also essential to consider that not all provitamin A carotenoids absorbed by humans are converted into retinol, i.e., some of them, such as β -carotene and β -cryptoxanthin, are absorbed and distributed by chylomicrons intact and accumulated in many tissues providing a protective effect against oxidative stress [56]. Rodrigues et al.[57] demonstrated that antioxidant capacity of carotenes against the peroxy radical is dependent on the opening of the β -ionone, the chromophore extension and addition of hydroxyl groups – *e.g.* all-*trans*- β -carotene have higher capacity to scavenge the peroxy radical than lutein but is less effective than lycopene and γ -carotene. So that, great part of bioaccessible carotenes from buriti, which would not be converted to vitamin A, will act as potent antioxidants at systemic, and local levels.

Results of cellular carotenoids uptake are presented in Fig. 5 and Table 2. Meanwhile, Fig. 5 presents the percentage of carotenoid taken from the micellar fraction; Table 2 shows the absolute amounts accumulated inside the cells. As one may notice, for both fruits, β -carotene was the most uptake and accumulated compound in the intestine cells. Nevertheless, pitanga presented an overall carotenoids uptake higher than buriti, which corroborates pieces of evidence that the food matrix affects the absorption of carotenoids. For instance, differences were also found regarding carotenoids uptake between cassava varieties (uptake of all-*trans*- β -carotene ranging 10–20%) and orange-fleshed sweet potato varieties (ranging 10–25%) [58], [59]. Despite the low micellarization of pitanga lycopene, its uptake from mixed micelles was relatively high (Fig. 5), similar to that observed in raw and processed tomato juice [60]. On the other hand, Fig. 5 also shows that lutein and zeaxanthin uptakes were low, compared to the high micellarization observed in Fig. 4 and to that reported for salad meals combined with unsaturated fatty acids [48].

Above data corroborate with the transporter mediated absorption of carotenoids in the intestine epithelium that facilitate carotenes passage and may undergo competition with other compounds from the food matrix [61]. On this way, our results indicate that all-*trans*- β -carotene might have facilitated transport across the brush border. Therefore, one may infer that the interactions between carotenoids may influence the cellular absorption. Indeed, a scavenger receptor class B type I (SR-BI) and a cluster determinant 36 (CD36) are proved to be involved in cellular uptake of β -carotene, α -carotene and β -cryptoxanthin [61]. Thus, we attribute the β -carotene higher uptake and accumulation in enterocytes to the SR-BI effect since it is expressed by caco-2 cells.

The saturation effect on caco-2 cells might explain the lower carotenoids uptake presented by buriti in relation to pitanga ($p < 0.05$, as shown in Fig. 5). However, the concentration of bioaccessible all-*trans*- β -carotene in the micellar phase of buriti is 150 times higher than pitanga. Thus, the carotenoids accumulated in the monolayers were still much higher for buriti than pitanga, in absolute amounts (Table 2). This saturation effect for carotenes and xanthophylls was demonstrated with isolated carotenoid compounds [62], showing that the higher the concentration of the carotenoid in the micellar fraction, the lower the absorption by the enterocyte-like caco-2 cells. In summary, our work demonstrated that pitanga and buriti fruits are rich sources of carotenoids of high bioaccessibility, then, they are potential fruits to be used as functional food products. Regarding the final amounts of carotenoids with the potential to be absorbed after the ingestion (Table 2), pitanga comes up as a source of xanthophylls, specifically β -cryptoxanthin and rubixanthin, while only a tiny part of lycopene is absorbed and can be used by the body. Likewise, the results presented in Tables 1 and 2 also indicate that buriti is an excellent natural source of vitamin A due to its high bioaccessible provitamin A content. Therefore, pitanga has the potential to present antioxidant activity against physiologic reactive species of oxygen as well may prevent macular degeneration, and we suggest further studies to confirm these functionalities. Similarly, buriti may have many health benefits like improving the immune system, preventing several cancers, avoiding eye diseases, and others that also should be tested to appraise how significant are these effects.

Table 2

– Final amount of carotenoids from pitanga and buriti pulps accumulated in caco-2 cells after 4h incubation with micelle fraction.

Carotenoid	Amount absorbed ² ($\mu\text{g/g}$ on wet weight basis)	
	Pitanga	Buriti
1. Violaxanthin	nd	nd
2. Lutein	3.99 ± 0.15^d	44.8 ± 3.9^b
3. Zeaxanthin	9.04 ± 0.03^c	nd
4. β-cryptoxanthin	21.5 ± 0.15^b	nd
5. 15-cis-β-carotene	nd	48.2 ± 3.3^b
13-cis-β-carotene	nd	13.7 ± 1.8^c
6. α-carotene	nd	44.3 ± 5.9^b
7. all-trans-β-carotene	37.9 ± 3.4^a	598 ± 20^a
8. 9-cis-β-carotene	nd	56.4 ± 1.9^b
9. rubixanthin	23.4 ± 2.78^b	nd
10. cis-rubixanthin	6.28 ± 0.71^{cd}	nd
11. γ-carotene	19.6 ± 2.3^b	nd
12. cis-γ-carotene	3.59 ± 0.48^d	nd
13. lycopene	2.78 ± 0.11^d	nd
¹ Data are mean $\pm$ standard deviation of three replicates, data are standardized by the mg of cellular protein per assay. Analysis of variance ANOVA (n = 3) and Tukey test for means comparison were carried out. Different letters in the column correspond to significant difference (p < 0.05) between carotenoids levels of same fruit.		

Finally, we provided evidence that the biochemistry of carotenoids and food matrix directly affect bioaccessibility. Our results showed that the hydroxylation and esterification of xanthophylls, carotenes *trans* isomerization of carbonic double bonds, presence and quantity of lipids from food matrix/meal, and interactions between different carotenoids were essential to explaining the digestion behavior of carotenoid-rich foods. In addition, our results remarked the need to quantify the amounts of bioactive compounds that the body can absorb rather than just measuring their content in the food matrix. Therefore, it is essential to develop products and processes that encompass, for instance, compound

delivery systems to reduce instability through digestion and increase the absorption in the small intestine. Highlighting, we point out that fruits such as pitanga and buriti may represent new raw materials for developing innovative products rich in bioactive compounds and health benefits.

Declarations

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

Credit authorship contribution statement

Paulo Berni: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing-Original draft preparation, Writing - Review & Editing. **Talita C. Negri:** Writing- partial draft preparation. **Stephanie S. Campoli:** Formal analysis, and Investigation. **Ericsem Pereira:** Writing- partial draft preparation. **Mark L. Faila:** Critical review of manuscript and Writing. **Solange G. Canniatti-Brazaca:** Writing - Review & Editing, Supervision, Project administration, Resources, Funding acquisition.

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Figures

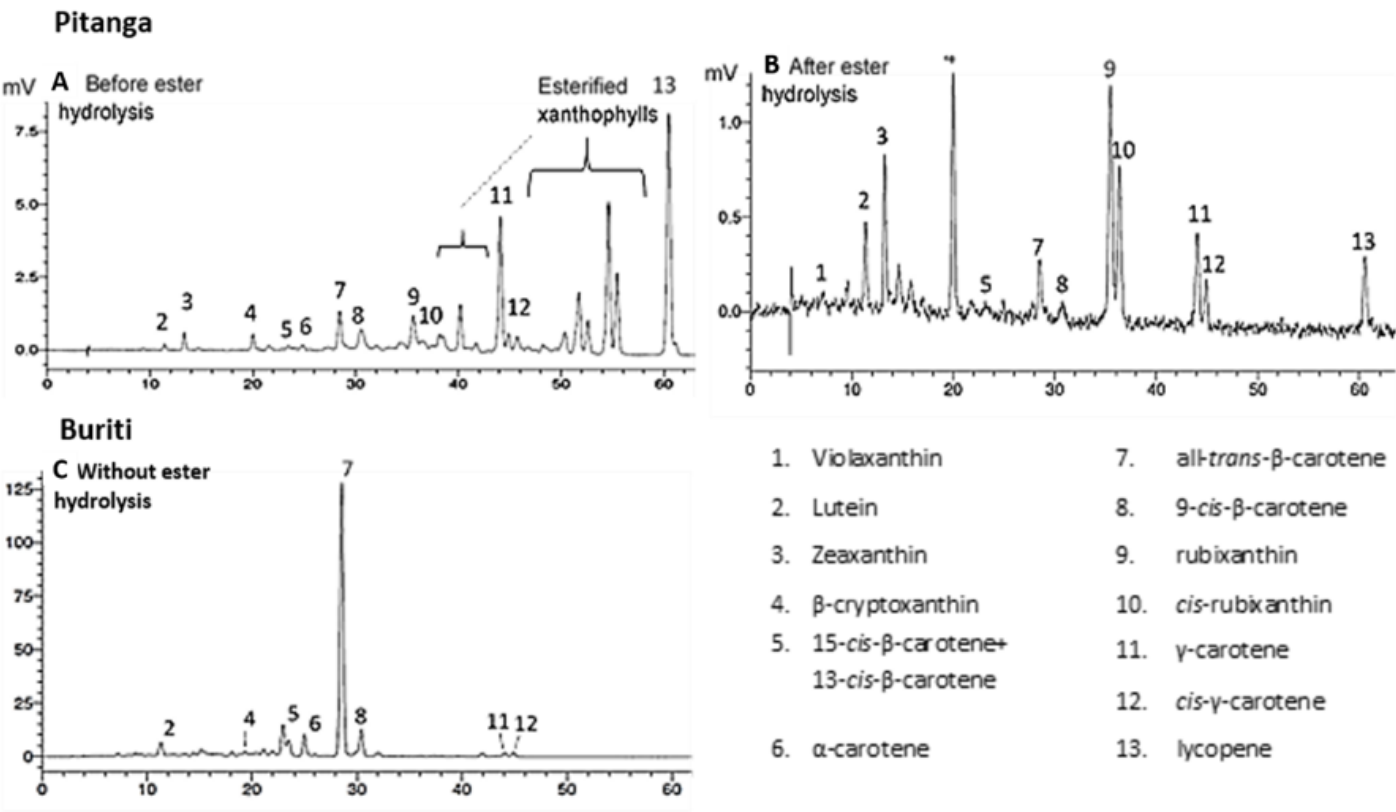


Figure 1

Representative chromatograms of carotenoids in pitanga and buriti fresh pulp. Chromatographic profiles for non-saponified (panel A) and saponified (panel B) extracts of pitanga, and buriti non-saponified extract (panel C) at 450 nm. Peaks correspond to absorption for 1.8 mg. 1.0 mg and 4.0 mg of fruit pulp for panels A, B and C, respectively.

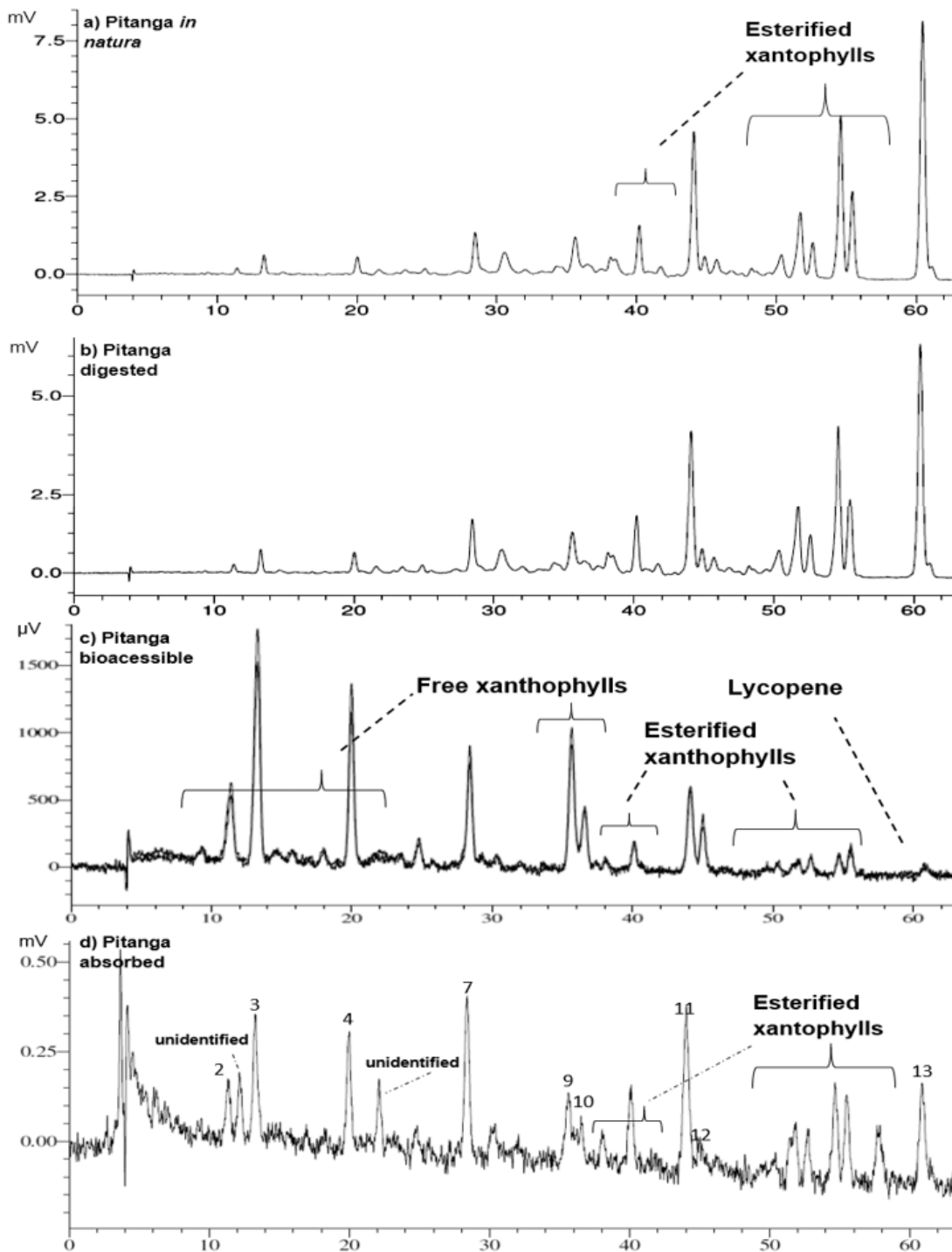


Figure 2

Representative chromatograms of *in vitro* digestions effects on the carotenoid profile of pitanga: a) Fresh pulp of pitanga, b) digested pitanga obtained after *in vitro* digestions, c) Micellar fraction separated from inaccessible digested residue, obtained after centrifugation and filtration, d) Enterocyte-like Caco-2 cells from T25 flasks after 4h incubation with 25% of micellar fraction in medium. Chromatograms are processed at 450 nm.

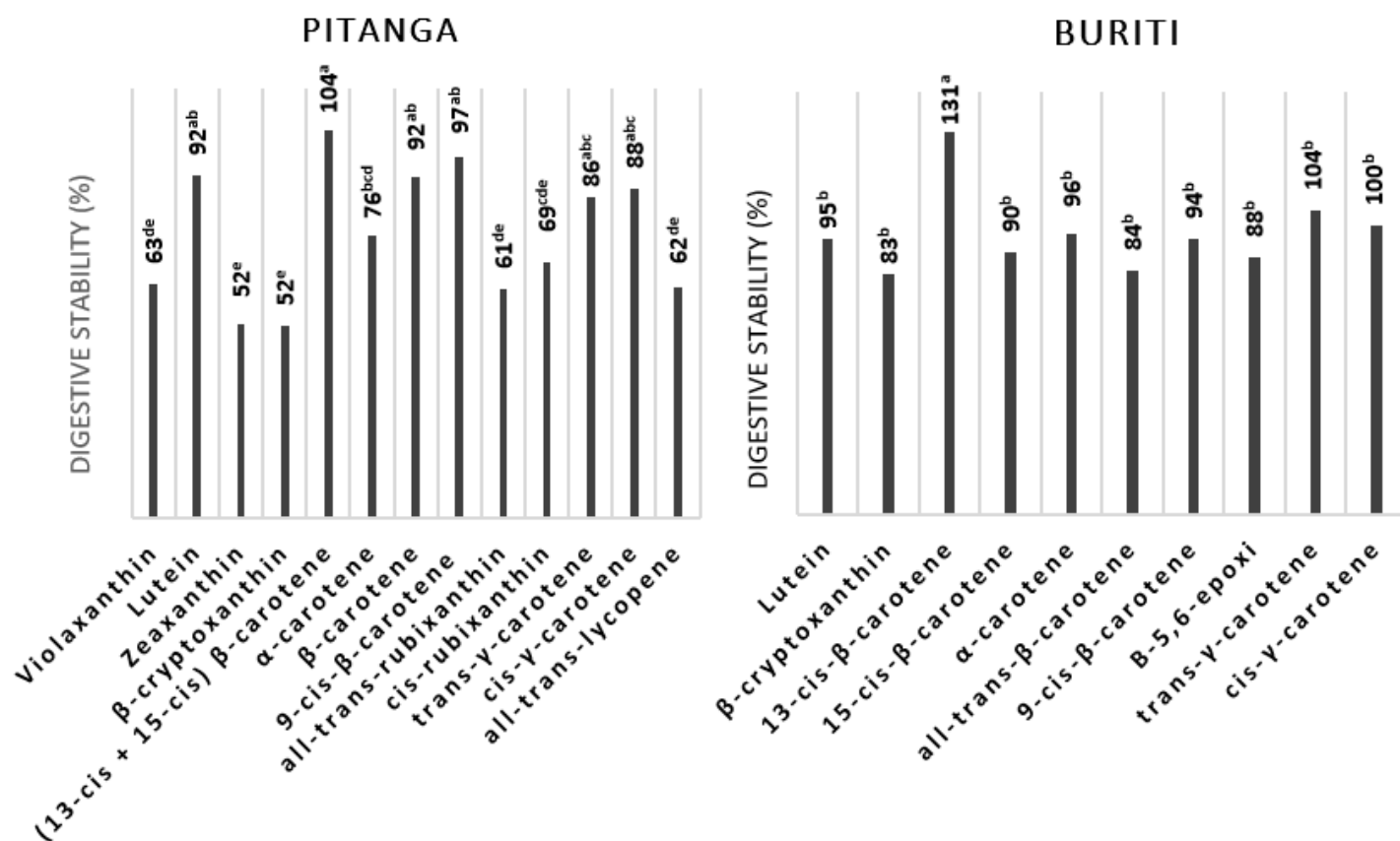


Figure 3

Stability of carotenoids after *in vitro* digestions of pitanga and buriti. Pitanga and buriti graphic represents percentage of carotenoid that remained in digested samples (n=3) after the analytical procedures. Analysis of variance ANOVA (n=3) and Tukey test for means comparison were carried out. Different letters above each bar correspond to significant difference ($P < 0.05$) between carotenoids bioaccessibility of same fruit.

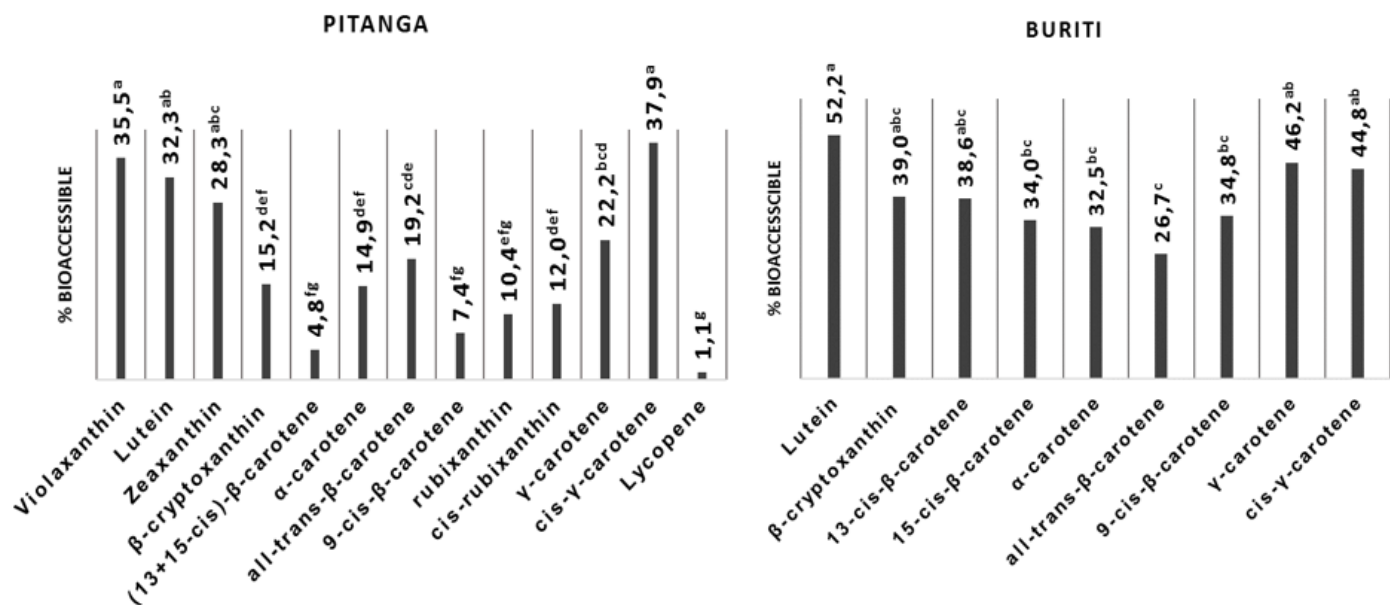


Figure 4

Bioaccessibility of carotenoids from pitanga and buriti. Data are related to percentage of carotenoid incorporated into mixed micelles after *in vitro* digestions. Data are mean $\pm$ standard deviation of three replicates. Analysis of variance ANOVA (n=3) and Tukey test for means comparison were carried out. Different letters above each bar correspond to significant difference ($p < 0.05$) between carotenoids bioaccessibility of same fruit.

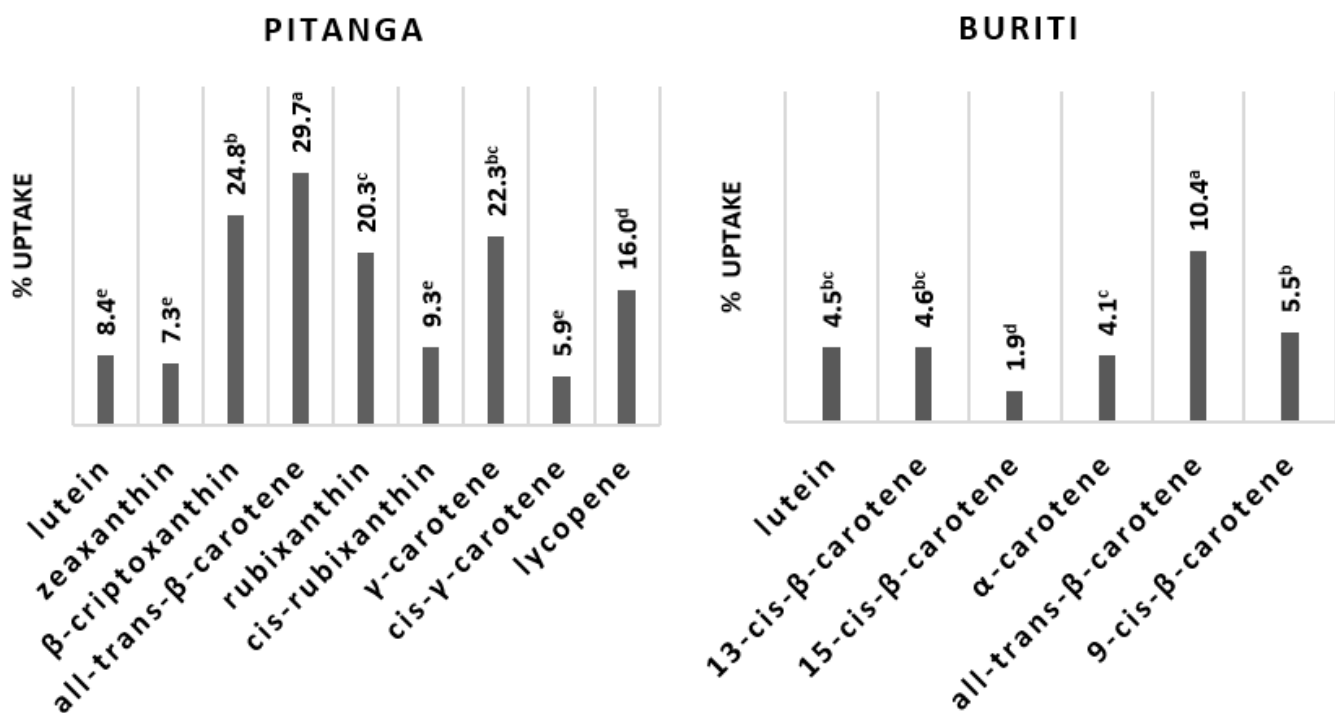


Figure 5

Carotenoid uptake by enterocyte-like Caco-2 cells culture. Data are related to the percentage of carotenoid that was taken from micellar fraction after 4h of exposure. Data are mean $\pm$ standard deviation of three replicates. Analysis of variance ANOVA (n=3) and Tukey test for means comparison were carried out. Different letters above each bar correspond to significant difference ($p<0.05$) between carotenoids from same fruit.