University of São Paulo
“Luiz de Queiroz” College of Agriculture

Food structure design to modulate bioaccessibility of carotenoids from brazilian native fruits after screening of eleven non-conventional tropical fruits

Paulo Roberto de Araujo Berni

Thesis presented to obtain the degree of Doctor in Science.
Area: Food Science and Technology

Piracicaba
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Paulo Roberto de Araujo Berni
Bachelor of Food Science

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Advisor:
Prof. Dr. **SOLANGE GUIDOLIN CANNIATTI BRAZACA**

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“The great book, always open and which we should make an effort to read, is that of Nature”

Antoni Gaudí

Contents

RESUMO	7
ABSTRACT	8
1 INTRODUCTION.....	9
2 SCREENING OF ELEVEN NON-CONVENTIONAL TROPICAL FRUITS – PROXIMAL COMPOSITION, FIBERS, ANTIOXIDANT POTENTIAL AND CAROTENOID BIOACCESSIBILITY	17
Abstract	17
2.1. Introduction	18
2.2. Materials and methods	19
2.3. Results and discussion.....	24
2.4. Conclusions	32
3 CAROTENOIDS FROM PITANGA (<i>EUGENIA UNIFLORA</i>) AND BURITI (<i>MAURITIA FLEXUOSA</i>) FRUITS: PROFILE, STABILITY, BIOACCESSIBILITY AND CELLULAR UPTAKE STUDIES BY <i>IN VITRO</i> DIGESTION COUPLED TO CACO-2 CELLS CULTURE	37
Abstract	37
3.1. Introduction	38
3.2. Materials and methods	40
3.3. Results and discussion.....	43
4 STRUCTURE DESIGN OF FRUIT MICROEMULSION DELIVERY SYSTEM FOR CAROTENOIDS FROM PITANGA (<i>EUGENIA UNIFLORA</i>) AND BURITI (<i>MAURITIA FLEXUOSA</i>)	61
Abstract	61
4.1. Introduction	62
4.2. Materials and methods	64
4.3. Results and discussion.....	69
4.4. Conclusions	83
5 CAROTENOID BEHAVIOR OF PITANGA (<i>EUGENIA UNIFLORA</i>) AND BURITI (<i>MAURITIA FLEXUOSA</i>) DURING MICROEMULSION PRODUCTION AND IN A DYNAMIC GASTROINTESTINAL SYSTEM	89
Abstract	89
5.1. Introduction	90
5.2. Materials and methods	93
5.3. Results and discussion.....	98
5.4. Conclusions	114
6 FINAL CONSIDERATIONS	121

RESUMO

Desenho estrutural da matriz alimentar para modulação da bioacessibilidade de carotenoides de frutas nativas do Brasil após a triagem de onze frutas tropicas não-convencionais

O Brasil é o país detentor da maior biodiversidade do planeta e um grande produtor de alimentos. Frutas tropicais, em especial as nativas brasileiras, podem conter quantidades consideráveis de carotenoides que possuem ação antioxidante, anti-inflamatória, provitamina A e anticâncer, como β -caroteno e licopeno. O desenho estrutural dos alimentos (*food structure design*), visa manipular a matriz alimentar com fins específicos, por exemplo, a preservação e manipulação da bioacessibilidade de carotenoides. Na presente tese buscou-se explorar frutas brasileiras não-convencionais no desenvolvimento de sistemas de entrega de carotenoides. Foi realizada uma etapa de triagem com 11 frutas, dentre as quais, 2 foram selecionadas, a pitanga (*Eugenia uniflora*) e o buriti (*Mauritia flexuosa*), e utilizadas na produção de microemulsões. Nesta triagem foram avaliadas a composição centesimal, o teor de fibras, o perfil de carotenoides e a bioacessibilidade destes carotenoides. O valor nutricional das frutas demonstrou seu potencial para utilização em produtos saudáveis, devido a seus teores elevados de fibras, minerais e carotenoides além do baixo teor calórico. A análise por HPLC-DAD permitiu identificar até 14 carotenoides nas amostras das 11 frutas estudadas na triagem. O estudo da bioacessibilidade dos carotenoides das 11 frutas demonstrou principalmente a superioridade da bioacessibilidade de xantofilas (variando de 10 a 52 %) em relação aos carotenos, e portanto a baixa bioacessibilidade de licopeno da pitanga (1,1%) e média bioacessibilidade de β -caroteno do buriti (26%). Pitanga e buriti tiveram o perfil de carotenoides detalhadamente acompanhados através da simulação *in vitro* da digestão associada à absorção intestinal por culturas de células Caco-2. Neste estudo, observou-se que embora as xantofilas sejam mais bioacessíveis, o tecido epitelial do intestino absorve preferencialmente carotenoides provitaminicos A, como o β -caroteno e a β -criptoxantina. Para produzir as microemulsões foram estudados processamentos (homogeneização de alta-velocidade - HSH e ultrassom - US) em combinação com uso de surfactantes (*Whey Protein Isolate* e Tween 80) e adição de óleo de milho como carreador dos carotenoides. Os experimentos mostraram que a interação entre US e HSH é capaz de romper as paredes celulares e liberarem os carotenoides com maior eficiência. Ficou demonstrado através de microscopia ótica e de fluorescência, tanto quanto pela análise de carotenoides, que foi possível manipular estruturalmente a matriz alimentar liberando os carotenoides de dentro das células vegetais e encapsulando-os dentro das gotículas de óleo, além de aumentar sua retenção após o processamento. As microemulsões obtidas sofreram efeito do tempo de processamento e do surfactante em relação à reologia, estrutura final da matriz, estabilidade ao armazenamento e estabilidade dos carotenoides ao processo. Por fim, foi utilizado um sistema dinâmico de simulação da digestão gastrointestinal para comparar o comportamento dos carotenoides oriundos das polpas integrais das frutas e das microemulsões selecionadas (Tween 80 a 2%, óleo de milho a 5% e processado por HSH-US 4 min-4 min). Os resultados demonstraram que foi possível aumentar a estabilidade à digestão e a bioacessibilidade dos carotenoides totais, do licopeno e do β -caroteno.

Palavras-chave: Frutas nativas; Caroteno; Licopeno; Células Caco-2; Microemulsão; Desenvolvimento de produtos; Bioacessibilidade; Biodisponibilidade

ABSTRACT

Food structure design to modulate bioaccessibility of carotenoids from brazilian native fruits after screening of eleven non-conventional tropical fruits

Brazil is the country with the greatest biodiversity on the planet and a major producer of food. Tropical fruits, especially natives from Brazil, may contain considerable amounts of carotenoids that have antioxidant, anti-inflammatory, provitamin A and anticancer actions, such as β -carotene and lycopene. The food structure design concept aims to manipulate the food matrix for specific purposes, *e.g.* the preservation and manipulation of carotenoid bioaccessibility. The aim of this thesis was to explore tropical fruits, native and exotic from Brazil in the development of delivery systems for carotenoids. A screening step was carried out with 11 fruits, among which 2 were selected, the pitanga (*Eugenia uniflora*) and buriti (*Mauritia flexuosa*) fruits that were used for the production of microemulsions. At the screening were evaluated the proximate composition, fiber contents, carotenoid profiles and bioaccessibilities. The nutritional value demonstrated that these fruits have high potential as raw-materials for healthy products due to their high fiber, minerals and carotenoid contents in addition to low energy value. Analysis by HPLC-DAD allowed the identification of 14 carotenoids in the 11 fruits studied for the screening. Results demonstrated the superiority of the bioaccessibility of xanthophylls (ranging 10 % – 52 %) in relation to carotenes, and the low bioaccessibility of lycopene from pitanga (1.1 %) and average bioaccessibility of β -carotene from buriti (26 %). Pitanga and buriti had their carotenoid profiles analyzed and monitored throughout an *in vitro* simulation of the digestion coupled with caco-2 cell cultures. Although xanthophylls are more bioaccessible, the intestinal epithelium absorb preferentially the provitamin A carotenoids, such β -carotene and β -criptoxanthin. In order to produce these microemulsions, high-speed homogenization (HSH) and ultrasound (US) were studied in combination with the use of surfactants (Whey Protein Isolate and Tween 80), and addition of corn oil as carotenoid carrier. The experiments have shown that the interaction of US and HSH is capable to break cell walls and release carotenoids with higher efficiency. Optical and fluorescence microscopy, as well as carotenoid analysis demonstrated that it was possible to manipulate the food matrix structure releasing the carotenoids from the plant cells and encapsulating them inside the oil droplets, what increased their retention after processing. The microemulsion were affected by time of processing and by surfactant related to their rheology, final structure, stability of emulsion and carotenoid stability to processing. Finally, a dynamic gastrointestinal system was used to compare the behavior of carotenoids from whole fruit pulps and selected microemulsions (2% Tween 80, 5% corn oil, processed by HSH-US 4 min -4 min). The results demonstrated that it was possible to increase the stability to digestion and bioaccessibility of total carotenoids, lycopene and β -carotene from the microemulsions.

Keywords: Native fruits; Carotene; Lycopene; Caco-2 cells; Microemulsion; Product development; Bioaccessibility; Bioavailability

1 INTRODUCTION

Malnutrition and chronic diseases that are related to food habits pose one of the biggest threats to global health and development, particularly in the developing world (WHO, 2018). The World Health Organization (WHO) proposes that a healthy and efficient food system, linked to healthy living habits, can significantly reduce the costs of treating chronic degenerative and non-communicable diseases while increasing people's quality of life, promoting productivity and generating employment and income. WHO (2018) encourage the development, production and dissemination of food products that contribute to a healthier diet. In this sense, intensification of fruit consumption is stimulated by consistent evidences of their potential to provide health benefits for people. The expansion of food diversity throughout the sustainable use of local biodiversity and traditional foods is intensively promoted by the scientific community (Rodriguez-Amaya et al., 2008; Heywood, 2011 Hough, 2014; Santeramo et al., 2018).

The knowledge about Brazilian biodiversity, and its use to improve people's health and social well-being, is strategic for promoting sustainable development. The fruit production sector has significant participation in agribusiness placing Brazil as the 3rd largest fruit producer in the world (Bueno and Baccarin, 2012; Silva and Abud 2017). However, among the twenty fruits most produced in 2016 (Kist et al., 2018) only three are native from Brazil, the pineapple, passionfruit and cashew nut. Many Brazilian fruits, which are little known or studied, have large amounts of bioactive compounds that have antioxidant, anti-inflammatory, anti-cancer properties and other actions. With the greatest biodiversity on the planet and one of the world's largest food producers, Brazil needs to innovate in the manufacture of food products from its plant resources (Nogueira, 2011, Raimundo et al., 2017).

Among the abundant bioactive compounds in Brazilian fruits there are carotenoids which are recognized as essential for a healthy life. They are found only in plants, algae and photosynthetic bacteria, and are responsible for the colors of red, orange and yellow tones in the great majority of foods, especially in fruits (Rodriguez-Amaya et al., 2008; Rao and Rao, 2007). Even with its numerous chemical forms, carotenoids can be divided into two large groups: carotenes and xanthophylls. These groups are differentiated by the presence of oxygen in the molecule, the carotenes do not have oxygen while the xanthophylls possesses (Fernández-García, 2012; Amorim-Carrilho et al., 2004).

β -carotene, α -carotene, γ -carotene, β -cryptoxanthin and β -zeacarotene, are the most common carotenoids that have vitamin A activity, since they can form in the body one or two

molecules of retinol (vitamin A) (Rodriguez-Amaya et al., 2008). Several ways of antioxidant action were identified for carotenoids and xanthophylls, like the unique capacity to capture oxygen singlet (Gülçin, 2012; Rao and Rao, 2007). The role of carotenoids in the prevention and remission of certain types of cancers has been demonstrated, with emphasis on their anti-inflammatory action and induction of physiological redox enzymes. Thus, carotenoids have significant bioactive functions and can not be disregarded when it comes to food, health and well-being (Miller et al., 1996; Zheng et al., 2013; Gülçin, 2012; Rodriguez-Concepcion et al., 2018).

Because carotenoids contains a conjugated system of double bonds, they are highly unstable, sensitive to heat, light, acids, oxygen and enzymes such as lipoxygenase, causing changes or may lead to partial degradation of their structures. The processing of carotenoid-rich foods can cause degradation of these compounds and impair their function. Degradation can act in two main ways: conversion of the *trans* forms into *cis* isomers that modifies their bioactivity; and oxidation reactions pathway that breaks down chemical structures producing low molecular weight residues, that also interfere in their bioactivity (Saini et al., 2015; Rodriguez-Amaya, 2001).

Carotenoids have to be bioaccessible for the body prior to provide the expected health benefits. Bioaccessibility, is defined as the portion of an ingested nutrient that will be available to be absorbed by the intestine after digestion. Synthetically, the steps necessary for the carotenoid bioaccessibility are: 1) release of carotenoids from the food matrix; 2) the released carotenoids have to be incorporated to the lipid phase of the digesta still in the stomach; 3) with the lipid droplets the carotenoids integrate the mixed micelles formed by the action of the bile salts; 4) these mixed micelles migrate to the intestinal lumen and come into contact with the microvilli of the enterocytes, and are absorbed by the intestinal cells (Yonekura and Nagao, 2007, Kopec and Failla, 2018).

The most common factors that negatively affect the stability of carotenoids in processed foods are heat treatment (mainly), homogenization and particle reduction, acidity, presence of oxygen and light. Most important factors in foods that negatively affect the carotenoid bioaccessibility are the physicochemical structure of the food matrix, especially the presence of fibers (dependent on the type of fiber), the chromoplast composition and structure (crystalloid) and the absence of lipids. Moreover, since carotenoids are highly lipophilic there are some barriers to overcome in order to efficiently incorporate them into novel functional foods: low solubility in water; crystallization capacity; chemical instability; and low bioaccessibility (McClements, 2015). Therefore, the processing and the food matrix from the

final products are fundamental for carotenoid bioaccessibility (Failla et al., 2014, Pugliese et al., 2013; Kopec and Failla 2018).

Considering that bioaccessibility and stability of carotenoids in fresh and processed fruits is generally low, it is possible to apply the processes and formulations to modulate it. For example, β -carotene and lycopene had their bioaccessibility increased by using optimal combinations of: cooking and oil incorporation; controlled heat treatment and suitable formulation; ultrasound processing and oil addition; cooking and mixing with excipient microemulsion (Anese et al., 2015; Berni et al., 2014; Buggenhout et al., 2012; Zhang et al., 2015). Thus, strategic formulation of products and optimal processing may be tools to increase carotenoid release, bioaccessibility, and consequently the action of promoting health (Buggenhout et al., 2012). Healthier ready-to-eat and fruit-derived products could be produced and formulated by a smart way. From the selection of the raw material combined with more adequate and efficient thermal and/or mechanical processes, would imply in obtaining products with better attributes for health, *i.e.* higher retention and bioaccessibility of the targeted carotenoid (Buggenhout et al., 2012). The use of these techniques are called food structure design and aims the protection, conservation, increasing bioaccessibility, and especially boosting health benefits (McClements, 2015; McClements et al., 2015)

Many bioactive compounds from plants are highly lipophilic, such as carotenoids, which causes many hindrance to incorporate these molecules in new functional foods, primarily low solubility in water and chemical instability (Recharla et al., 2017). Several techniques seek to protect, preserve, enhance solubility, increase bioaccessibility and bioactivity of carotenoids. The most studied and applied strategy is the encapsulation of these highly lipophilic molecules in emulsion based systems at micro and nano scales (Recharla et al., 2017; McClements, 2015). Successful encapsulation of purified carotenoids in micro and nano-emulsions have been done by many research groups (Gomes et al., 2017; Davidov-Pardo et al., 2016; Gul et al., 2015; Zhang et al., 2015; de Paz et al., 2013; Hejri et al., 2013).

At this thesis are provided efforts on developing products with structured food matrix that modulate the stability and bioaccessibility of carotenoids from Brazilian fruits. The research was performed in four phases: 1) screening of carotenoid-rich fruits from a set of eleven pre-selected; 2) defining the carotenoid profile and bioaccessibility of the two chosen fruits; 3) developing products with the two fruits by applying the food structure design approach; 4) and evaluating the impact of processing and formulation on the carotenoid stability and bioaccessibility.

At least three topics approached in this work should be highlighted. Firstly, the exploration of eight fruits that are native from Brazilian biomes – Buriti (*Mauritia flexuosa*), Pitanga (*Eugenia uniflora*), Araçá-boi (*Eugenia Stipitata*), Seriguela (*Spondias purpurea*), Cambuiti-cipó (*Sageretia elegans*), Jaracatiá (*Jaracatia spinosa*), Capeba (*Odontocarya acuparata*) and Pitangatuba (*Eugenia neonitida*) – and three exotic fruits that are well adapted to Brazilian climate – Acerola (*Malpighia emarginata*), Dovialis (*Dovyalis abyssinica*) and Abriçó-da-praia (*Mimusopsis comersonii*). In addition, the food structure design is an innovative approach in the food product development field, that is aligned with the current demands of consumers for healthier food and improved quality of life. Moreover, the methodologies applied for assessing the carotenoid bioaccessibility – *i.e. in vitro* digestion, uptake by Caco-2 cells culture and dynamic gastrointestinal system – are top of the art techniques in this research field.

This thesis is organized in four chapters, each one related to four phases of the present research, and to the manuscripts submitted for publication. At the first chapter are presented the nutritional value of the 11 fruits that integrated the screening and the carotenoid contents and bioaccessibility by *in vitro* digestion of 9 that were not selected for the product development. At the second chapter, pitanga and buriti are thoroughly studied regarding their carotenoid profile, bioaccessibility and uptake by caco-2 cells culture. The third chapter presents the food structure design of microemulsions of pitanga and buriti pulps together with oil and surfactant, produced by highspeed homogenization and ultrasound processing. Finally, the fourth chapter reveals how the processing and formulation impact carotenoid release, retention, encapsulation, stability to digestion and bioaccessibility throughout the dynamic gastrointestinal system.

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2 SCREENING OF ELEVEN NON-CONVENTIONAL TROPICAL FRUITS – PROXIMAL COMPOSITION, FIBERS, ANTIOXIDANT POTENTIAL AND CAROTENOID BIOACCESSIBILITY

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Abstract

Eleven non-conventional tropical fruits were evaluated regarding their nutritional value, antioxidant potential, carotenoid contents and bioaccessibility. The fruits were chosen due to their spread through the Brazilian territory: araçá-boi (*Eugenia Stipitata*), jaracatiá (*Jaracatia spinosa*), cambuí (*Sageretia elegans*), seriguela (*Spondias purpurea*), capeba (*Odontocarya acuparata*), pitangatuba (*Eugenia selloi*), pitanga (*Eugenia Uniflora*), buriti (*Mauritia flexuosa*), acerola (*Malpighia emarginata*), dovialis (*Dovyalis abyssinica*) and abricó-da-praia (*Mimusopsis comersonii*). Results have shown that these fruits are rich in dietary fibers and minerals, have high moisture content, and are low in proteins and lipids, excepting buriti that have 19% of lipids. Twelve carotenoids were analyzed and results varied greatly between fruits ranging 0.04 – 104 µg/g wet weight. Xanthophylls have great presence, being higher than carotenes for araçá-boi, seriguela, pitangatuba and dovialis. Bioaccessibility also varied greatly between fruits and between different carotenoids ranging 2 – 75% . Although the clear fruit matrix effect, xanthophylls were more bioaccessible than carotenes, while lycopene and γ-carotene presented the poorest bioaccessibility. The present study is fundamental to expand the knowledge about the fruit properties, carotenoids bioaccessibility and potential benefits for health, as well to preserve natural resources and promoting new fruits for human nutrition.

Keywords: Brazilian native fruit; Bioavailability; Xanthophylls; Carotenes; *in vitro* digestion;

2.1. Introduction

The great biodiversity found in South American biomes, especially the ones inside Brazilian territory (such as Atlantic Forest, *Cerrado* and Amazon Forest), represents an important source of new plants for human nutrition, new flavors and new materials for food industry [1]. The recognition that healthy diets can effectively prevent the development of certain diseases provided a whole new perspective for food and nutritional sciences, food market and, mainly for people's food habits. Many studies consistently link the intake of carotenoids from fruits and vegetables with the improvement of the eye health, the cognitive function, the cardiovascular health, the immune system functions, and the prevention of type 2 diabetes and some types of cancer (breast, cervical, ovarian, colorectal) [2,3]. Many Brazilian fruits have been presenting good results when tested for these properties and bioactive compounds (antioxidant, anti-inflammatory, anti-cancer and provitamin A) [1,4,5]. This report resume the exploration of eleven tropical fruits that have their geographical distribution widespread in Brazilian territory: araçá-boi (*Eugenia Stipitata*), jaracatiá (*Jaracatia spinosa*), cambuíti (*Sageretia elegans*), seriguela (*Spondias purpurea*), capeba (*Odontocarya acuparata*), pitangatuba (*Eugenia selloi*), pitanga (*Eugenia Uniflora*), buriti (*Mauritia flexuosa*), acerola (*Malpighia emarginata*), dovalis (*Dovyalis abyssinica*) and abricó-da-praia (*Mimusopsis comersonii*). According to the Re flora program and herbarium [6] some of these fruits are native from Brazilian biomes (aráçá-boi, jaracatiá, cambuíti, capeba, pitangatuba, pitanga and buriti). The other four fruits were introduced in Brazil and now they can naturally occur in many places, especially at southeast and northeast regions and along to the Brazilian coast.

Seriguela, pitanga, buriti and acerola already have commercial production and distribution in Brazil for internal consume *in natura* and for agroindustry processing as pulp, extracts (for cosmetics and supplements), oil (buriti), jelly and other products. Brazil also exports a minority of these products and fruits. There is effort for improvement of the commercial production and agroindustry of araçá-boi, jaracatiá, and dovalis. However these fruits are little know and integrates only local markets, ecotourism and ethnic foods. Cambuíti, capeba, pitangatuba and abricó-da-praia, despite they have high yield per plant, it is only consumed fresh by local people or are completely ignored for consumption [7 – 9].

The research with Brazilian fruits are getting more attention as there are reported interesting data regarding their bioactive compounds and health benefits. Previous works reported great amounts of provitamin A carotenoids in buriti [5], identified a diversity of carotenes (*e.g.* lycopene) and xanthophylls (*e.g.* rubixanthin) in pitanga [10] and provided

evidence that araçá-boi could be used as nutraceutical ingredient in the production of functional foods due to its carotenoid pattern [11]. The genus *Eugenia* has been presenting many fruits with high antioxidant activity against biological radicals and anti-inflammatory activity [1]. Acerola presents very high antioxidant activity due to its high contents of vitamin C and polyphenols [4]. Nutritional value of tropical fruits are of great interest to local communities that ingest these fruits regularly, *e.g.* buriti can be source of minerals, fibers, vitamin A and important fat acids in the diet [12].

Carotenoids are among the most important compounds in fruits that provide the mentioned health benefits. In plants carotenoids have primary and secondary functions – *e.g.* they provide photoprotection (primary) especially in the leaves and play important role of fruit communication attracting seed dispersing animals (secondary). So that they are responsible for yellow to red color in fruits that also stimulate the palate in humans. Carotenes and xanthophylls occur in the plants in several different forms: water soluble complexes with proteins; esterified with fat acids; solubilized in lipids. The human digestion and absorption of carotenoids from fruits suffer influence of the carotenoid species that are present, the fruit matrix, their chemical state, the amount ingested and absorption modifiers. These factors can be studied by *in vitro* methodologies (including *in vitro* digestion) and allows to estimate their bioaccessibility, *i.e.* the amount of the carotenoid released from the food matrix during digestion and made available for absorption [2, 3].

Therefore, this research aimed to expand the basic knowledge about the tropical fruits from Brazil. Eleven fruits were studied regarding their proximate composition, dietary fibers, antioxidant potential, carotenoids composition and their bioaccessibility. Especial emphasis is given to carotenoids since these compounds in fruits are among the main providers of health benefits.

2.2. Materials and methods

2.2.1. Fruit Samples

The eleven tropical fruits included in this study (Fig 1) were chosen due to their availability, yield, yellow to red color of their pulps when mature, and that are native and/or well adapted to Brazilian climate. Araçá-boi, jaracatiá, cambuí, seriguela, capeba, pitangatuba, dovalis and abricó-da-praia were collected (around 0.5 to 2 Kg of each) at *Sítio de Frutas Raras*, Campina do Monte Alegre, São Paulo, Brazil (-23.5359370, -48.5124060). Whole and ripe fruits were collected, washed and transported in Styrofoam boxes with ice. Ripe pitanga

and acerola were harvested (around 3 Kg of each) from the orchard located at the Luiz de Queiroz College of Agriculture (ESALQ), Piracicaba, São Paulo, Brazil. Buriti pulp was produced by the co-op Sertão Veredas located in Chapada Gaúcha, north of Minas Gerais state, Brazil. The farmers collected ripe buriti fruits, peeled them, added warm water, and crushed them to separate the seeds and pulp. They were frozen and shipped (around 5 Kg) to Piracicaba, São Paulo. Excepting buriti, the fruits were sanitized and pulped in the Department of Agri-food Industry, Food and Nutrition (LAN/ESALQ). Araçá-boi, jaracatiá, seriguela, capeba, pitangatuba and dovalis had their seeds discarded. Their pulp and peel were homogenized with liquid nitrogen in a analytic mill (A11 Basic Mill IKA®). Cambuí was pulped in the same way, however the seeds have not been removed, since they are too small and are ingested when they are eaten. Abriçó-da-praia had their seeds and hard skin removed, and since its pulp is very soft, it was only mashed and stored. Whole pitanga and acerola were pulped in a juicer (Philips Walita®) where their seeds were retained. All the samples were stored in 50 mL tubes with blanket of nitrogen gas, sealed, packed in black bags and stored frozen at -25°C until the analysis.

2.2.2. Reagents

All-*trans*-Violaxanthin (VioX), all-*trans*-lutein (Lut), all-*trans*- α -carotene (α -C), all-*trans*- β -carotene (trans- β C), all-*trans*- γ -carotene (trans- γ -C) and all-*trans*-lycopene (Lyc) standards for HPLC, and also the enzymes for *in vitro* digestion, were purchased from Sigma-Aldrich (St Louis, MO, USA). Only chromatographic grade organic solvents were used for carotenoid extraction and HPLC analysis (Tedia®, Fairfield, OH, USA). All other reagents were analytical grade.

Figure 1 – Fruits of the study. The pictures of araçá-boi, jaracatiá, cambuí, seriguela, dovialis, capeba, pitangatuba, acerola, pitanga and abricó-da-praia are available on-line [7] and published with permission. Buriti picture are available online and have creative commons license [13]

2.2.3. Proximate composition and dietary fibers

It was carried out classic protocols for analyzing the proximate composition. Moisture by drying samples (105°C), ash by incinerating samples (550°C) and proteins by Kjeldhal followed the AOAC [14] established protocols. Lipid content was determined by gravimetry after solvent extraction [15]. Soluble and insoluble dietary fiber analysis was done by the enzymatic-gravimetric method [16]. This protocol was chosen since these fruits have very low amounts of starch and protein (< 2% wet weight). Total carbohydrates was calculated by difference and energy content converted from the proximate analysis results following the AOAC [14] recommendation.

2.2.4. Radical scavenger capacity

For assessment of the radical scavenger capacity it was used the protocol described in Rufino *et al.* [4]. Samples were extracted with solution of MeOH 50% (60min) at room temperature, centrifuged (24500 g for 15min) and supernatant collected. Residues were extracted with acetone solution 70% (60 min) at room temperature and supernatants were collected after centrifuging. Both extracts were combined and had the volume adjusted. ABTS and DPPH assays were performed following the protocol [4]. Results were calculated using standard curves of Trolox and data expressed in μM of Trolox equivalents/g of wet weight.

2.2.5. Carotenoid extraction and saponification

For extracting the carotenoids from fruit samples it was realized the procedures described by Kimura *et al.* [17]. In summary, homogenized aliquots (0.2 – 1 g, depending of the fruit color) were extracted with cold acetone and 3g of celite using an porcelain mortar and pestle, until the samples gets colorless. The extracts were filtered under vacuum and partitioned in separatory funnel to 15 mL of petroleum ether. Following, a saponification step was carried out to hydrolyze the xanthophyll esters. The extracts were kept 16h in the dark mixed with a solution of KOH (10%) in methanol containing 0.01% of BHT. The flasks received a blanket of nitrogen gas to avoid carotenoid oxidation. Then, the extracts were washed 5 times with distilled water, passed through funnel with Na_2SO_4 and then dried under nitrogen flux. Carotenoids from micellar fraction were extracted as described in Berni *et al.* [18] – aliquots of 5–10 mL were agitated 1 min in vortex with 10 mL of THF/MeOH (1:1, v/v) followed by addition of 10 mL of hexane and then centrifugation at 800 g for 10 min at 4 °C. The upper layer was collected and the saponification step was carried out as described above. The dried extracts were resuspended in MeOH:MTBE (1:1, v/v), total solvent volume was dependent on

the carotenoid concentration (0.2 – 4 mL). Extracts were filtered (PTFE 0.22µm) before injection into the HPLC system.

2.2.6. Carotenoid analysis by HPLC-DAD

Fruit carotenoids were analyzed by a HPLC-DAD system (Shimadzu® LC-20A Prominence) with a polymeric YMC™ C30 column (150 mm x 4.6 mm, 5 µm particle size). The analytic condition is described in Kimura *et al.* [17] consisting in: MeOH and MTBE as mobile phase in linear gradient starting at 90:10 (MeOH:MTBE) to 40:60 in 60 min, returning to initial conditions and kept for 15 min; flow rate was 0.8 mL/min; column temperature was 30°C; injection volume ranged 10 µL – 60 µL. Standard curves of VioX, Lut, α -C, *trans*- β C, *trans*- γ -C and Lyc were done for carotenoid identification and quantification. For identifying carotenoid without its respective standards we compared their diode array absorption spectra and elution order with the certified reference material (CRM485) reported in Kimura *et al.* [17]. All-*trans*-zeaxanthin (ZeaX), all-*trans*- β -cryptoxanthin (β CX), 9-*cis*- β -carotene (9-*cis*- β C), 13-*cis*- β -carotene (13-*cis*- β C) and 15-*cis*- β -carotene (15-*cis*- β C) were calculated using the *trans*- β C standard, since they carry the same chromophore and similar max absorption peak (λ_{max}). The *cis*- γ -carotene was calculated using the *trans*- γ -C curve.

2.2.7. Carotenoid bioaccessibility by *in vitro* digestion

In vitro digestion was carried out according to Garret, Failla and Sarama [19]. Briefly, fruit samples were processed with a solution of basal salts (NaCl 120 mol/L, CaCl₂ 6 mmol/L and KCl 5 mmol/L) in high speed homogenizer (5000 rpm, 1 min). Excepting buriti, it was added 2% (wt/wt) of soybean oil to the fruit samples as minimum lipids needed for mixed micelles formation. Artificial saliva containing 106 units/mL of α -amylase (Sigma® A3176) was added (6 mL) for simulating the oral phase. The mixtures were incubated 37 °C, 10 min under agitation (rocker shaker 120 rpm). The pH was adjusted to 2.5 and 2 mL of pepsin solution (Sigma® P7000; 50000 units/mL) was added. Flasks were incubated for 1 h, 37 °C, 120 rpm. The pH was raised to 6.0 to stop the gastric phase. Then, 3 mL of bile extract solution (Sigma® B8631; 40 mg/mL), and 2 mL of pancreatin (Sigma® P1750, 4000 units/mL) and lipase (Sigma® L3126, 1000 units/mL) solution were added. Before incubation of the intestinal phase for 2h, 37°C, pH was adjusted to 6.5. Aliquots of the digesta were centrifuged at 5000 g, 60 min, 4°C until aqueous phase separation. The upper phase contains the mixed micelles formed with the bioaccessible carotenoids. The ratio between carotenoids from mixed micelles and the initial content of the fruit pulps predicts their bioaccessibility. All procedures to avoid carotenoid

degradation during digestions were taken – blanket of nitrogen gas in the tubes, protection against direct light, ice basket during the manipulation of samples and low room temperature. Micellar phases were stored at -25°C until carotenoid analysis by HPLC-DAD.

2.2.8. Statistics

Analyses were done in triplicate and data was tested for significant differences using ANOVA and Tukey statistics at 95% reliability ($p < 0.05$). Software used for the ANOVA and Tukey tests was the Statistical Assistance software Assistat 7.7 beta (UFCEG, Campo Grande, PB, Brazil).

2.3. Results and discussion

This manuscript is part of a bigger research that aims to identify new fruits for innovation on food product development. We were looking for fruits with high carotenoid contents and bioaccessibility. Another characteristics of the fruit were also seek, *e.g.* their nutritional composition, potential for commercial use, different taste and consumer's interest. So that proximate composition and antioxidant potential were assessed along with carotenoid content and bioaccessibility. Data presented here is the first step of this research project.

The results of the proximate composition from the eleven fruits are listed in table 1. The energy provided by 100g of each fruit ranged from 15 Kcal (araçá-boi) to 198 Kcal (buriti). Usually fruits have very low energy value, what can be verified by the general high moisture with low contents of lipids and sugars (Table 1). Inversely, buriti presented the highest lipid content and lowest moisture, therefore is the most caloric fruit in this study. Araçá-boi, pitangatuba and pitanga presented high moisture, respectively, 93.4, 93.8 and 83.5 %. These three fruits from the same genus (*Eugenia*) are very appreciated for consumption due to its juicy pulp. Jaracatiá presented the highest ash content. it was not found any data in the literature regarding the proximate composition of this fruit. Since ash results indicates the content of minerals, especially the ones that are micronutrients for humans, like calcium and magnesium, it is important further studies to analyze the mineral profile of jaracatiá. The protein content usually is low in fruits, so that the highest protein contents (around 1%) were from jaracatiá and acerola.

Regarding soluble and insoluble fibers abricó-da-praia is the fruit that presented the highest concentration of these components (table 1). Dietary fibers are very important for feces formation and transit in the intestine, so that they can affect the digestion and bioaccessibility of many nutrients. Carbohydrates results exclude the dietary fibers, so that its values represent

the soluble sugars of fruits. The fruits with the higher carbohydrates (table 1) are seriguela, pitanga and abricó-da-praia, and therefore with the most sweet taste.

The radical scavenging capacity of the unconventional tropical fruits is presented in the figure 2. Acerola presented the highest equivalents of Trolox for the DPPH assay and the second place for the ABTS. In a study of 18 non-traditional fruits from Brazil, Rufino *et al.* [4], reported that acerola is very rich in vitamin C, a strong antioxidant that acts especially in the DPPH assay. However in their study many other Brazilian fruits – *e.g.* yellow mombin (*Spondias mombin*), cashew apple (*Anacardium occidentale*), umbu (*Spondias tuberosa*) and açai (*Euterpe oleracea*) – presented higher antioxidant capacity than acerola due to their polyphenolic compounds [4]. The polyphenolic composition also can explain why the cambuí in the ABTS assay had the highest result (figure 2). This fruit is red-to-purple colored, what is typical from fruits that are rich in anthocyanins. If assuming similarities between cambuí and açai, it is noteworthy that açai had higher antioxidant potential (ABTS) than other 14 Brazilian fruits analyzed [4]. An multivariate analysis of 44 tropical fruits (native and exotic from Brazil) regarding their antioxidant potential resulted in a homogeneous distribution of the majority fruits. However some fruits distanced from the main group due to specific components. Namely, vitamin C in acerola, flavonoids in araçá (*Psidium guineenses*) and pitanga, and monomeric anthocyanins in açai [20].

Excluding acerola and cambuí due to their significative higher radical scavenging capacity, all the other fruits remained in a similar extent for trolox equivalents in both tests, DPPH and ABTS. The ranged observed of 1.77 – 7.8 μM trolox/g (DPPH) and 8.6 – 39.8 μM trolox/g (ABTS) also are in agreement to the literature [4,11,20]. Many other studies have been showing high antioxidant capacity for Brazilian native fruits and identifying some fruits as sources of many bioactive compounds [1]. There is a need to move forward to proof the health benefits of these fruits establishing them as functional foods and elucidating their mechanisms of action as well the impact of processing.

Table 1 – Proximate composition and dietary fibers results for the unconventional tropical fruits

Fruits	Energy (Kcal/100g)	Moisture ¹ (%)	Protein (%)	Ash (%)	Lipids (%)	Soluble fibers (%)	Insoluble fibers (%)	Carbohydrates (%)
Araçá-boi	15.46	93.4a	0.41c	0.21g	0.24c	0.10f	2.78e	2.91
Jaracatiá	44.8	82.4e	1.03a	1.78a	0.24c	0.45c	4.50d	9.63
Cambuí	52.65	80.5f	0.48c	0.48ef	0.69c	0.15ef	6.56c	11.13
Seriguela	73.97	77.4g	0.47c	0.62d	0.26c	0.80b	3.05e	17.43
Capeba	45.25	88.4c	0.23d	0.58de	0.68c	0.10f	0.30f	9.68
Pitangatuba	22.15	93.8a	0.17d	0.10h	0.43c	0.09f	0.99f	4.40
Dovialis	42.04	88.6c	0.19d	0.66d	0.42c	0.23def	0.53f	9.38
Acerola	30.91	91.2b	1.08a	0.31g	0.53c	0.24def	1.144f	5.45
Abriçó-da-praia	62.49	69.5h	0.71b	1.20b	1.17b	0.99a	14.12a	12.28
Buriti	198.13	63.2i	0.68b	0.92c	19.20a	0.39cd	9.94b	5.70
Pitanga	54.32	83.5d	0.62b	0.42f	0.40c	0.28de	2.71e	12.07

¹Data are mean of three replicates (% of wet weight). 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 fruits.

Figure 2 – Radical scavenger capacity of unconventional tropical fruits. Results are equivalents of Trolox ($\mu\text{M/g}$ wet weight). Analysis of variance ANOVA ($n=3$) and Tukey test for means comparison were carried out. Different letters correspond to significant difference ($p<0.05$) between fruits

Figure 3 – Representative chromatograms of carotenoid extracts from unconventional tropical fruits.

Overall observation on the carotenoid content of analyzed tropical fruits (figure 3, Table 1) demonstrates that carotenoids with provitamin A activity are the majority – *trans*- βC , $\alpha\text{-C}$,

β -CX, 9-*cis*- β C, 13-*cis*- β C, 15-*cis*- β C. The *trans*- β C is present in all samples and is the main carotenoid of 8 fruits from the 11 analyzed. Pitangatuba, seriguela and dovalis present xanthophylls as the major carotenoids, specifically β -CX. This profile of carotenoid distribution among tropical fruits is well demonstrated by the database of carotenoid contents published by Dias *et al.* [21]. At this database, *trans*- β C is reported in almost all tropical and subtropical fruits listed, followed by large presence of xanthophylls, especially β -CX and Lut, while Lyc occurs only in some fruits [21]. Pitanga and buriti results for carotenoids analysis are not presented in this chapter because they were selected for food product development. The study regarding pitanga and buriti carotenoids can be detailed read on next chapter. Capeba is the fruit presenting the higher contents of all carotenoids that were identified and calculated. Also, *trans*- γ -C, *cis*- γ -C and Lyc were identified in capeba, these are uncommon carotenes in fruits. The values of *trans*- β C found (Table 1) also coincide with the ranges presented at Dias *et al.*[21] for acerola, seriguela, araçá-boi, and pitanga (despite pitanga's data is not shown here). The other 7 fruits were not found at this database.

By searching the scientific names of jaracatiá, cambuí, capeba, pitangatuba and abricó-da-praia it was not found results of carotenoids from these fruits. It is important to mention that naturally occurring carotenoids have great variation between samples due to ripening, sunlight incidence and soil composition [2]. The RAE (Table 1) was calculate according to FAO [22] and shows that the consumption of these fruits would contribute to the vitamin A intake, excepting cambuí and abricó-da-praia. The consumption of 100 g of Capeba would surpass 100 % of RDI (Recommended Daily Intake) of 450 μ g/d RAE for children 4-6 years of age [22].

In Table 1 there is the bioaccessible amounts of each carotenoid found in the mixed micelles formed after *in vitro* digestion, and in Figure 4 there is the bioaccessibility (%), *i.e.* the efficiency of micellarization of carotenoids as the ratio of the initial amounts in fresh fruit and the bioaccessible fraction. Digested capeba presented the bioaccessible amounts of all carotenoids higher than any other fruit in the Table 1. Among the fruits that most provide bioaccessible carotenes the first are capeba and jaracatiá, Brazilian native fruits, followed by seriguela and acerola, exotic fruits. Pitangatuba appears, after capeba, as a greater provider of bioaccessible xanthophylls (the sum of β -CX, VioX, Lut and ZeaX).

Table 2 – Carotenoid composition found for the unconventional tropical fruits and the bioaccessible amounts of each carotenoid

Fruits	trans-βC ¹		α-C		β-CX		9-cis-βC		13-cis-βC		15-cis-βC		
	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	
<i>Araçá-boi</i>	2.10c	0.29b	0.65b	0.30c	2.44d	0.35d	0.18cd	0.04c	0.16c	0.03c	0.11d	0.05c	
<i>Jaracatiá</i>	10.6b	1.91b	1.55b	0.09de	0.53ef	0.04e	0.39cd	0.035c	0.85bc	0.026c	0.63bcd	0.02c	
<i>Cambuítí</i>	0.70c	0.073b	0.36b	nd	0.05e	0.01e							
<i>Seriguela</i>	2.89c	0.84b	0.97b	0.23cd	5.94c	1.29c	0.07d	nd	0.13c	0.03c	0.08d	0.02c	
<i>Capeba</i>	104.1a	32.6a	47.5a	14.85a	13.99a	3.20a	18.37a	7.01a	12.1a	6.05a	8.81a	3.69a	
<i>Pitangatuba</i>	3.35c	1.11b	2.45b	0.69b	4.48c	1.89b	1.83b	0.47b	1.56b	0.53b	1.29b	0.49b	
<i>Dovialis</i>	2.4c	0.25b			9.0b	1.25c	0.9c	nd	0.7bc	0.04c	0.3cd	0.04c	
<i>Acerola</i>	11.7b	1.52b	1.24b	0.06e	1.92de	0.22de	0.73cd	nd	1.44b	0.05c	1.19bc	0.04c	
<i>Abricó-da-praia</i>	0.11c	0.016b	0.04b	0.009e									
	VioX		Lut		ZeaX		trans-γ-C		cis-γ-C		Lyc		RAE ² (μ/100g)
	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	Fresh	Bioac.	
<i>Araçá-boi</i>	1.04b	0.24b	1.60b	0.15d	0.55b	0.25c							31.5
<i>Jaracatiá</i>			0.33ef	0.035de									104.8
<i>Cambuítí</i>			0.59de	0.101c									7.5
<i>Seriguela</i>			1.63b	0.80b	0.22b	n.d.							54.2
<i>Capeba</i>	30.98a	2.07a	2.21a	1.64a	19.41a	4.93a	11± 0.5	0.42± 0.03	7.3± 2.4	0.25± 0.02	6.2± 0.5	0.14± 0.02	1290
<i>Pitangatuba</i>	4.36b	0.69b	0.84cd	0.40c	4.44b	1.37b							76.3
<i>Dovialis</i>													65.4
<i>Acerola</i>			1.00c	0.03e									235
<i>Abricó-da-praia</i>			0.164f	0.066d									1.08

¹Data are mean of three replicates (μg/g of wet weight). 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 fruits, nd = not detected. Retinol activity equivalents calculated according to FAO[22]

¹Data are mean of three replicates (μ g/g of wet weight). 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 fruits. nd = not detected. Retinol activity equivalents calculated according to FAO[22]

Figure 4 – Bioaccessibility of carotenoids of unconventional tropical fruits. Results are the ratio between the carotenoid from fresh fruits and the amounts micellarized after *in vitro* digestions (%). Analysis of variance ANOVA (n=3) and Tukey test for means comparison were carried out. Different letters correspond to significant difference ($p < 0.05$) between carotenoids from each fruit

It was not found studies that determined bioaccessibility of carotenoids from these fruits. Therefore we compared the bioaccessible amounts with other common tropical fruits. While Capeba provide approx. 33 $\mu\text{g/g}$ for *trans*- βC and 3 $\mu\text{g/g}$ for $\beta\text{-CX}$ in the bioaccessible fraction, oranges provided 0.02 $\mu\text{g/g}$ for *trans*- βC and 0.04 $\mu\text{g/g}$, mandarins 0.1 $\mu\text{g/g}$ for *trans*- βC and 1.8 $\mu\text{g/g}$ [23], mango 8.9 $\mu\text{g/g}$ for *trans*- βC and papaya 2.4 for *trans*- βC [24]. A beverage (50:50 v/v of water and pulp) made with caja (*Spondias mombim*), also a brazilian native fruit from the same genus of seriguela (*Spondias purpurea*), formed mixed micelles during *in vitro* digestion containing: 0.06 $\mu\text{g/g}$ of *trans*- βC , 0.06 $\mu\text{g/g}$ of $\alpha\text{-C}$, 0.15 $\mu\text{g/g}$ of $\beta\text{-CX}$, 0.08 $\mu\text{g/g}$ of Lut and 0.03 ZeaX, excluding the esterified xanthophylls [25]. Therefore, our data of bioaccessible amounts in the mixed micelles after *in vitro* digestion are slightly higher than the found in literature for another fruits, what can be explained by the high initial content in the fresh tropical fruits analyzed here.

The bioaccessibility of carotenoids from each fruit is presented in the figure 4. For the araçá-boi fruit, ZeaX, 15-*cis*- βC and $\alpha\text{-C}$ were ~45% bioaccessible, being at least 25% higher than the other carotenoids. Jaracatiá had low carotenoid bioaccessibility when compared to the other fruits – ranging from 2% for 13-*cis*- βC to 18% for *trans*- βC . There were no significant difference between the bioaccessibility of Lut, $\beta\text{-CX}$ and *trans*- βC from cambuití. Seriguela presented a range of 18% to 49% bioaccessibility, being Lut the most bioaccessible. The *in vitro* digestion of capeba revealed 75% of Lut bioaccessibility followed by the *cis*- βC isomers (38-50%), $\alpha\text{-C}$ and *trans*- βC (~31%), $\beta\text{-CX}$ and ZeaX (22 - 26%), and finally the Lyc, *trans*- $\gamma\text{-C}$, *cis*- $\gamma\text{-C}$ and VioX had the lowest bioaccessibility (2-6%). Pitangatuba presented the lowest bioaccessibility for VioX (15%) and the highest for Lut (45%). For dovalis the bioaccessibility was 13% for $\beta\text{-CX}$, 8% for *trans*- βC followed by the other carotenes, but not significantly. Acerola had very low bioaccessibility of its carotenoids, being $\beta\text{-CX}$ and *trans*- βC the most efficiently incorporated into mixed micelles, with 11% and 13% respectively. Finally, abricó-da-praia presented Lut as the most bioaccessible (39%), $\alpha\text{-C}$ (24%) and *trans*- βC (14%). These results are very consistent with literature for the range of bioaccessibility of several carotenoids from many different fruits [24, 26 – 28]. Likewise, in accordance that usually xanthophylls are more bioaccessible [30, 31] than carotenes, and that Lyc and VioX in most cases have very low bioaccessibility [26, 28]. It is important to mention that the *cis* isomers of *trans*- βC and *trans*- $\gamma\text{-C}$ found in the micellar phase could be affected by the carotenoid extraction and saponification steps, since at this stage the carotenes are already released and can suffer stress during this long analysis period, despite all the precautions that were taken (BHT, light protection and room temperature controlled under 25°C).

More examples of carotenoid bioaccessibility from tropical fruits could be mentioned. Papaya grow in India can show bioaccessibility of 8.2% for *trans*- β C and 3.4% for Lyc [31] or 35% for *trans*- β C [24]. The differences are probably due to year and location of production, varieties and maturity stage. Veda, Platel and Sirinivasan [24] reported the bioaccessibility of *trans*- β C ranging 24% - 39% in six different varieties of mango. *In vitro* digestions of seven different varieties of peppers provided results of bioaccessibility with many differences between than, although bioaccessibility of β -CX (30% - 112%), Lut (36% – 106%) and ZeaX (34% - 106%) results were always higher than *trans*- β C (6% - 16%). Lycopene bioaccessibility both for tomatoes and papaya was < 5% when analyzed by the same *in vitro* digestion protocol [30]. Therefore in plant foods the carotenoid bioaccessibility are very affected by food matrix and particular characteristics, like maturity and growing conditions.

2.4. Conclusions

Eleven unconventional tropical fruits were studied as new plants for human nutrition. Some of these fruits have their proximate composition, dietary fibers and carotenoid contents reported for the first time. The fruits presented high contents of fibers and low energy values, excepting buriti due to its 19% of lipids. Acerola and cambuí have much higher radical scavenging capacity than the other fruits. Jaracatiá, seriguela, capeba, pitangatuba and acerola highlighted due to their carotenoid diversity and concentration. The *in vitro* digestion results presented variations between fruits mainly due to their food matrix, although generally xanthophylls were more bioaccessible than carotenes. Lyc from capeba presented low bioaccessibility as expected. The scientific knowledge together with the commercial exploitation of tropical fruits, especially the Brazilian natives, can stimulate sustainable development, better food habits, protection against biopiracy and the innovation in food systems.

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3 CAROTENOIDS FROM PITANGA (*EUGENIA UNIFLORA*) AND BURITI (*MAURITIA FLEXUOSA*) FRUITS: PROFILE, STABILITY, BIOACCESSIBILITY AND CELLULAR UPTAKE STUDIES BY *IN VITRO* DIGESTION COUPLED TO CACO-2 CELLS CULTURE

Chapter submitted to Journal of Agricultural and Food Chemistry

Abstract

There were analyzed the carotenoid profile, stability during *in vitro* digestion, bioaccessibility (micellarization due to bile salts) and absorption (uptake by Caco-2 cells cultures) of pitanga and buriti fruits. Pitanga showed a more complex and diverse carotenoid profile while buriti had a higher carotenoid concentration. In general, the xanthophylls had lower stability during *in vitro* digestion than carotenes, excepting lycopene which was the least bioaccessible. The *cis* isomers of β -carotene showed an increase during simulated *in vitro* digestion. Regarding bioaccessibility, violaxanthin, lutein and zeaxanthin from pitanga were significantly more bioaccessible than other carotenes, excepting the *cis*- γ -carotene. Pitanga's lycopene and *cis* forms of β -carotene had the lowest bioaccessibilities. Xanthophylls and γ -carotene forms of buriti were significantly more bioaccessible than the other carotenoids for this fruit. The amounts of bioaccessible carotenoids shown that pitanga provides more xanthophylls while buriti provides more amounts of carotenes. The Caco-2 cells experiment has shown that an important fraction of xanthophylls are absorbed in their esterified forms. β -carotene had the highest uptake by Caco-2 cells for pitanga and buriti. Therefore, β -carotene is prevailing inside enterocytes after 4h exposition to digesta. Pitanga presented higher cellular absorption of carotenoids than buriti ($p < 0,05$). This chapter evidence the essential need to better understand the bioaccessibility of carotenoids from diverse food matrix.

Keywords: Carotenes; Xanthophylls; *Eugenia uniflora*; *Mauritia flexuosa*; Bioavailability; Caco-2 cells

Graphical abstract

Highlights

- Carotenoid profiles, their bioaccessibility and intestinal uptake from pitanga and Buriti were measured
- Xanthophylls from pitanga presented higher bioaccessibility than carotenes
- Carotenes presented higher cellular uptake from micellar fraction than xanthophylls
- Carotenoids from buriti had lower absorption by caco-2 cells than pitanga

3.1. Introduction

Food and nutritional sciences point out the health benefits of fruit bioactive compounds, specially carotenoids. Moreover, food researchers are elucidating the health-related mechanisms of carotenoids therewith promoting new ways of its consumption, processing and innovation.¹ Diverse antioxidant properties in carotenoids have been identified, highlighting unique capacity to quench singlet oxygen.² Strong epidemiological evidence show that consumption of lycopene, α -carotene and β -carotene leads to a reduction in the risk of cancer, such as breast and prostate tumors.³ Recently low carotenoid concentrations were identified in older individuals and patients with cardiovascular disease, besides an widespread carotenoid deficiency in the general population.⁴ Not all carotenoids act in the same way and are available for absorption in the human digestive system. In fact, bioaccessibility is dependent on food

matrix, intrinsic chemical structure, and carotenoid x carotenoid interactions.⁵ Therefore, it is essential to better understand carotenoid composition and bioaccessibility from new sources.

Brazil is known for its great biodiversity including several native fruits that are rich in bioactive compounds, specially carotenoids. Despite this abundance of potential functional fruits, there is a lack of scientific knowledge about their composition and health benefits. Some of these Brazilian native fruits have diverse carotenoid profiles and concentrations. This is the case of pitanga (*Eugenia uniflora*) and buriti (*Mauritia flexuosa*). Pitanga occurs naturally on the Brazilian *restinga* and other coastal semi-deciduous forests, its largest farming is at Northeast region.⁶ Pitanga fruit is used as juice, frozen pulp, and nectar that are rich in bioactive carotenoids, such as lycopene and xanthophylls^{7,8,9} Buriti is a palm fruit native in the Amazon forest, and grows in Northeast, Central-west and Southeast regions of Brazil.^{6,10} Buriti fruit has many interesting properties such as high content of polyphenolic and carotenoid compounds, mainly all-*trans*- β -carotene. Moreover, its oil has *in vitro* antibacterial activity, assists in wound healing, and is used to make cosmetics and therapeutic products.^{11,12,13}

Bioaccessible carotenoids have first to be released from the food matrix, integrated into water soluble mixed micelles in the small intestine and absorbed by passive diffusion in enterocytes due to its dispersion in the chyme aqueous phase.¹⁴ Many phenomena can influence these steps. Globular shape of chromoplasts from mango and papaya presents higher carotenoid liberation and bioaccessibility than tubular shape in tomatoes and carrots.¹⁵ Unsaturated fatty acids promote bioaccessibility and basolateral secretion of carotenoids.¹⁶ Amount and properties of pectin affect several events involved in carotenoid micellarization that may increase or decrease bioaccessibility.¹⁷ Therefore, bioaccessibility and stability of carotenoids in foods are greatly impacted by perishability, storage, preservation, and ways of consumption. Moreover, home or industrial processing of vegetable foods play a key role in bioaccessibility because it may break cellular structures liberating carotenoids for digestion.^{16,18,19} Thus, this study contributes providing information about carotenoids bioaccessibility of these unique fruits that helps understand carotenoid bioaccessibility in general foods.

3.2. Materials and methods

3.2.1. Fruit Samples

Pitanga (*Eugenia uniflora*) was harvested from the orchard located at Department of Agri-food industry, food and nutrition of the Luiz de Queiroz College of Agriculture (LAN/ESALQ), Piracicaba, São Paulo, Brazil. Red ripe pitanga was collected, selected, washed and pulped in a juicer (Philips Walita®), then frozen and stored. Buriti (*Mauritia flexuosa*) pulp was produced by the co-op *Sertão Veredas* located in *Chapada Gaucha*, north of Minas Gerais state, Brazil. The farmers collected ripe Buriti fruits, peeled them, added warm water, and crushed them to separate the seeds and pulp, which was frozen and shipped to *Piracicaba, São Paulo*. All the samples were stored in 50 mL tubes with blanket of nitrogen gas, sealed, packed in black bags and stored frozen at -25°C until the time of conducting the analysis. The samples were frozen under these conditions until analyses were done. All the analytical procedures were carried out at the Food Analyses and Nutrition laboratory, LAN/ESALQ-USP.

3.2.2. Reagents

Commercial carotenoid standards, β -apo-8'-carotenal and enzymes for *in vitro* digestion were purchased from Sigma-Aldrich (St Louis, MO, USA). Specific reference numbers of each enzyme are informed in the text. Organic solvents for extraction and HPLC analysis were chromatographic grade (Tedia®). Caco-2 cells cultures were purchased from *American Type Culture Collection* (ATCC®HTB37™). *Dulbecco's Modified Minimum Essential Medium* – DMEM, penicillin-streptomycin, fungizone, non-essential amino acids solution and L-glutamine were purchased from GIBCO™ (Made in Brazil) while heat inactivated fetal bovine serum was from Sigma-Aldrich (St Louis, MO, USA)

3.2.3. Carotenoid extraction and saponification

Aliquots for each sample changed according the step and appearance of carotenoid concentration, *i.e.* ranged 0.1-0.2 grams of fresh fruit, 5 mL of digested food, 6 – 10 mL of micellar fractions and cellular pellets of T25 flasks. For extracting carotenes and xanthophylls at same time it was chosen the method described in Tibak *et al.*²⁰ with modifications. Briefly, thawed samples were extracted in 50 mL centrifuge tubes adding 15 mL of hexane, ethanol and acetone solution (2:1:1, v/v/v) with 0.1% BHT. Tubes were mixed with a vortex and taken for 5 min in ultrasound bath and then back to the vortex for 1 minute. To the extract was added 3 mL – 5 mL of saturated salt solution and mixed with a vortex again for 30 seconds, then tubes

were centrifuged for 5 minutes at 800 g, 4°C. Upper phase was collected with a pipette and taken to dry with nitrogen gas inside the hood. For ester hydrolysis, saponification procedures were carried out with independent extracts by combining equal volumes of methanolic KOH solution (10 %, 0.1 % BHT) with the extracts.²¹ The mixtures were left overnight in dark at room temperature. Upper phase was collected and washed three times with distilled water to remove remaining KOH. To the final extract was added anhydrous sodium sulfate (~ 0.2 g) to soak remained water then taken to dry with nitrogen gas in the hood. The dried extract was dissolved in methanol and MTBE (1:1 v:v), total volume dependent on the appearance of carotenoid concentration (0.2 – 4 mL), filtered in Millex® PTFE, 0.22 µm pore size. Finally, vials were placed in the HPLC-DAD system for injection. β-apo-8'-carotenal was added randomly to samples before extraction (10 µg/mL in methanol). Average recovery of β-apo-8'-carotenal was 98.6 % ± 17.2 that allow to estimate the overall quality of carotenoid extraction and analysis.

3.2.4. Carotenoid analysis by HPLC-DAD

Chromatographic analysis of carotenoid is presented in Kimura *et al.*²² and carried out as follows. Liquid chromatographic system coupled to a diode array detector (HPLC-DAD) Shimadzu® (LC-20A Prominence) was employed. The mobile phase consisted of methanol and MTBE gradient starting at 90:10 (Methanol:MTBE) to 40:60 in 60 minutes, linear gradient, returning to initial conditions and kept for 15 minutes for re-equilibration, the flow was 0.8 mL / min, injection volume changed according to the experimental step and carotenoid concentration ranging 10 µL – 40 µL. The column used was the polymeric YMC™ C₃₀ (150 mm x 4.6 mm, 5 µm particle size). Identification of carotenoids was carried out combining commercial standards, elution order on C₃₀ column compared to a certified reference material (CRM485)²², diode array absorption spectra^{21,23} and comparisons with publications that confirmed identity of carotenoids in HPLC-MS for the same fruits.^{9,12} Standard curves of all-*trans*-violaxanthin, all-*trans*-lutein, all-*trans*-α-carotene, all-*trans*-β-carotene, all-*trans*-γ-carotene and all-*trans*-lycopene were carried out and used for carotenoid calculation. The respective r^2 values for the standard curves were 0.9452, 0.9915, 0.9998, 0.9958, 0.9965 and 0.9949. Zeaxanthin, β-cryptoxanthin, *cis* and *trans* rubixanthin and *cis* isomers of β-carotene were quantified by all-*trans*-β-carotene standard curve as they share same chromophore and similar max absorption peak (λ_{max}).

3.2.5. Carotenoid bioaccessibility by *in vitro* digestion

For the bioaccessibility analysis, an *in vitro* simulated digestion model was used as described in Failla¹⁴ and Chitchumroonchokchai, Schwartz and Failla²⁴, with slight changes. Oral, gastric and intestinal phases of *in vitro* digestion are summarized next. Fruit samples (1 – 2 g) were homogenized with 10 mL of basal salts solution (BSS, NaCl 120 mol/L, CaCl₂ 6 mmol/L and KCl 5 mmol/L) in 50mL centrifuge tubes. The same BSS was used for volume adjustment when needed. Because we previously determined only 0.4% (wet weight) of lipids in pitanga pulp, by Folch *et al.*²⁵ protocol, 2% (wt/wt) soybean oil was added for mixed micelles formation, but no oil was added to the buriti (19.2% lipid) during the *in vitro* digestion. For the oral phase 6 mL of previously prepared artificial saliva containing 106 units/mL of α -amylase (Sigma[®] A3176) was added, and incubated at 37°C for 10 min in a rocker shaker (120 rpm). Following, pH was adjusted to 2.5 with HCl 1 M and 2 mL of pepsin solution was added (Sigma[®] P7000; 50000 units/mL in HCl 100 mM). Total volume was adjusted to 40 mL and the tubes were incubated for 1 h, 37 °C, 120 rpm. The pH was raised to 6.0 with 1 M NaHCO₃ to stop the gastric phase. Porcine bile extract solution (3 mL; Sigma[®] B8631; 40 mg/mL in 100 mM NaHCO₃) and 2 mL of pancreatin (Sigma[®] P1750) and lipase (Sigma[®] L3126) solution at ~4000 units/mL and ~1000 units/mL in 100 mM NaHCO₃, respectively, were added. Before incubation for 2h, 37°C, pH and volume were adjusted to 6.5 and 50mL respectively. After the intestinal phase digestion, homogenized aliquots (12 mL) were centrifuged at 5000 g, 60 min, 4°C. The supernatant (aqueous fraction) was collected and sterile filtered (Millipore[®], 0,22 μ m pore size) generating the micellar fraction, *i.e.* bioaccessible carotenoids. The ratio between carotenoids incorporated into mixed micelle and the determined in the fresh fruit pulp predicts the carotenoid bioaccessibility. The remaining digesta was analyzed for their carotenoid concentration. The ratio between carotenoids found in digesta and the determined in the fresh fruit pulp represents the individual carotenoid stability. In all steps and storage samples were covered with nitrogen gas, sealed with parafilm[®], protected against direct light and high room temperature. Samples of digested and bioaccessible fraction were stored at -26°C until carotenoid analysis by HPLC-DAD as described above.

3.2.6. Carotenoid uptake by Caco-2 cells culture

Caucasian colon adenocarcinoma cells (Caco-2 from *American Type Culture Collection* ATCC[®]HTB37[™]) were cultivated for the evaluation of carotenoid intestinal absorption. The procedures of cells culture were the described by Garret, Failla and Sarama (1999) and Chitchumroonchokchai, Schwartz and Failla (2004). Passages 22-27 of Caco-2 cells were seeded in T25 flasks (25 cm² of area growth) at 2 – 3 x 10⁵ cells/flask. Cultures were maintained

at 37°C, humidified atmosphere, 95% air and 5% CO₂. *Dulbecco's Modified Minimum Essential Medium* (DMEM, GIBCO™) enriched with L-glutamine (1%), non-essentials amino acids (1%), penicillin-streptomycin (1%), fungizone (0.2%) and heat inactivated fetal bovine serum - FBS (15%) was utilized to maintain the cells growing until the culture acquire confluence (~6 days). After confluency FBS was reduced to 10%. Media was changed every other day until cells differentiate in enterocytes, 10-14 days. The quality of cellular cultures was checked visually by inverted microscope. Attachment, cell growth, absence of contamination and number of domes were considered for controlling the viability of monolayers that are ready for experiment. Monolayers were washed with *Minimum Essential Medium* (MEM, GIBCO™) to carry out experiments. A fresh complete DMEM, pH 6.5, containing 25% of the micellar fraction obtained from the *in vitro* digestions was added to the monolayers (final volume 5 mL) and incubated for 4 hours. Thereafter, Caco-2 monolayers were washed once with 2 mg/L of albumin in cold PBS (pH 7.0) and twice with only cold PBS (pH 7.0), harvested with rubber scrapper, collected and centrifuged at 500 g/10 min, 4°C. Cells pellets were covered with nitrogen gas, sealed and stored at -26°C until carotenoid extraction. Protein content of cell samples was determined by a Bradford rapid assay (BIO RAD: Quick start™ Bradford) using bovine serum albumin as a standard.

3.2.7. Statistics

Carotenoid determination was done in triplicate with homogenized fresh fruit pulp then experiments for bioaccessibility were carried out independently also in triplicate. Data were analyzed for significant differences using ANOVA between carotenoid species and fruit. Means were compared using the Tukey test and significant at 95% reliability ($p < 0.05$). Software used for the ANOVA and Tukey tests was the Statistical Assistance Software Assistat 7.7 beta (UFCEG, Campo Grande, PB, Brazil).

3.3. Results and discussion

3.3.1. Carotenoid compounds of pitanga and buriti

The carotenoid analysis of pitanga and buriti fruits by chromatography enabled to separate and positively identify 14 carotenoids presented in table 1. They were identified through a combination of commercial standards, chromatographic elution sequence in a C₃₀ column, UV-vis spectra with the diode array detector and literature comparisons.^{9,12} Violaxanthin, lutein, α -carotene, all-*trans*- β -carotene, γ -carotene and lycopene were quantified

by its own standard curves while zeaxanthin, β -cryptoxanthin and rubixanthin were quantified by all-*trans*- β -carotene standard curve since some carotenoids have the same chromophore in their chemical structure and similar UV-vis spectra.²¹ Because accurate carotenoid identification and calculations are a complex task, we presented only results of the carotenoids that were confidently identified, calculated and those we could track through the *in vitro* digestion experiment.

Table 1. Parameters for carotenoid identification¹

Carotenoid	RT (min)	$\lambda_{\max}$ (nm) ¹	% III/II ¹
1. Violaxanthin	6.4	415/439/468	94
2. Lutein	11.4	421/444/472	66
3. Zeaxanthin	13.2	(423)/ 446/474	28
4. β -cryptoxanthin	19.8	(425)/450/477	29
5. 15- <i>cis</i> - β -carotene ²⁺	23.2	339/(423)/448/473	11
13- <i>cis</i> - β -carotene ²	23.4		
6. α -carotene	24.7	421/445/473	65
7. all- <i>trans</i> - β -carotene	28.2	(425)/451/477	26
8. 9- <i>cis</i> - β -carotene	30.0	338/(420)/447/472	20
9. rubixanthin	35.6	436/459/490	54
10. <i>cis</i> -rubixanthin	36.5	355/436/459/490	38
11. γ -carotene	44.1	438/461/492	57
12. <i>cis</i> - γ -carotene	44.9	294/355/(438)/464/495	54
13. lycopene	60.3	445/471/503	75

^a $\lambda_{\max}$ and % III/II were obtained from the photodiode array detector of the peak in their current mobile phase. ^b There were partial coelution of 15-*cis*- β -carotene and 13-*cis*- β -carotene.

Figure 1. Representative chromatograms of pitanga and buriti fresh pulps and the effect of ester hydrolysis on the carotenoid profile of pitanga: a) before the saponification step, mass injected in the HPLC system correspond to 1.8 mg of fresh fruit; b) after the saponification procedure, mass injected in HPLC system correspond to 1 mg of fresh fruit. c) Buriti mass injected in HPLC system correspond to 4 mg. Chromatographic conditions are described in methods. Chromatograms are processed at 450 nm.

Esterified xanthophylls were observed mostly in pitanga and minor presence in buriti samples. Representative chromatogram of pitanga (Fig. 1a) shows the presence of esterified xanthophylls and the influence of the saponification step for ester hydrolysis (Fig. 1b). Rubixanthin, β -cryptoxanthin, zeaxanthin and violaxanthin were in this order the most esterified xanthophylls detected in pitanga. High degradation of lycopene, β -carotene and γ -carotene happened during the saponification step (Fig. 1a and 1b). Xanthophylls esterified with fat acids are common in foods, specially vegetables, hence saponification reaction is needed to quantify it by HPLC-DAD. However, this mighty reaction also can cause destruction or structural transformations of carotenoids, specially the carotenes.²⁶ For buriti (Fig. 1c) no relevant amounts of the esterified xanthophylls was observed and previous test of saponification only increased the *cis* forms of β -carotene. Therefore, data of saponified samples was used only for pitanga xanthophylls. Several small non-identified peaks were observed in buriti HPLC-DAD analysis that possibly are from epoxy forms of carotenes previously identified and reported by Rosso and Mercadante.¹²

3.3.2. Carotenoid contents of pitanga and buriti

Table 2 presents data of carotenoid contents for both fruits. When compared with 23 vegetables that are good sources of all-*trans*- β -carotene listed in Fernández-García *et al.*²⁷, buriti still at least 4.9 times higher than any vegetable. More than 78% of the identified carotenoids in buriti are the *trans* and *cis* forms of β -carotene. Buriti has 8 carotenoids having provitamin A activity – that contains the β -ionone ring on its chemical structure²⁸ – in very high concentration, ranging from 19 to 434 $\mu\text{g/g}$ on wet weight basis (Table 2). It also includes a high content of lutein, 37.8 $\mu\text{g/g}$ on wet weight basis (Table 2), an important component for eyes health that may prevent macular degeneration.²⁹ Our results are similar to others found in literature, such 360 $\mu\text{g/g}$ of all-*trans*- β -carotene and 82 $\mu\text{g/g}$ of α -carotene presented in Rodriguez-Amaya *et al.*³⁰ The *cis*- β -carotene isomers naturally occurs in this fruit in high quantity, *e.g.* 59 $\mu\text{g/g}$, 18.5 $\mu\text{g/g}$ and 8.8 $\mu\text{g/g}$ respectively for the 13-*cis*, 9-*cis* and 15-*cis* β -carotene reported in Rosso and Mercadante¹², and agree with our findings. However, this palm fruit is collected inside the forests after it falls down and its edible pulp is extracted using warm water. These procedures associated to long time of transportation and storage followed by aggressive solvent extractions for analysis can generate the *cis* isomers.³¹ These reasons can explain the higher amount of 9-*cis* and 15-*cis* β -carotene found in our samples.

Pitanga results present a more complex and diverse carotenoid profile and content than buriti (Table 2 and Fig. 1). Pitanga contains lycopene as a major carotenoid that is at least twice higher than the 6 xanthophylls it brings together, an uncommon characteristic among plant foods (Table 2). All carotenoids found in pitanga in our study were already identified by HPLC-MS by Azevedo-Meleiro and Rodriguez-Amaya⁹, excluding the minor β -carotene-5,6-epoxide and *cis*-lycopene that we did not confirm present. Furthermore, the carotenoid content determined in pitanga fruits grown in Piracicaba-SP (Table 2) match with the results reported by Porcu and Rodriguez-Amaya²³ in pitanga grown in Campinas-SP, the same climate and mesoregion. Their study shows great variation of carotenoids contents in pitanga due to ripeness, climate and growing region. Noteworthy, carotenoid in fruits are part of a secondary plant metabolism that are very dynamic and dependent on plant variety, sunlight, soil, climate and maturity.³² Range variations of carotenoid and distribution through carotenoid species associated with food matrix plays an important role on bioaccessibility.^{1,5} Therefore, it is crucial to accurately determine carotenoids composition on fresh foods before going through bioaccessibility studies.

Table 2 – Initial carotenoid contents in fresh pulp, bioaccessible amounts of carotenoids obtained in micelle fraction after *in vitro* gastrointestinal digestion and its absorption by Caco-2 human intestine cells ¹

Carotenoid	Pitanga			Buriti		
	Fresh (µg/g)	Bioaccessible (µg/g)	Absorbed ² (ng/mg protein/g)	Fresh (µg/g)	Bioaccessible (µg/g)	Absorbed ² (ng/mg protein/g)
1. Violaxanthin	0.55±0.12 ^h	0.19±0.02 ^d	nd	nd	nd	nd
2. Lutein	2.33±0.18 ^f	0.75±0.08 ^c	3.99±0.15 ^d	37.8±2.8 ^{de}	19.7±0.32 ^b	44.8±3.9 ^b
3. Zeaxanthin	4.03±0.74 ^e	1.11±0.02 ^b	9.04±0.03 ^c	nd	nd	nd
4. β-cryptoxanthin	11.4±0.25 ^c	1.74±0.07 ^a	21.5±0.15 ^b	21.5±0.91 ^e	8.34±0.14 ^d	nd
5. 15- <i>cis</i> -β-carotene ³ + 13- <i>cis</i> -β-carotene ³	2.01±0.02 ^f	0.10±0.02 ^d	nd	56.8±8.4 ^{bcd}	21.6±1.03 ^b	48.2±3.3 ^b
6. α-carotene	2.58±0.17 ^f	0.39±0.01 ^d	nd	42.3±2.65 ^{cd}	14.3±0.30 ^c	13.7±1.8 ^c
7. all- <i>trans</i> -β-carotene	2.58±0.17 ^f	0.39±0.01 ^d	nd	65.0±5.63 ^b	20.9±1.38 ^b	44.3±5.9 ^b
8. 9- <i>cis</i> -β-carotene	3.98±0.26 ^e	0.77±0.19 ^c	37.9±3.4 ^a	434±16.3 ^a	115±4.17 ^a	598±20 ^a
9. rubixanthin	1.82±0.08 ^{fg}	0.14±0.07 ^d	nd	60.8±0.23 ^{bc}	21.2±1.20 ^b	56.4±1.9 ^b
10. <i>cis</i> -rubixanthin	14.2±0.37 ^b	1.48±0.15 ^a	23.4±2.78 ^b	nd	nd	nd
11. γ-carotene	6.23±0.11 ^d	0.75±0.21 ^c	6.28±0.71 ^{cd}	nd	nd	nd
12. <i>cis</i> -γ-carotene	1.42±0.17 ^{fgh}	0.31±0.01 ^d	19.6±2.3 ^b	19.8±4.15 ^e	8.86±0.31 ^d	nd
13. lycopene	0.73±0.05 ^{gh}	0.27±0.01 ^d	3.59±0.48 ^d	20.9±4.10 ^e	9.12±0.19 ^d	nd

¹Data are mean ± standard deviation of three replicates (µg/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. ² Results were standardized by mg of cell protein (2.8 mg of protein per 25cm²) per g of fresh sample. ³There was partial coelution of 15-*cis*-β-carotene and 13-*cis*-β-carotene, it was possible to identify and calculate them separately only for buriti samples.

3.3.3. Stability of carotenoids during simulated digestions

Figure 2 shows the carotenoid profile of pitanga *in natura* without saponification (2a), the carotenoids stability after *in vitro* digestion (2b), the bioaccessibility (2c), *i.e.* carotenoids that were released from food matrix and incorporated into mixed micelles, and the carotenoid uptake by human intestine cells Caco-2 (2d). Comparing first and second chromatograms (Fig. 2a and 2b) may be noted that carotenoid maintained relatively stable during *in vitro* digestion. Carotenoids from pitanga were statistically less stable than buriti ($p < 0.05$). In general, xanthophylls were less stable than carotenes, *e.g.* in pitanga only 52 % of β -cryptoxanthin and zeaxanthin remained after *in vitro* digestion (Fig. 3). Remaining carotenes ranged from 62 % for lycopene in pitanga to 96 % for α -carotene in buriti. *Cis* isomers of β -carotene showed slightly increase during simulated digestion – 104 % of stability for 15-*cis* and 13-*cis* β -carotene in pitanga and 131 % of stability for 13-*cis* β -carotene in buriti (Fig. 3). Although, carotenoid stability during *in vitro* digestion usually exceeds 75 %¹⁴, Courraud *et al.*³³ reported all-*trans*- β -carotene stability varying 31%, 100% and 40% for commercial standard, carrot juice and spinach, respectively. Courraud *et al.*³³ also reported that all-*trans*- β -carotene instability occurs mainly in gastric phase due to its high acidity. Therefore, carotenoid can be differently affected by the digestive environment and the food matrix. This may explain why carotenoids from pitanga had lower stability than buriti, since pH of pitanga whole pulp was 2.8 and of buriti 4.8. Moreover, carotenoids solubilized/emulsified in lipids are more likely protected against degradation²⁴ thus carotenoids from buriti were more stable during the *in vitro* digestion simulations.

3.1.1. Bioaccessibility of carotenoids after *in vitro* digestion

Comparison of chromatograms in figures 2a and 2c shows the carotenoids efficiency of micellarization, *i. e.* bioaccessibility. Xanthophylls being less hydrophobic than carotenes due the oxygen in their chemical structure are easily incorporated into mixed micelles³⁴, that is why the higher peaks in the chromatogram Fig. 2c corresponds to the violaxanthin, lutein, zeaxanthin and β -cryptoxanthin. Markedly lycopene has almost no incorporation into mixed micelles (Fig. 2c), in fact, its bioaccessibility was 1.1% (Fig. 4). Further, small peaks of esterified xanthophylls in micellar phase (Fig. 2c) indicate that esterification seems to impair their incorporating into mixed micelles when compared to free xanthophylls. The minor breaking of esterification by digestive enzymes seems to be irrelevant.^{16,17,34}

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.

Violaxanthin, lutein and zeaxanthin from pitanga were significantly more bioaccessible than other carotenes excepting *cis*- γ -carotene whereas lycopene along with *cis* forms of β -carotene had lowest bioaccessibility (Fig. 4). For buriti same pattern was observed, xanthophylls and γ -carotene forms being significantly more bioaccessible than the other present carotenoids (Fig. 4). These evidences show that some competition may happen between carotenoids to get into micelles during the intestinal phase of digestions.

Overall bioaccessibility of carotenoids from buriti was significant higher than pitanga ($p < 0.05$). Indeed, pitanga composition is much different from buriti. Pitanga has a considerable amount of fibers, low amount of oil, high humidity and acidity while buriti is an oily palm fruit. Presence of 19% of fat in buriti fruit against only 2.4% in pitanga – initial plus added oil for *in vitro* digestions – indicates also the influence of carotenoid:lipid ratio on bioaccessibility. Lipids in diet, mainly unsaturated fat acids, increases the micellarization and absorption of carotenes while fat acid saturation has minimal influence on the xanthophylls bioaccessibility.¹⁶ Our results (Fig. 4) corroborate with Failla *et al.*¹⁶ study that reported bioaccessibilities in a salad meal containing 3% wt/wt of different fat sources ranging 45-57% for lutein and zeaxanthin, 9-20% for all-*trans*- β -carotene and 1.5-5.3% for lycopene. For the best of our knowledge this is the first time carotenoids bioaccessibility from these unique plants was assessed. The assessment of carotenoid bioaccessibility provide essential information about potential health benefits of functional fruits. Usually, when any fruit is analytically proven to contain some bioactive components, many nutritional recommendations stimulate that fruit's consumption. Bioaccessibility data can help professionals, companies and policymakers to properly guide dietary changes to specific needs.

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 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.^{35,36} Sölyom *et al.*³⁵ reported antioxidant capacity of carotenoid rich micellar fraction by Trolox equivalent antioxidant capacity (TEAC) 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 possessing a reactive opened-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.² β -cryptoxanthin have anti-inflammatory effects, enhance the immune system and may reduce the risk of osteoporosis.^{37,38} Thereby pitanga may have much more potential as a functional fruit due to β -cryptoxanthin, zeaxanthin and lutein than lycopene bioaccessible amounts.

Regarding buriti, all-*trans*- β -carotene is the most abundant in the micellar phase. With two β -ionone rings, all-*trans*- β -carotene is converted to retinol by BCO1 and BCO2 enzymes in enterocytes twice more than other carotenenes.²⁸ Except lutein, all carotenenes from buriti have vitamin A activity, rendering buriti an interesting food for combating vitamin A deficiency in many areas of Brazil and the world. The mean requirement ingestion of retinol equivalent (RE), for adult women established by FAO³⁹ is 300 μ g RE/day. Buriti have 96.7 μ g RE/g calculated according to FAO³⁹, that considers an averaged carotenoid bioaccessibility of 25%. However, we presented that buriti have a range of 26 – 46 % bioaccessibility for its provitamin A carotenoids. Not all provitamin A carotenoids absorbed by humans are converted into retinol, *i.e.* some carotenoids such β -carotene and β -cryptoxanthin are absorbed and distributed by chylomicrons intact and accumulated in many tissues providing a protective effect against oxidative stress.⁴⁰ Rodrigues *et al.*³⁶ demonstrated that antioxidant capacity of carotenenes against the peroxy radical is dependent on the opening of the β -ionone, the chromophore extension and addition of hydroxyl groups. For example, 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, information regarding antioxidant potential of carotenoid rich fruits should be followed by comprehensive work about their bioaccessibility.

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.

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) during 4 h. Results of cellular uptake of carotenoids are presented in percent taken from media at figure 5, and in absolute amount accumulated inside the cells at table 2. β -carotene had the highest uptake and accumulation in the intestine cells for both fruits. Pitanga presented an overall uptake of carotenoids higher than buriti. There is evidences that food matrix may affect the absorption of carotenoids from foods. Previous works using the same protocol for *in vitro* digestion and cellular experiment also found differences between cassava varieties (uptake of all-*trans*- β -carotene ranging 10-20 %) and orange-fleshed sweet potato varieties (ranging 10-25%).^{41,42} Despite the very low micellarization of lycopene, its uptake from mixed micelles was relatively high (Fig. 5). Our data for carotenoid uptake by caco-2 cells compared to others found in literature, is similar in the case of lycopene^{43,44} while lutein and zeaxanthin values presented corroboration⁴⁵ and discrepancies.⁴⁶

Differences of carotenoid uptake by Caco-2 cells between green, red and yellow bell peppers, and green and red chili peppers were also noted in a great extent for β -carotene

(ranging 1.3 – 31%) in the work of O’Sullivan *et al.*⁴⁵. In agreement to our observations, they detected that although lutein (a xanthophyll) was the most abundant carotenoid in the micelles, cellular uptake of β -carotene were the highest. Therefore, interactions between carotenoids may influence the cellular absorption. Moreover, our results indicate that all-*trans*- β -carotene may have facilitated transport across the brush border. In fact, 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.⁴⁷ We attribute the β -carotene higher uptake and accumulation in enterocytes to the SR-BI effect since it is expressed by caco-2 cells.⁴⁷ Thus, our data corroborate that transporter mediated absorption in the intestine epithelium facilitates carotenes passage, and that competition with other compounds from the food matrix may affect carotene absorption.

Buriti presented lower carotenoid uptake than pitanga ($p < 0,05$, Fig. 5) that may be explained by a saturation effect on caco-2 cells. Note that the concentration of bioaccessible all-*trans*- β -carotene in the micellar phase was 150 times higher than pitanga. O’Sullivan *et al.*⁴⁵ demonstrated this saturation effect for carotenes and xanthophylls – *i.e.* as much higher was the concentration of the carotenoid in the micellar fraction, lower was the absorption by the enterocyte-like caco-2 cells. However, despite this saturation effect carotenoid accumulated in Caco-2 cells were higher for buriti (Table 2). Lycopene was in low concentration in the micellar phase due to very low micellarization (1.1%), however it had a relative high uptake (16%), what also can be explained by this concentration effect.

In summary, our work presents pitanga and buriti fruits as very rich sources of carotenoids highly bioaccessible and consequently great potential to be recommended and commercialized as functional fruits. Pitanga comes up as a source of xanthophylls, specifically rubixanthin, while only a small part of lycopene is absorbed and can be used by the body. Therefore pitanga has a potential to present antioxidant activity against physiologic reactive species of oxygen as well may prevent macular degeneration. We suggest further studies to confirm these functionalities. Likely, buriti is a good natural source of vitamin A due to high bioaccessible provitamin A content. Hence, buriti may have many health benefits like improve immune system, prevent several cancers, avoid eyes diseases and others, that also should be tested to appraise the extent of these effects.

The assays provided evidence that the biochemistry of carotenoids and food matrix are the main effectors of bioaccessibility. Hydroxylation and esterification of xanthophylls, carotenes *trans/cis* isomerization, presence and quantity of lipids from food matrix/meal, as well the interactions between different carotenoids are essential to explain what happens inside

the digestive system with carotenoids from fruits. Also, it is remarkable that beyond the simple quantification of bioactive compounds in fruits we must know how much of that compound can be absorbed by the body. Thus it is important to develop products and process that focus on reducing instability through digestion and increase absorption in small intestine, like compound delivery systems. Ultimately, pitanga and buriti should be more explored regarding their bioactive compounds and functionalities, because they can represent a new raw-material for development of innovative products.

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3.2. References

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4 STRUCTURE DESIGN OF FRUIT MICROEMULSION DELIVERY SYSTEM FOR CAROTENOIDS FROM PITANGA (*EUGENIA UNIFLORA*) AND BURITI (*MAURITIA FLEXUOSA*)

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Abstract

A structural approach was used to produce a fruit microemulsion product in order to extract and encapsulate the naturally occurring carotenoids from buriti (*Mauritia flexuosa*) and pitanga (*Eugenia uniflora*). Buriti is a fruit produced by an Amazonian palm tree, its pulp is very rich in carotenoids, mainly β -carotene. Pitanga originates from the Brazilian Atlantic forest and has high amounts of lycopene. These hydrophobic plant pigments have antioxidant, anti-inflammatory, and anticancer activity. Their health benefits are limited by their physical location and structure in fresh fruits and products. For the structural design of buriti and pitanga microemulsions, fruit pulps were submitted to the following experiments: 1) experimental design testing high-speed homogenization (HSH) and ultrasound (US) processing for carotene release; 2) oil-in-water (O/W) microemulsion direct formation by adding corn oil, Tween 80 or Whey Protein Isolate (WPI) to fruit pulp and processing the resulting mixtures; 3) microstructure and rheologic study of fresh pulps and microemulsions. Main results showed that US processing has more impact on tissue fragmentation, cell disruption and carotene release than HSH and it is indispensable for fruit microemulsion formation. Microscopy studies clearly elucidated that most carotenes are entrapped inside cell walls and must be released for incorporation into lipid microcapsules. After microemulsion formation, surfactant not only establishes bonds to the internal oil and external water, but it also interacts with the carbohydrate from cell walls (mainly cellulose) that is in suspension, forming a gel-like structure; this was demonstrated by Laser Scanning Confocal Microscopy (CLSM).

Keywords: Lycopene; Beta-carotene; Tropical fruits; Fruit Beverage; Smoothie; Surfactant

Graphical abstract

Highlights

- High-speed homogenization and ultrasounds release carotenoids from cell structures
- Processing fruits, oil and surfactant together transfer and encapsulate carotenoids
- Fruits microemulsions present gel-like networks revealed by CLSM microscopy
- Fruits matrices interact differently with the surfactant defining rheological behavior
- Tween 80 microemulsion hold out 3 weeks more than WPI without phase separation

4.1. Introduction

The emergence of new scientific knowledge, market needs and environmental demands are pushing the food industry to very innovative approaches. Strategic formulation and structural design are strong tools to provide the health benefits consumers most want in new products. On this way, Buggenhout *et al.* (2012) asserted that integrating the effects of processing and formulation on structure, texture, biochemistry and nutritional quality can lead to the production of attractive, ready-to-eat products with health-promoting properties. This kind of approach in product development has to keep the focus on functionality, *e. g.* bioactive

compounds delivery in the human body.

Fruits are increasingly known as a rich source of nutrients and bioactive compounds that enhance wellness and are capable of preventing diseases. Some tropical fruits, and particularly, in this case, those that are native from Brazil, contain very high amounts of carotenoids (Rodriguez-Amaya, Kimura, Godoy & Amaya-Farfán 2008). These hydrophobic plant pigments – *e.g.* β -carotene and lycopene – have antioxidant, anti-inflammatory, and anticancer activity. In addition, several of the most abundant carotenoids in many fruits and vegetables show provitamin A activity (*e.g.* β -carotene, α -carotene and β -cryptoxanthin) (Fernández-García *et al.* 2012). Buriti (*Mauritia flexuosa*) is the fruit produced by an Amazonia palm tree. Its pulp is very rich in carotenoids and particularly all-*trans*- β -carotene which represents approximately 400 $\mu\text{g/g}$ fresh weight (de Rosso & Mercadante, 2007). This is probably the highest concentration of this provitamin A carotenoid found in fruits. Pitanga (*Eugenia uniflora*), also known as Brazilian cherry, originates from Atlantic forest and has a very diversified carotenoid profile including lycopene and rubixanthin (approx. 71 $\mu\text{g/g}$ and 11 $\mu\text{g/g}$, respectively) in ripened fruit (Porcu & Rodriguez-Amaya 2008).

Carotenoid health benefits only occur if they are available for being absorbed throughout the human digestive system and reach biologic tissues, *i.e.* if their bioaccessibility is assured. They have first to be released from the food matrix, be integrated into water-soluble mixed micelles in the small intestine and be absorbed by passive diffusion in enterocytes (Failla & Chitchumroonchokchai, 2005). Carotenoids bioaccessibility is highly dependent on the physical structure of the plant as well on the interactions of carotenoids with a meal or food matrix components, such fibers and oils. For instance, the globular shape of chromoplasts in mango and papaya allows an easier release of carotenoids compared to tubular-shaped ones in tomatoes and carrots (Schweiggert, Mezger, Schimpf, Steingass & Carle 2012), while polyunsaturated long-chain fatty acids promoted the bioaccessibility of carotenoids from raw vegetables salad (Failla, Chitchumroonchokchai, Ferruzzi, Goltz, & Campbell, 2014). The high amount of pectin can decrease carotenoid bioaccessibility by changing the viscosity of gastrointestinal medium, while when present in low amounts it can increase bioaccessibility due to stimulation of bile salts binding (Cervantes-Paz *et al.*, 2016). Industrial processing of vegetable foods plays a key role in bioaccessibility because it may break cellular structures releasing carotenoids for digestion (Buggenhout *et al.* 2012).

It is well established that the microstructural characteristics of the food matrix and the lipids present there drive carotenoid bioaccessibility, meaning that it can be modulated by

processing and formulation (Lemmens *et al.* 2014, Sensoy, 2014). Anese *et al.* (2015) reported that ultrasound (US) application on tomato pulp caused an increase in viscosity and a slight increase in lycopene bioaccessibility while reducing initial lycopene concentration and negatively affecting stability during storage. McClements, Decker and Weiss (2007), reported that emulsion-based delivery systems are more suitable for lipophilic bioactive compounds, such as carotenoids. McClements and Xiao (2014), introduced the idea of excipient emulsions, an emulsion not loaded with a bioactive itself but that will increase bioaccessibility of a lipophilic compound when co-ingested with a vegetable source, like carotenoids from fruits. Bioaccessibility of lycopene from tomatoes (raw, boiled and juice) was enhanced by excipient O/W emulsions and was influenced by lipid droplet size as well by the particle size of tomatoes plant tissue (Li *et al.* 2017; Salvia-Trujillo & McClements 2016). Therefore, carotenoid entrapment within plant cell structures is a major obstacle limiting their release and subsequent incorporation into the micelle fraction after digestion. However, after release, free carotenoids are very exposed to degradation by external factors – such as oxygen, light and variations of pH. Combining a process to release the great amount of carotenoids in pitanga and buriti plant cells along with encapsulating them inside lipid droplets seemed thus to be a good strategy to create a functional food product, such as a tropical fruit functional beverage.

Despite the knowledge that carotenoids ingestion is positively associated with reduced risk of various chronic diseases and therefore with the quality of life, it is still lacking a variety of functional foods as product options in the market. There are many technical challenges in the development of health-promoting plant-based new products with high concentration and bioaccessibility of carotenoids. Our research aimed at developing a stable microemulsion product – similar to a smoothie – from buriti and pitanga, with the ability to protect carotenoids and increase their bioaccessibility. The present paper presents the structural design of this product. Our hypothesis was that producing a microemulsion-based product by directly processing fruit pulps together with a surfactant would transfer the carotenoids from the matrix to lipid microcapsules while stabilizing the whole system. Results also show the effect of different types of surfactants, high-speed homogenization (HSH) and ultrasound (US) on a matrix of the raw material and microemulsion's behavior.

4.2. Materials and methods

4.2.1. Fruit pulps

Pitanga was grown and harvested from the orchard in a farm located at Paraibuna, São

Paulo, Brazil, (-23.275394, -45.423188), and commercialized by *Sítio do Bello*, a company specialized in tropical fruits mainly originated from Brazil. Red ripe pitanga was harvested, selected, sanitized and pulped using a crushing machine for pulps at *Sítio do Bello*. There was no water added for processing and seeds were separated during the process. Pulp was packed in sealed bags, frozen at -26 °C and sent in a thermal box to Piracicaba, São Paulo, Brazil. The present lot of Buriti was grown and collected at Urucaia valley, Minas Gerais, Brazil, in the crop of 2016. Buriti pulp was processed by the co-op Copabase located at Arinos, Minas Gerais, Brazil and was commercialized by the co-op network Central do Cerrado. Farmers collected ripe fruits that fall down to the ground since buriti is a palm tree and the fruits have a strong peel protection. Fruits were soaked in clean water (3:1, fruit:water) during 12 h and pulp was taken off by a crushing machine for pulps (Bonina®, 0.5 DF, Brazil, 0.5 mm pore size). Frozen pulps were shipped to Piracicaba, São Paulo, Brazil by plane and stored in -80 °C freezer for 20 days. Pitanga and buriti pulps were freeze-dried at -76 °C for 3 days and packed in vacuum-sealed bags and then shipped by plane to Braga, Portugal, where they were stored frozen at -25 °C until the time of conducting the experiments. All the experiments and analytical procedures were carried out at the Centre of Biological Engineering, University of Minho, Braga, Portugal.

For experimental tests, dried samples (freeze dried pitanga and buriti pulp) were re-suspended in distilled water. The whole pitanga contains 83 % of water and 0.4 % of lipids while buriti fresh pulp contains 63 % and 18 % lipids (w/w) – moisture determined by drying overnight at 105 °C and lipids in an automatic soxhlet equipment (Soxtec 8000, Foss, Denmark). To compare the experimental results between pitanga and buriti, the water content in whole pulp was standardized to 93 % that allowed to have equal final microemulsion composition for oil, water and fruit dry matter (see microemulsion formulation below). After this equalization, the final natural occurring oil content of whole pulp was 0.1 % and 3.64 % (w/w) for pitanga and buriti respectively.

4.2.2. Reagents

Surfactant Tween 80 was purchased from Panreac AppliChem (Germany) and Whey Protein Isolate (WPI) from Arla Foods (Denmark). Nile red stain (TCI, Tokyo, Japan) and Calcofluor white Calcofluor white (Sigma-Aldrich, EUA). Corn oil (Fula®, Sovena, Portugal) was used without further purification.

4.2.3. Microemulsion processing and formulation

In order to be encapsulated, carotenoids have first to be released from chromoplasts and

cellular microstructures. For this purpose, a high-speed homogenizer (Ultra-Turrax homogenizer, T 25, Ika-Werke®, Germany) and an ultrasound processing probe (Vibra-cell™, VCX500, Sonics®, USA) were applied. Whole pulp samples (10 mL) were processed in 50 mL centrifuge tubes kept inside an ice-bath all the time to avoid overheating. Samples were submitted for processing by HSH during 4 min at 10,000; 15,000 and 20,000 rpm combined with US treatment with a 20 kHz probe and 40 % amplitude during 4 and 8 min. Additionally, only US treatment for 4 min and only HSH (15,000 rpm/4 min) were tested.

Only food grade ingredients were used in formulations of the fruit microemulsions. Oil in water microemulsions were produced by processing whole fruits pulp, surfactant (Tween 80 or WPI), corn oil and water in the same proportions for both fruits. The final microemulsion product was composed by 5 % oil, 91 % water, 2 % dried pulp (of pitanga or buriti) and 2 % surfactant of final weight. Formulation principles, oil type and proportions were based on the work of Zhang *et al.* (2015) and Salvia-Trujillo & McClements (2016). Briefly, microemulsions were made by preparing surfactant solution in distilled water (final concentration of surfactant in microemulsion was 2% w/w) and mixing 3.2 mL of this solution with 1.5 mL re-suspended whole pulp of each fruit in 50 mL conical tubes. Corn oil was added in exact amounts to reach 5% (w/w) of final total lipids contents in both cases, having in mind that buriti has an initial lipids content higher than pitanga. Mixtures were then taken to HSH (15,000 rpm) and processed immersed in ice to avoid overheating. The speed of 15,000 rpm was chosen due to the results from carotenoid release and time processing (please see section 2.7 experiment design). Briefly, samples were taken for US processing (20 kHz probe, 40 % amplitude), and kept in ice during the time of US application (please see section 2.7 experiment design). Both types of HSH and US probes were placed at the sample's geometric center. Samples were kept in ice in a Styrofoam box to avoid heat exchange and light.

4.2.4. Carotenoid release from the fruit matrix

In order to prove carotenoid release due to tissue disruption, processed whole pulps were diluted 1:10 (v/v), vortexed and filtered through a 100 µm stainless steel sieve; this means that only fragments smaller or equal to fruit cellular units were caught. Samples were analyzed initially, *i.e.* in control and processed fruit pulps considered as total carotenoids, and after filtering through a 100 µm cutoff sieve. We assumed that carotenoids in the filtrate are released due to tissue and cell wall disruption, since cellular units of pitanga and buriti ranged 50-300 µm size. The filtered fraction was assessed for turbidity and carotenoid content. Turbidity was measured simply by diluting 1:3 (v/v) and reading absorbance at 540 nm (standard wavelength

for turbidity) and 450 nm (main absorption of carotenoid red color). For carotenoid assessment, aliquots of control and processed samples before and after filtering were taken and had their carotenoid extracted and quantified using a small scale method based on Amorim-Carrilho, Cepeda, Fente and Regal (2014). Aliquots (50 – 300 μ l) were well mixed in the vortex for 1 min with 1.5 mL of acetone/ethanol/hexane (50:25:25, v/v/v) solution containing 0.01 % of BHT. Distilled water was added until a final volume of 2 mL and centrifuged for 10 min at 10,000 rpm. This provokes phase separation and carotenoids moved to the upper phase that was collected. Then 1.5 mL of distilled water was carefully added to the carotenoid-rich extracts. In case of forming an emulsion between the solvent and the aqueous phase, a pinch of sodium chloride ($\approx$ 20 mg) was added and the upper phase was collected again. Sodium sulfate anhydrous ($\approx$ 20 mg) was added to the final extract to bind water residues and the final volume was adjusted (1 – 2 mL). The each sample was placed in a 96-well microtiter plates and its absorbance was read by an ELISA (Synergy HT™, Biotek®, USA) reader at 450 nm, and the β -carotene standard curve was determined.

4.2.5. Rheological analyses

Rheological shear measurements were carried out at 25 °C, with a Hybrid rheometer DHR-1 (TA Instruments USA), using parallel plates (40 mm diameter and 100 μ m gap). Flow curves of samples were obtained by a three-shear rate sweeps (up-down-up) program, using a shear-rate range between 1 and 100 s⁻¹. The three steps program was carried out in order to eliminate time-dependence, allowing the system to reach the steady state.

4.2.6. Analyses of microstructure

Brightfield and fluorescence microscopy images were analyzed using an epifluorescence microscope (Olympus BX51) coupled with a DP71 digital camera and three sets of filters (DAPI – 360-370/420; FITC – 470-490/520; and TRITC – 530-550/590) (Olympus Portugal SA, Porto, Portugal). All images were acquired using the Olympus cellSens software. For better observation aliquots were diluted 1:10 (v/v) in distilled water and 5 – 20 μ L of each sample was used under the lamina coverslip. Photographs of at least 3 different areas from every replicate were taken.

For fruits cellular characterization a desktop Scanning Electron Microscope (SEM) coupled with energy-dispersive X-ray spectroscopy (EDS) analysis (Phenom ProX, The Netherlands) and the corresponding ProSuite software were used. Dried samples of buriti and

pitanga whole pulps were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were coated with 2 nm of Au for improved conductivity. The aluminium pin stub was then placed inside a Phenom Standard Sample Holder (SH). The analysis was conducted at 5 kV and 3.3 spot size.

Fluorescence images were acquired using a Confocal Scanning Laser Microscope (CLSM) (Olympus BX61, Model FluoView 1000). Calcofluor white was used for detection of cellulose fibers in microemulsions (laser excitation line 405 nm and emission filters BA 430–470, blue channel); for detection of encapsulated carotenoids laser excitation line 488 nm and emissions filters BA 519–590, green channel, were used in agreement to Kilcrease *et al.* (2013). Nile Red was used for detection of lipids droplets location and size (laser excitation line 559 nm and emissions filters BA 575–675, red channel). Sample aliquots (100 µL) were stained with 10 µL of Calcofluor white and/or 10 µL of Nile Red, held for 15 min at room temperature in the dark. After this, 5 µL of the stained aliquots were placed on microscope slides, under coverslips, then added one drop of immersion oil (Olympus, Portugal) and evaluated by microscope. Images were acquired with the program FV10-Ver4.1.1.5 (Olympus). Selected images were presented either as single CLSM optical sections or maximum intensity type constructions.

4.2.7. Experiment design and statistical analyses

Aiming at reaching the best process for tissue fragmentation and cells disruption, a central composite non-factorial surface design was run (2 factors, 11 runs, 3 replicates at the central point) where HSH intensity tested were 10,000; 15,000 and 20,000 rpm during 4 min while US (20 kHz probe, 40% amplitude) processing varied from 0 (no processing), 4 and 8 min. For microemulsion processing studies, HSH time and intensity were fixed (4 min, 15000 rpm), while the US was the dependent variable in a fully random experiment. Data were analyzed for significant differences using ANOVA, means were compared using the Tukey test at a level of significance of 95 % ($p < 0.05$). Statistica 13[®] (Dell Inc., USA) software was used for surface design, ANOVA and Tukey tests. Statistics results are in the Figures/Tables when necessary and mentioned in the text when conclusions are taken.

4.3. Results and discussion

4.3.1. Pitanga and buriti pulp characteristics

In plants, carotenoids are located inside chromoplasts. These organelles have several shapes and can store different carotenoid compounds depending on species and variety (Kilcrease, Collins, Richins, Timlin, & O'Connell, 2013). Figure 1 shows general tissue and cellular characteristics of pitanga and buriti. Scanning electron and brightfield microscopy (Figure 1, A1 and B1) showed that buriti cell units are oval shaped – measuring around 180 μm length and 50 μm width - very turgid and have flexible walls. Pitanga cellular units are the opposite, they are smaller - around 50 μm size - cellular shapes are geometric, cell walls are straight and look dryer and rigid (Figure 1, B1 and B2). By the micrographs in Figure 1, it is possible to see the entrappent of the buriti lipids full of carotenoids (A2), shining due to auto-fluorescence (A3) and leaking out when cell wall is broken (A1 and A2). The SEM-EDS equipment allows to analyse the elemental composition of the sample in at a unique spot, thereby the proportions found between the carbon (C) and oxigen (O) elements also indicates where is the cell walls (73.8:24.4, w/w) and the lipids (86.9:13.0, w/w) poured out from cells. Pitanga whole pulp presents many tissue fragments, as can be seen in Figure 1 (B2), that shows fluorescence but the carotenoid location is hard to define (B3). Many studies about carotenoid bioaccessibility demonstrated strong hindrance of carotenoid release due to chemical structures, location, chromoplast shape and plant cell composition that would limit potential health benefits (Schweiggert *et al.* 2012, Buggenhout *et al.* 2012). Therefore, the cell wall is the first barrier that has to be overcome to release carotenoids and encapsulate them.

Figure 1. Structural characteristics of fruit pulp: Scanning Electron Microscopy (SEM) of buriti (A1) and pitanga (B1) cellular units; Brightfield micrographs of cellular clusters presenting entrapped carotenes, mainly yellow β -carotene (A2) and red lycopene (B2); Green Fluorescent filter 470-490/520 indicating carotenoids presence and location (A3, B3).

4.3.2. Tissue disruption and carotenoid release

Microscopy analyses (Figure 2) showed that HSH and US have an impact on particle size, distribution and cellular disruption. These results suggest that cell wall breakage with HSH needs more intensity/time to be effective. The micrographs observed with the carotenoid release and turbidity data (Table 1) show that HSH causes lower cellular disruption than US. Also, there is an interaction effect between HSH and US when applied in combination. Besides, sonication is more effective if used after HSH initial treatment due to plant tissue fragmenting and cell wall cracking. This effect is better observed in buriti (Figure 2, A) than in pitanga fruit pulp, yet pitanga seems to be more easily reduced to smaller fragments (Figure 2, B).

The greater the intensity of HSH applied with higher US time exposure, the greater the turbidity (Table 1, Figure 2), that leads to smaller fragments in higher concentration, *i.e.* effective cell disruption. However, total carotenoid content is reduced due to processing when compared to control ($p < 0.05$, Table 1) and the carotenoid release is not directly proportional to parameters used ($p < 0.05$). In fact, carotenoids are very unstable to light, oxygen, heat and acid conditions, once outside cellular structures. Despite ultrasound effect of acoustic cavitation leads to cell rupture and enhancement of plant metabolites extraction, high ultrasound intensities can cause significant degradation of the antioxidant compounds including carotenoids – *e.g.* carotenoids recovery after ultrasound treatment can range from 60 – 80 % in microalgae (Saini & Keum, 2018). In fact, treatments for carotenoid release, and consequent bioaccessibility increase are not always positive. Anese *et al.* (2015) unsuccessfully applied ultrasound treatment to increase the bioaccessibility of lycopene from tomatoes and had a decrease in lycopene concentration during storage because of its exposition to environmental stresses.

Figure 2. Microphotographs of buriti (left side) and pitanga (right side) re-suspended pulps: A1 and B1) not processed; A2 and B2) homogenized by HSH (15,000 rpm / 8 min); A3 and B3) sonicated whit US (20 kHz probe, 40 % amplitude, 8 min); A4 and B4) HSH followed by the US.

Table 1 – Effect of processing on tissue fragmentation and carotenoid release¹

Processing HSH – US	Buriti				Pitanga			
	Total Carotenoids (µg/mL)	Free Carotenoids (µg/mL)	Turbidity at 540nm (abs)	Turbidity at 450nm (abs)	Total Carotenoids (µg/mL)	Free Carotenoids (µg/mL)	Turbidity at 540nm (abs)	Turbidity at 450nm (abs)
Control (0 – 0)	253.7 ±6.6	54.7 ±1.6	1.80	2.35	59.6 ±1.1	15.9 ±0.2	0.64	0.81
10,000 – 0'	149.7 ±4.6	43.6 ±1.0	2.07	2.79	47.4 ±1.0	18.7 ±0.4	0.93	1.19
15,000 – 0'	105.9 ±2.0	40.6 ±0.6	2.08	2.82	50.2 ±0.4	21.7 ±0.5	0.82	1.07
20,000 – 0'	227.2 ±9.7	54.9 ±1.2	2.11	2.89	47.1 ±0.8	22.4 ±0.2	0.98	1.30
10,000 – 4'	209.4 ±5.6	72.2 ±1.6	2.17	2.94	55.3 ±0.6	21.3 ±0.4	0.98	1.25
15,000 – 4'	218.3 ±6.1	81.4 ±1.7	2.28	3.09	43.0 ±0.5	20.4 ±0.5	0.99	1.31
15,000 – 4'	188.5 ±4.2	68.4 ±1.4	2.20	2.99	54.2 ±0.3	21.2 ±1.1	1.03	1.31
15,000 – 4'	213.7 ±6.9	84.5 ±2.9	2.20	2.98	55.7 ±0.8	21.1 ±1.2	1.09	1.44
20,000 – 4'	174.1 ±5.0	75.7 ±1.6	2.18	2.98	52.7 ±0.6	21.5 ±0.4	1.03	1.33
10,000 – 8'	177.3 ±3.8	84.1 ±2.0	2.13	2.93	46.8 ±0.8	21.2 ±0.2	0.97	1.25
15,000 – 8'	115.8 ±2.3	63.9 ±1.1	2.01	2.69	49.2 ±0.5	17.9 ±0.4	0.94	1.26
20,000 – 8'	175.4 ±3.4	74.9 ±1.7	2.12	2.88	44.7 ±0.5	23.8 ±0.1	0.89	1.20

¹Processing parameters: HSH is on rpm while US intensity was 20 kHz, 40 % amplitude varying time (min). Experiment is central composite non-factorial surface design with 3 replicates in central point. Total carotenoids were read in triplicate, data is means ± SD. US processing effects were statistically significative for free total carotenoid (L and Q, $p < 0,05$) and turbidities (Q, $p < 0,05$) from buriti. For pitanga only US effect on turbidity was significative (Q, $p < 0,05$). For all variables processing was statistically diferent ($p < 0,05$) from the control samples.

4.3.3. Whole pulp and microemulsion rheology

Figure 3 shows the flow curves of pitanga and buriti whole pulp, revealing that viscosity (η) decreases with the increase of shear rate ($\dot{\gamma}$). In all cases, a shear-thinning behavior was observed that may be regarded as arising from modifications in the macromolecular organization of the solution as the shear rate changes. Pitanga whole pulp viscosity increased with the processing treatment (independently of intensity and time applied), indicating a structural change of samples.

In the case of buriti whole pulp, there are viscosity increments as the intensity of HSH increases, however when combined with US the increase of viscosity is less pronounced. Indeed, 20,000 rpm of HSH during 4 min without US processing presented the highest viscosity for buriti whole pulp. Leverrier *et al.* (2016) showed that insoluble solids content and organization in an apple model suspension have a first-order effect on the rheological behavior of the pulp causing the non-newtonian shear-thinning characteristics observed; particle size and shape had only a second order effect. Polysaccharides and the aqueous phase or other soluble particles forms a gel-like network that breaks under high shear rates, and then reorganize when flow curve cycle slow down (Leverrier, Almeida, Espinosa-Muñoz, & Cuvelier, 2016; Butler & Snook, 2018).

Effect of surfactant on rheological behavior was observed only in buriti microemulsion, showing that the fruit matrix determines the thixotropic behavior. The viscosity reduction intensifies after increasing shear rate – specifically after 10 s^{-1} for WPI microemulsion – representing how the microemulsion structures highly influence rheology. In complex systems, like foods, emulsions can be stabilized by particles and form many structural arrangements (layers, complexes, aggregates, networks) (Dickinson, 2012). Before initial shearing, the ability of structural arrangements to start flowing depends on the potential energy barrier that keeps particles organized. At low shear rates, the structure of the suspension resists deformation until the destruction of internal networks is completed. In summary, the whole pulps have their rheology strongly determined by particle interactions, mainly polysaccharides from cell walls (like pectin and cellulose), while the microemulsions rheological properties are a consequence of particles interaction with the emulsified oil droplets surface – *i.e.* of the microemulsions structural arrangement.

Figure 3 – Flow curves of whole pulp (upper charts) and fruit microemulsion (bottom). Whole pulp labels of treatments are HSH – US (rpm x 1000 – min) while labels of fruit microemulsions are Tween 80 (T80) or WPI followed by treatment HSH – US (rpm x 1000 – min).

4.3.4. Microstructure and stability of fruit microemulsions

Preliminary tests were performed for fruit microemulsion production in two steps – first producing stable excipient O/W microemulsions followed by incorporation of pre-fragmented fruit pulp. However, not all tests ended in homogeneous systems after mixing, some were ineffective to form the microemulsion. Finally, the one-step direct processing of a mixture of whole fruit pulp (2 % dry matter) mixed with a surfactant (2 % of Tween 80 or WPI) and corn oil (5 %) in 91 % water was able to produce homogenous fruit microemulsions (Figure 5). Experiments showed that the microstructural organization of the final product is a direct effect of fruit type and surfactant used, while size and homogeneity are mainly influenced by time/intensity of processing (Figure 4). Also, for control samples, Figure 4 shows that energy input – mainly due to the US use – reduce oil droplet size from $\approx 0.9\text{-}10\ \mu\text{m}$ to almost reaching the nanoscale $\approx 0.3\text{-}1.0\ \mu\text{m}$. The fruit matrix, buriti or pitanga, interacted differently with surfactants, forming a network organization where fruit fibers entrapped oily microcapsules. In microemulsions many surfactants – such as caseinate, whey protein, lecithin or Tween 80 - interact externally with matrix polysaccharides – like cellulose, pectin and fucoidan - adsorbing to the surfaces of lipid droplets and/or altering the colloidal interactions between the lipid droplets, causing many structural and functional effects (Chang & McClements, 2016; Hu *et al.* 2016; Zhang *et al.* 2015).

In the case of pitanga, the final microemulsion presented a more homogenous gel-like microstructure, while the oily microcapsules entrapped within the cellular fibers net have a smaller size compared to buriti (Figure 4). Figure 4 also shows that there is a slight influence of surfactant on the microstructure. WPI seems to have lower and weaker interaction with fibers, presenting more scattering and homogeneous gel-like structures. Tween 80 presents bigger agglomerates (and in higher numbers) than WPI, however it seems to let fewer oil droplets free (Figure 4). For both fruits, the processing intensity only reduced particle size and increased homogeneity while keeping the main organization (Figure 4).

Figure 4 – Brightfield micrographs of: A) control samples - microemulsion composed of water 93 %, corn oil 5 % and Tween 80 2 % (v/v). Magnification lens 60x; B) and C) buriti and pitanga microemulsion, the composition described in methods. Magnification lens 10x; D) and E) buriti microemulsion, Tween 80 and WPI as surfactants. Magnification lens 10x; F) and G) pitanga microemulsion, Tween 80 and WPI as surfactants.

The surfactant interaction with fruit fibers showed to be fundamental to the final product stability (Figure 5). In the first 24 h all fruit microemulsions were stable, but before 7 days stored at 7-10 °C, microemulsion instability starts to appear, first in WPI samples. Buriti products made with WPI separated in two phases for both US (4 min) and HSH (4 min) + US (8 min) process applied. For pitanga with WPI, all microemulsions lost stability before 7 days of storage, independently of the process applied. Only Tween 80 microemulsions resisted until 28 days of storage at 7-10 °C, however, after that period instability signs appeared such as creaming/oily phase separation. The conclusion is that Tween 80-based microemulsions were more stable, independently of the fruit used. The process that presented the highest stability after 28 days was HSH (15,000 rpm/4 min) + US (20 kHz, 40 % amplitude/4 min) for both buriti and pitanga fruits. Instability of O/W emulsions, which includes gravitational separation, *i.e.* creaming, flocculation and coalescence, all leading to phase separation (McClements & Gumus 2016), hinders their use in food products. These problems are naturally occurring in emulsions due to the energy loss of the systems, however, the presence of polysaccharides can help stabilizing the whole system or, on the contrary, may induce instability, depending on the nature of surfactant-polysaccharides interactions (Dickinson, 2013). This is the reason why we tentatively tried to elucidate the type of interactions between the microemulsions and the fruit matrix.

Figure 5 – Stability of final buriti and pitanga microemulsions stored at 7-10 °C during 28 days. Each photo is set, from left to right – 3 tubes using WPI as surfactant and 3 tubes with Tween 80 (T80)– as follows: US processing (20 kHz, 40% amplitude/4 min); HSH (15,000 rpm/4 min) + US (20 kHz, 40% amplitude/4 min); HSH (15,000 rpm/4 min) + US (20 kHz, 40% amplitude/8 min)

4.3.5. Elucidation of the interaction of microemulsion with the fruit matrix

Since the main objective is to obtain a structure where the naturally occurring carotenoids are transferred from the plant cells into the oil microcapsules formed, our hypothesis was that cell wall disruption along with carotenoids lipophilic properties would promote their encapsulation. Therefore, we used CLSM microscopy to reveal microstructure and carotenoid location. Carotenoids autofluorescence can be observed by CLSM microscopy with excitation at 488 nm and an emission filters of 515-590 nm, and micrographs indicate its exact location inside cellular structures (Kilcrease *et al.*, 2013). The same principle coupled to

Raman fluorescence spectroscopy allowed Wan Mohamad *et al.* (2017) to quantify *in situ* β -carotene (detected at 532 nm) inside oil droplets from an O/W microemulsion stabilized by WPI. In Figure 6 (micrographs A2, B2, C2 and D2), green autofluorescence is a strong evidence that carotenoids from fruits migrated into oily microcapsules - stained with Nile Red (Figure 6 A3, B3, C3 and D3), confirming our hypothesis that direct processing of pitanga and buriti microemulsions would encapsulate their natural carotenoids.

Amiri-Rigi and Abasi (2016) were able to increase lycopene extraction from tomato products residues up to 35 % recovery applying a microemulsion-based system - saponin as surfactant combined with ultrasound treatment; it is noteworthy that lycopene bioaccessibility usually is very low, around 0.1-3 %, mainly because of its entrapment inside cellular structures (Salvia-Trujillo & McClements, 2016). Zhang *et al.* (2016) successfully used excipient emulsions to increase β -carotene bioaccessibility and they have shown by CLSM microscopy that carrot naturally-occurring carotenoids migrated into the oil droplets increasing their accessibility to the digestive system. The main physic-chemical characteristic of carotenoids is their lipophilicity due to their structure: a long carbon chain with conjugated double bonds. Based on our results and related literature, there is enough evidence to show that direct production of fruit-based microemulsions readily extracts and encapsulates carotenoids from the fruit matrix.

The observed interaction between surfactant and fruit matrix is the key to the final product's microstructure. In our experiments, oil microcapsules and cellulose exhibited different interactions depending on fruit and surfactant used. Figure 6 shows the polysaccharides net formed mainly due to cellulose fibers – marked with calcofluor white stain – and probably pectin that is not observed in the micrographs. The net formed by oil droplets and the fruit fibers behave as a gel-like structure being able to change the rheology and properties of the whole system. Buriti present longer and thicker cellulose fibers than pitanga, clearly organized in blocks of intertwined fibers. Pitanga microstructure is more spread, gel-like, homogenous and formed with thin cellulose fibers compared to buriti. Micrographs of buriti microemulsion with Tween 80 present less free oil droplets than the ones stabilized by WPI. Inversely, pitanga microemulsion made with Tween 80 presents much more free oil droplets than the WPI-based product.

Zhang *et al.* (2015) showed that lactoferrin and Tween 80 stabilized-microemulsions did not interact with low methoxyl pectin at neutral pH, while sodium caseinate-stabilized microemulsion did interact with pectin at study conditions promoting a flocculation effect. Since the microemulsions were prepared with whole fruit pulp, they presented the acidity of the

original fruit. Thus the pH of pitanga and buriti microemulsions were 1.8 and 3.5, respectively, measured by pHmeter. The surfactant aggregates with cellulose (figure 6) and probably pectin observed are gel-like structures and have some rheologic effects, like an increase in viscosity and microemulsion stability. Other works showed microemulsions interactions with polysaccharides (xanthan gum and locust bean gum) with microscopy photos that have an organization of the microstructure that is similar to the one we observed in our products (agregation, flocculation and gel-like structures), and similar rheological effects (Chung, Degner & McClements, 2013; Wu & McClements 2015). Therefore, we may conclude that the obtained microstructure is mostly an effect of surfactant-fibers interactions, which have a great importance in the final functional properties, since carotenoid-rich microcapsules need to be spread and free to be attacked by digestive enzymes for carotenoid accessibility.

Figure 6 – CLSM microscopy of buriti and pitanga microemulsions. Calcofluor white in the first column marks cellulose fibers. Auto-fluorescence of carotenoids in the second column (excitation 488 nm and emission filter BA 519–590nm). Nile Red in third column mark lipid droplets. The last column is an overlap revealing the 3 dimensions microstructure.

4.4. Conclusions

A structure design approach to encapsulate carotenoids from pitanga and buriti was successfully applied. Both high-speed homogenization and ultrasounds were able to brake fragments of fruit tissues and disrupt cellular walls releasing carotenoids, mainly when these processing techniques were used in combination. However, the main results showed that ultrasound processing has a more pronounced impact on tissue fragmentation, cell disruption and carotene release than high-speed homogenization and therefore it is an indispensable step for fruit microemulsion formation. Microscopy clearly shows that most carotenes are entrapped inside cell walls and must be released for incorporation into lipid micelles. The high-speed homogenizer and ultrasound treatments released up to 50 % of the initial amount of carotenoids. After microemulsion formation, the surfactant does not link only to the internal oil and external water, it also interacts with the carbohydrates from cell walls (mainly cellulose that is in suspension), forming a gel-like structure – that was demonstrated by CLSM microscopy. Tween 80 and WPI were used as surfactants due to their food grade status and for being widely used in the food industry. Surfactant interactions with the food matrix depend on the fruit used. In both cases Tween 80 was more stable over time than WPI, but buriti rheology was much more influenced by surfactant and processing than pitanga. Tween 80-stabilized microemulsions present softer viscosity than WPI-based microemulsions for both fruits. The gel-like structure formed is the main factor for final product stability and rheology, and may modulate carotenoid encapsulation and release during digestion, thus influencing bioaccessibility. The obtained buriti and pitanga microemulsions have a high potential for the development of new products with more bioaccessible β -carotene and lycopene.

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Conflicts of interest: none

4.5. References

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5 CAROTENOID BEHAVIOR OF PITANGA (*EUGENIA UNIFLORA*) AND BURITI (*MAURITIA FLEXUOSA*) DURING MICROEMULSION PRODUCTION AND IN A DYNAMIC GASTROINTESTINAL SYSTEM

Chapter submitted to Journal of Food Science and Technology

Abstract

Uncommon tropical fruits are emerging as raw-material for new food products with health benefits. This work aimed to formulate and process microemulsions from pitanga (*Eugenia uniflora*) and buriti (*Mauritia flexuosa*) fruits. Since these fruits are very rich in carotenoids, it was sought to release, encapsulate and protect their carotenoids along with increasing bioaccessibility, specifically of lycopene and β -carotene. Pitanga and buriti microemulsion were produced by applying a direct processing (high speed homogenization – HSH at 15000 rpm and Ultrasound – US with 20 kHz probe at 40 % amplitude) of the whole pulp together with surfactant (T80 or WPI at 2%) and corn oil (5%). All treatments (HSH – US for 0-4, 4-0, 4-4, 4-8 min-min) applied were able to increase the amount of carotenoid released. However, the processing also decreases total amount of carotenoids in whole pulp of studied fruits. The significant impact of processing during microemulsion production was not severe. The overall data suggest that surfactant and oil presence during processing may protect the carotenoids in fruits and microemulsions. Final recovery of total carotenoids, after passing the samples through a dynamic gastrointestinal system, was higher for microemulsions than for whole pulps. Results of total carotenoids, β -carotene and lycopene indicate that high losses happened during jejunum and ileum phases. The present paper confirms that is possible to increase β -carotene and lycopene bioaccessibility from fruits by direct processing microemulsions ($p < 0.01$). The microemulsion processing and formulation chosen was able to improve final bioaccessibility of total carotenoids from buriti, β -carotene from pitanga and buriti, and of lycopene from pitanga.

Keywords: Dynamic digestive system; Tropical fruits; Bioavailability; Beta-carotene; Lycopene; Structure design

Graphical Abstract

Highlights

- Ultrasound is effective for carotenoid release from pitanga and buriti matrices, however stimulate its degradation
- Surfactant protected the carotenoids against processing degradation
- Carotenoids from pitanga and buriti were efficiently encapsulated, ranging 25 – 80 %
- Microemulsion protected carotenoids against degradation and losses during digestion by a dynamic gastrointestinal system
- Microemulsion increase total carotenoids, β -carotene and lycopene bioaccessibility up to 4 times

5.1. Introduction

There is a growing demand for plant based products that promote health and well-being. Thereby, fruits represents the most popular option for new products due to their ‘naturally healthy’ image and their bioactive compounds composition (*e. g.* carotenoids). Several fruits are being used by functional foods industry for developing new healthy products, like smoothies, yogurts, beverages, ice-creams and even as natural colorants. This can incorporate new flavors as well functionalities to diet (Sun-Waterhouse 2011, Khan *et al.* 2013). Carotenoids are present in many common foods and its ingestion can provide protection for the

eyes, improvement in vision health, promotion of the immune systems, reduction of the overall risk of chronic diseases and avoid abortion (Saini *et al.* 2015).

In this sense, the biodiversity of fruits native from Brazil can be rich source of innovation and opportunities, growth of income, value adding and scientific contribution. Pitanga (*Eugenia uniflora*) belongs to the botanic family of *Myrtaceae*, grows on tropical and subtropical regions, and it is originally from Brazilian *restinga*. The fruit is very valued due to its strong red color, juicy pulp, unique aroma, sour and sweet taste. Northeast region of Brazil have the largest production, being that only Pernambuco state produces annually up to 1.700 tons/year, that is basically commercialized *in natura* or processed as fruit pulp. Pitanga has a very high concentration of antioxidant compounds like flavonoids, vitamin C and carotenoids – mainly lycopene in concentration levels around 70 µg/g of fresh pulp (Negri *et al.* 2016; Porcu and Rodriguez-Amaya 2008). Buriti is a palm fruit from *Arecaceae* family, it naturally grows on *amazônia*, *caatinga* and *cerrado* biomes. Buriti fruit is very rich in lipids, around 20% of fresh pulp, that are extracted and commercialized as crude or refined oil. Usually buriti oil is consumed in foods or applied in cosmetics due to healing properties of the high concentration of carotenoids, tocopherol and fat acids. Probably buriti is the plant with the highest content of all-*trans*-β-carotene in the nature, reaching levels of 373 µg/g of whole pulp (de Rosso and Mercadante 2007; Negri *et al.* 2016)

The preserving techniques, processing, storage and final product matrix/formulation have great impact on stability and bioaccessibility of carotenoids, and consequently on the intended health benefits (Dias *et al.* 2014; Failla *et al.* 2014). Carotenoid bioaccessibility is defined as the portion that are ready for being absorbed by intestinal cells after food digestion, *i. e.* carotenoids incorporated into water soluble mixed micelles produced by bile salts and lipids (Failla and Chitchumroonchokchai 2005). Food matrix factors that are determinant for bioaccessibility are: intracellular form of carotenoid (*e.g.* crystalline or micellar); kind of fiber present (soluble or insoluble); amount and kind of lipids present (*e.g.* unsaturated long chain fat acids increase bioaccessibility) (Failla and Chitchumroonchokchai 2005; Saini *et al.* 2015). The presence, amount and type of lipids is crucial. Also, interactions of components originally present in the meal or produced inside the digestive system still can influence bioaccessibility – *e.g.* fibers, carotenoid x carotenoid interactions, kind of lipids ingested with the carotenoid source (Sensoy 2014; Saini *et al.* 2015; Fernández-García *et al.* 2012).

Once carotenoids are released from plant cells they are subject to structural modifications and degradation that can impair bioaccessibility (Yonekura and Nagao 2007).

Thus, every event after cells fragmentation due to processing, mastication or stomach digestion will influence final bioaccessibility in different ways. Therefore, processing, implies changes in the food matrix that causes two main effects: carotenoids can degrade losing their health effect, but, in the other hand, bioaccessibility can be improved/impaired due to structural changes. Indeed, losses of bioactive compounds and/or increase in bioaccessibility due to processing are oftentimes reported (Pugliese *et al.* 2013; Dias *et al.* 2014; Failla *et al.* 2014; Anese *et al.* 2015; Berni *et al.* 2015).

Generally, industrial processing of plant based products do not take into account the retention and bioaccessibility of bioactive compounds. The standard processing of soups and sauces for example treat all ingredients at same way, applying high temperature for long time that results in decrease of nutritional, functional and sensorial quality of final products (Buggenhout *et al.* 2012). Carotenoid bioaccessibility in foods is very low, but it is possible to strategically formulate and process them in order to increase their release, bioaccessibility and consequently their health benefits (Buggenthout *et al.* 2012). For instance, bioaccessibility of β -carotene, lutein and lycopene were augmented due to cooking process as frying (Berni *et al.* 2014), microemulsion addition (Li *et al.* 2017) or optimal combination of adequate process/formulation (Buggenhout *et al.* 2012), respectively. However, still exist many gaps to be filled about this topic that represents a very promising field for research and food innovation.

Many bioactive compounds are highly lipophilic, such as carotenoids, which causes many hindrance to incorporate these molecules in new functional foods, primarily low solubility in water and chemical instability (Recharla *et al.* 2017). Several techniques combine structure design approaches and/or delivery systems engineering. They aims to protect, preserve, enhance solubility, increase bioaccessibility and bioactivity of lipophilic bioactive compounds. The most studied and applied strategy is the encapsulation of lipophilic bioactive compounds in emulsion based systems at micro and nano scales (Recharla *et al.* 2017; McClements 2015). Successful encapsulation of purified carotenoids in micro and nanoemulsions that increase their solubility in water, stability and bioaccessibility have been done by many research groups (Gomes *et al.* 2017; Davidov-Pardo *et al.* 2016; Gul *et al.* 2015; Zhang *et al.* 2015; de Paz *et al.* 2013; Hejri *et al.* 2013).

Therefore, our approach attempt to manipulate structure and composition of fruit matrix in order to encapsulate carotenoids leading to higher retention and bioaccessibility of carotenoids. We produced a microemulsion-based product by directly processing fruit pulps of pitanga and buriti together with a surfactant that stabilized the whole system. Our hypothesis was that combined process and formulation would transfer the carotenoids from the matrix to

lipid microcapsules while protecting their carotenoids and increasing their bioaccessibility. The present chapter focus on carotenoid contents, retention, efficiency of encapsulation and bioaccessibility throughout a dynamic digestive system. Data also show the effect of the surfactants Tween 80 (T80) or Whey protein isolate (WPI), and processing by high-speed homogenization (HSH) and ultrasound (US) on carotenoid behavior over emulsion formation.

5.2. Material and Methods

5.2.1. Pitanga and buriti fruit pulp

Buriti pulp was commercialized by the co-op network Central do Cerrado, Brasília, Distrito Federal, Brazil. Fruits were soaked in clean water, 3 parts of water to 1 part of fruit (v/v), during 12 hours and pulp was taken off by a crushing machine for pulps (Bonina®, 0,5 mm pore size). Pitanga pulp was acquired from the tropical fruits company Sítio do Bello, Paraibuna, São Paulo, Brazil. Red ripe pitanga was harvested, selected, sanitized and pulped at Sítio do Bello using a crushing machine for pulps. There was no water added and seeds were separated during the processing of pitanga. Both fruits pulps were packed in sealed bags, frozen at -26 °C and sent in thermal box to Piracicaba, São Paulo, Brazil, and immediately stored in -80 °C freezer. Pulps were freeze dried at -76°C during 3 days and packed in vacuum-sealed bags and then shipped by plane to Braga, Portugal, where they were stored frozen at -26 °C until the time of conducting the experiments. Dried samples of lyophilized pitanga and buriti pulp were re-suspended in distilled water until the final water content of 93 % of wet weight. After this equalization, final natural occurring lipids contents in whole pulp were 0.1 % and 3.64 % respectively for pitanga and buriti.

5.2.2. Reagents

Commercial standards of β -carotene, lycopene and enzymes for the dynamic *in vitro* digestion were purchased from Sigma-Aldrich (St Louis, MO, USA). Specific reference numbers of each enzymes are informed in the correspondent methods. Organic solvents for extraction and HPLC analysis were chromatographic grade (Chromasolv™, Muskegon, MI, USA). Surfactant Tween80 (T80) was purchased from Panreac AppliChem (Germany) and Whey Protein Isolate (WPI) from Arla Foods (Denmark). Corn oil (Fula®, Sovena, Portugal) was used without further purification.

5.2.3. Microscopy

Brightfield images were analyzed using an epifluorescence microscope (Olympus BX51) coupled with a DP71 digital camera and three sets of filters (DAPI – 360-370/420; FITC – 470-490/520; and TRITC – 530-550/590) (Olympus Portugal SA, Porto, Portugal). All images were acquired using the Olympus cellSens software. For better observation aliquots were diluted 1:10 (v/v) in distilled water and 5 – 20 μ L of each sample was used under the lamina coverslip. Photographs of at least 3 different areas from every replicate were taken.

Fluorescence images were acquired using a Confocal Scanning Laser Microscope (CLSM) (Olympus BX61, Model FluoView 1000). Calcofluor white (Sigma-Aldrich, EUA) was used for detection of cellulose fibers in microemulsions (laser excitation line 405 nm and emission filters BA 430–470, blue channel); for detection of encapsulated carotenoids laser excitation line 488 nm and emissions filters BA 519–590, green channel, were used in agreement to Kilcrease *et al.* (2013). Nile Red (TCI, Tokyo, Japan) was used for detection of lipids droplets location and size (laser excitation line 559 nm and emissions filters BA 575–675, red channel). Images were acquired with the program FV10-Ver4.1.1.5 (Olympus).

5.2.4. Microemulsion formulation and processing

First step of producing the microemulsion is to release the carotenoids from fruit chromoplasts. For this purpose, samples were submitted to processing by a high-speed homogenization (HSH) equipment (Ultra-Turrax[®] homogenizer, T 25, Ika-Werke, Germany) at 15000 rpm during 0 min and 4 min combined with ultrasound (US) treatment with a 20 kHz probe (Vibra-Cell[™], Sonics[®], EUA), 40 % amplitude during 0, 4 and 8 min. The samples were processed in 50 mL centrifuge tubes and kept inside ice-bath all the time to avoid overheating and carotenoid degradation. To assess treatment impact, total carotenoids, as well β -carotene and lycopene by HPLC-DAD were analyzed in whole, processed, and in filtered pulp after processing (stainless steel sieves, particle size < 100 μ m).

Microemulsion formulation was based on the work of Zhang *et al.* (2015) and Salvia-Trujillo and McClements (2016). Only food grade ingredients were used in formulations of the fruit microemulsions. Oil-in-water (O/W) microemulsions were produced by processing together fruit pulp, surfactant (T80 or WPI), corn oil and water at equal proportions for both fruits. Briefly, microemulsions were made by preparing surfactant solution (T80 or WPI) in distilled water, and then mixing with whole pulp and corn oil. Final microemulsion product was composed by 5 % corn oil, 91 % distilled water, 2 % of surfactant and 2 % of pulp (in dry basis). Processing parameters for microemulsion and the selected one for dynamic gastrointestinal system study were chosen based on previous data of total carotenoids, rheologic behavior, and

on microemulsion stability during storage, all results that compose another manuscript of our work (unpublished). Mixtures were first processed by HSH (varying 15000 rpm during 4 min) and immediately taken to US sonication (20 kHz probe, 40 % amplitude, varying time). Both probes for HSH and US processing were placed at sample geometric center. Samples were kept all the time in ice inside a Styrofoam box to avoid heat and light.

5.2.5. Carotenoid extraction for analysis

For carotenoid assessment aliquots were took and had their carotenoid extracted and quantified using an small scale method based on Amorim-Carrilho *et al.* (2014) review, described concisely as follow. Aliquots (50 – 300 µl) were vigorously mixed in vortex for 1 min with 1.5 mL of Acetone/Ethanol/Hexane (50/25/25 v/v/v) solution containing 0.01% of BHT. Distilled water was added until 2 mL of final volume and centrifuged for 3 min at 1000 rpm. This provoke phase separation and carotenoid drives to upper phase. Carotenoid rich extracted were collected, then 1.5 mL of distilled water was added carefully. In case of forming an emulsion a pinch of sodium chloride ($\approx$ 20 mg) was added and the upper phase is collected again. Sodium sulfate anhydrous ($\approx$ 20 mg) was added to final extracts for binding water residues. For extraction of carotenoids from jejunum and ileum filtrates obtained after dynamic simulation of digestion, as well the undigested residues, the same procedures were used with the adjustment of volumes and keeping the same sample/solvent ratio. Extracts were collected and then dried under nitrogen flow, resuspended in petroleum ether (for spectrophotometric measurement of total carotenoids) or in methanol/MTBE (50/50 v/v) for HPLC-DAD analysis of β -carotene and lycopene.

To evaluate retention and release of carotenoids due to processing, samples were analyzed: initially, *i.e.* the control sample; in the HSH and US processed fruit pulp; and after filtering throughout 100 µm cutoff sieve, that estimates free carotenoids. Microemulsions were analyzed freshly and in the upper phase after centrifuging for 5 min at 8385 g, that allowed to estimate efficiency of encapsulation, since unencapsulated carotenoids are attached to cell fibers in pellets. Each step of dynamic digestion simulation – gastric, duodenum, jejunum filtrate, ileum filtrate and final unfiltered residue – had the carotenoids contents measured to evaluate stability of carotenoids to digestion and final bioaccessibility.

5.2.6. Carotenoid analysis by spectrophotometer and HPLC-DAD

Total carotenoids - In a 96 well microplate reader (Synergy™ HT, Bio-tek®) 300 µL of extracts, as well β-carotene standard curves, had absorbance measured at 450 nm. Two curves were prepared, where first ranged 0.18 - 1.42 µg/mL of β-carotene in 8 points and second ranged 2.7 – 6.3 µg/mL of β-carotene in 5 points, respectively R^2 was 0,9849 and 0,9635, and were used for whole pulps, < 100 µm filtered samples, microemulsion ready and after centrifugation.

β-carotene and lycopene - Chromatographic analysis of carotenoid is presented in Kimura *et al.* (2007). The HPLC-DAD system used was a Shimadzu® Nexera X2 (modules: degasser DGU 20A 5R, pump LC-30AD, autosampler SIL-30AC, oven CTO-20AC and detector DAD SPD-M20A) with a polymeric YMCTM C₃₀ (150 mm x 4.6 mm, 5 µm particle size). The mobile phase was methanol and MTBE at 90:10 (Methanol:MTBE, v/v) in linear gradient to 40:60 in 60 min, returning to initial conditions and kept for 15 min for re-equilibration; the oven temperature was 30° C while autosampler chamber was kept in 8° C; flow was 0.8 mL / min and injection volume changed according to appearance of carotenoid concentration ranging 10 µL – 40 µL. Identification of β-carotene and lycopene was based on commercial standards, and diode array absorption spectra. Chromatograms were taken at fixed wavelength of 450 nm for β-carotene and 470 nm for lycopene integration and calculation. Standard curves of β-carotene (from 0.9 µg/mL to 4.5 µg/mL) and lycopene (from 1.09 µg/mL to 2.74 µg/mL) were performed, and the respective r^2 values for the standard curves were 0.9988 and 0.9918.

5.2.7. Dynamic gastrointestinal system

A dynamic gastrointestinal system equipment and protocol, described in Pinheiro *et al.* (2016), was used to evaluate carotenoids stability to digestion and bioaccessibility comparing *in natura* samples and microemulsions. In summary, this model simulates the main events that occur during digestion – *i.e.* simulation of stomach, duodenum, jejunum and ileum digestions by separated reactors. The temperature is maintained to 37 °C and physiological pH of each step is controlled by addition of HCl or NaHCO₃. The peristaltic movements are mimicked with water jacket. The compartments are connected by silicone tubes and the flow throughout the system is controlled manually using 10 mL plastic syringes. The micellar phase is separated by filtration through a hollow-fibre device (SpectrumLabs Minikros®, M20S-100-01P, USA) placed after jejunum reactor and another one after the ileum reactor. The micellar phase is collected directly in a ellenmeyer setted in ice to stop further reactions. At this filtrate are the mixed micelles containing the bioaccessible carotenoids.

The system runs with initial 40 mL of samples, and the experiment was done in triplicate. Gastric and intestinal secretions were freshly prepared and secreted inside the reactors by syringe pumps at pre-set flow rates. Gastric enzyme solution was made by adding 0.0176 g of pepsin (3409 U), 0.0741 g of lipase (54 U) in 100 mL of gastric salts solution (NaCl 5 g/L, KCl 0.6 g/L and CaCl₂ 0.25 g/L), pH adjusted to 1.5 with HCl 1M, and flow rate was 0.33 mL/min. Duodenum secretion was made by combining 34 mL of pancreatin solution (2.8 g in 40 mL distilled water), 20 mL of small intestine salts solution (NaCl 4.8 g/L, KCl 2.2 g/L, CaCl₂ 0.22 g/L and NaHCO₃ 1.5 g/L) and 66 mL of bile solution (4 g/100 mL of distilled water), final pH adjusted to 7.5 with NaHCO₃ 1M, flow rate 0.6 mL/min. Jejunum secretion was prepared with 40 mL of the bile solution diluted 1:10 with small intestine salts solution, flow rate was 2.13 mL/min. Finally, in the ileum was secreted only the small intestine salts solutions at 2 mL/min flow. The system runs 5h of each digestion. At 90 min is collected an aliquot of the stomach, and at 120 min is collected an aliquot of the duodenum. The aliquots helps to follow the stability to digestion. The ratio between initial amount of carotenoids and the amounts in the micellar phases indicates the bioaccessibilities (jejunal, ileal and total). The residue also is collected and analysed composing the results of carotenoid recovery.

5.2.8. ζ -potential

The ζ -potential of the particles in the samples as they passed through the various stages of the dynamic gastrointestinal system were measured according to Pinheiro *et al.* (2013) using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK). The measurement was performed at 25 °C assuming the Smoluchowski model and using a disposable folded capillary cell (DTS1070), as also an automatic voltage. Zeta analysis was carried out by zetasizer software through an Auto Mode - General Purpose model. Initial samples were diluted 1:1000 (v/v), while chyme and digesta were diluted 1:10 (v/v) in appropriate buffer solution (pH 2 for stomach sample and pH 7 for initial and small intestine samples) at room temperature. Each individual ζ -potential measurement was determined in triplicate.

5.2.9. Statistics

Experiment data was analyzed for significant differences using ANOVA, means were compared using the Tukey test and significant at 95 % reliability ($p < 0.05$). Software used for the surface design, ANOVA and Tukey tests was the Statistica 13[®] (Dell Inc.).

5.3. Results and discussion

5.3.1. Carotenoid release and impact of processing

For the purpose of producing a microemulsion while transferring the carotenoids from pitanga and buriti to the oil droplets, the main obstacle is to release the carotenoids as much as possible. An experiment was carried out to test carotenoid release and establish the parameters for the microemulsion production. Results of total carotenoid release and retention after processing are presented in Figure 1. The observed behavior is that all treatments applied are able to increase the ratio of initial carotenoid/free carotenoid, *i.e.* release. However, at the same time processing decreases the total amount of carotenoids from studied fruits. All treatments tested, especially when combined HSH-US, was successfully capable to break cells and release its intracellular content as it can be seen by empty cells and small fragments observed in Figure 2. After testing different treatments it was chosen to discard the HSH without US (T-U 4'-0') process because the results of carotenoids release are unexpressive, and the microemulsion formation in further tests was not homogeneous. The US treatment at 24 kHz was able to fragment plant tissue, break the cellular wall and release lycopene crystalloids from tomatoes – demonstrated by light microscopy images very similar to ours – with slight decrease of lycopene concentration (Anese *et al.* 2015)

Figure 1 – Evaluation of carotenoid release and impact of processing pitanga and buriti whole pulps by HSH and US. Results are total carotenoids determined spectrophotometrically. Acronyms meaning treatments HSH-US (T-U) and time of processing (min-min). Different letters for the same series represents significant differences between treatments ($p < 0.05$).

Carotenoids are found in plants, and are stored inside chromoplasts that varies in shape and size related to their carotenoid composition (Kilcrease *et al.* 2013; Saini *et al.* 2015). The carotenoids are stored in two main forms, lipid-dissolved or liquid-crystalline, that have influence on bioaccessibility due to their readiness to being digested and micellized during human digestions (Schweiggert *et al.* 2012). Images from light microscopy on Figure 2 show these differences in buriti (lipid-dissolved) and in pitanga (liquid-crystalline), as well the carotenoid entrapment inside cellular structures. In some fruits like mango and papaya, β -

carotene and xanthophylls are in the both states, lipid-dissolved and liquid-crystalline, together with crystalloid lycopene (not easily seen by light microscopy), all inside of globular chromoplasts (Schweiggert *et al.* 2012). This seems to be the case of pitanga that is rich in lycopene, β -carotene and xanthophylls (Porcu and Rodriguez-Amaya 2008). The entrapment of the carotenoids is numerically perceived in Figure 1 by the data of carotenoid release from control samples. Pitanga whole pulp has around 25 % of its carotenoid released from cellular walls while buriti has only 15%. In the whole pulp, the free carotenoids exist due to pulp's taking of, and are dispersed in the aqueous fraction inside lipid droplets or as crystalloids.

Carotenoid retention are strongly affected by kind of processing, temperature, time of exposure and intensity of the energy applied (Dias *et al.* 2014; Berni *et al.* 2015; Frias *et al.* 2010). The processing goal was to get the higher content of free carotenoids. On this way, only HSH (15000 rpm, 4 min) followed by US (20 kHz, 40% amplitude, 8 min) in pitanga presented higher quantity of free carotenoids compared to control (Figure 1). Nevertheless, the extent of this increment is low (14 $\mu\text{g/mL}$) despite statistically significant. The food matrix have a protection rule on retention, but once carotenoids are released they are more subject of degradation and environmental stresses. Carail *et al.* (2015) investigated the kinetics of all-*trans*- β -carotene degradation due to US processing (20 kHz, same used in our work), they demonstrated that degradation is high in presence of water and oxygen, and have more influence of time of exposure than US intensity.

Figure 2 – Microemulsion formation: carotenoid location in cell; carotenoid release; formed microemulsion (T80, 4 min of HSH and 4 min of US); microemulsion (Nile red stained and carotenoid autofluorescence) interaction with fruit cellulose (calcofluor white stained).

5.3.2. Microemulsion development

To produce the microemulsion it was applied a direct processing of the whole pulp together with surfactant (T80 or WPI at 2% of final weight) and corn oil (5% of final weight). T80 and WPI surfactants were already used to produce excipient emulsions using olive oil or corn oil that successfully increased bioaccessibility of lycopene and β -carotene from tomato and carrots (Salvia-Trujillo and McClements 2016; Li *et al.* 2017; Zhang *et al.* 2016). Figure 2 shows the oily microcapsules formed due to surfactant action that stabilize the system. Many works have been demonstrated the efficacy of US to break cell walls and improve carotenoid extraction to oil. For example, Goula *et al.* (2017) were able to extract up to 93.8% of total carotenoids from pomegranate peel using US assisted extraction with sunflower oil as extracting solvent, *i. e.* breaking cellular walls and transferring carotenoid to oil. Also, US is effective tool for producing stabilized microemulsion of β -carotene in oil (de Paz *et al.* 2013; Kentish and Feng, 2014).

CLSM image (figure 2) clearly show the microstructure formed. It is possible to observe that surfactant interacts externally with tissue fragments and fibers that comes from cellular walls. Carotenoids autofluorescence can be observed by CLSM microscopy with excitation at 488 nm and an emission filters of 515-590 nm (same we used at our analysis), and micrographs indicate its exact location inside cellular structures (Kilcrease *et al.* 2013). The same principle coupled to Raman fluorescence spectroscopy allowed Wan Mohamad *et al.* (2017) to quantify *in situ* β -carotene (detected at 532 nm) inside oil droplets from an O/W microemulsion stabilized by WPI. The CLSM micrograph (Figure 2) reveals this gel-like structure since the oil droplets are stained with Nile red, that combined with green autofluorescence from carotenoids, exhibit a green circle with an yellow interior. Calcofluor white stained the cellulose fibers from fruit matrix, that is seen as a blue net binding the oil droplets and forms agglomerates dispersed in the aqueous phase. This outline of fluorescence is a strong evidence that confirms our hypothesis that direct processing of whole pulp, surfactant and oil is able to release, transfer and encapsulate carotenoids from fruits inside oil droplets. For better understand we refer to our parallel work (unpublished) where microstructure study is detailed discussed and reported. Study of the interaction between microemulsions made with different surfactants (sodium caseinate, Tween 80 or lactoferrin) with different levels of low methoxy pectin showed the occurrence of oil droplets aggregation, flocculation and gel-like structures by CLSM images very similar to the ones presented in this paper (Zhang *et al.* 2015). These gel forming capacity due to surfactant interaction with polysaccharides, was also demonstrated in sodium caseinate oil-in-water emulsion with cellulose, exploring the cellulose for increase whole system stability

(Hu *et al.* 2016). Micro and nano emulsions have been extensively explored as delivery systems for carotenoids such as lutein, lycopene, β -carotene and overall total carotenoids (Davidov-Pardo *et al.* 2016, McClements and Gumus 2016; Zhang *et al.* 2016; Salvia-Trujillo and McClements, 2016; Liu *et al.* 2015). However, for the best of our knowledge, this is the first time a microemulsion is produced by directly processing surfactant, oil and carotenoid rich fruit pulp aiming to encapsulate their carotenoid and increase bioaccessibility of lycopene and β -carotene.

5.3.3. Tracking the carotenoid over the microemulsion production

In this section we focus on retention and encapsulation efficiency of total carotenoids, lycopene and β -carotene during the microemulsion processing as well as the effect of HSH-US (T-U) treatment at different times and the differences between the surfactants used (T80 or WPI). On Figure 3 results indicate slight degradation of total carotenoids being significant only for pitanga WPI and buriti T80 microemulsions that were US processed for 8 min. The overall data of total carotenoids, when compared to previous retention study (Figure 1), suggest that surfactant and oil presence during processing may protect total carotenoids, since the impact is much less noted. The energy input is probably caught by the emulsion formation, by size reduction of the oil droplets and by the surfactant stabilization of the whole system avoiding the hydroxylation, isomerization and cleavage phenomena since these main forms of carotenoid chemical degradation are energy dependent (Carail *et al.* 2015; Sun *et al.* 2010). Further, Carail *et al.* (2015) demonstrated that degradation caused by ultrasound needs that carotenoids have contact to oxygen and/or to the radicals hydroxyl ($\text{OH}^\bullet$) and hydrogen peroxy radicals ($\text{HO}_2^\bullet$) formed due to sonolysis of water, effects that surfactant cover may prevent. Efficiency of encapsulation of US exclusive processing is lower than HSH combined with US in all cases, exception for pitanga WPI microemulsion processed 4 min for HSH and for US (figure 3). Surfactant effect is significant only for pitanga ($p < 0.05$), where WPI have higher efficiency of encapsulation (figure 3).

Figure 3 – Effect of treatment and surfactant on total carotenoid content and its encapsulation for pitanga and buriti fruits. Results are total carotenoids determined spectrophotometrically. Acronyms meaning treatments HSH-US (T-U) and time of processing (min-min). Different letters for the same series represents significant differences between treatments ($p < 0.05$).

Regarding the amount of total carotenoid emulsified there are significative differences for pitanga T80, pitanga WPI and buriti WPI microemulsions ($p < 0.05$). These differences may be related to total carotenoid degradation, *i. e.* similar to demonstrated at Figure 1, along with the carotenoid encapsulation also occurs some degradation. It is noteworthy that pitanga and buriti are very complex matrices and have a diverse profile of carotenoids (Azevedo-Meleiro and Rodriguez-Amaya 2004; de Rosso and Mercadante, 2007) in our preliminary study of pitanga and buriti carotenoids profile and bioaccessibility it were identified up to 14 different carotenes and xanthophylls (chapter 3). The enclose of carotenoids into the oil droplet may not obey a balanced incorporation, so that carotenoid x carotenoid interactions plays an important rule. The encapsulation of carotenoids inside oil droplets can be compared to their incorporation into the mixed micelles formed during intestinal digestion. Many reports shows greater micellarization of xanthophylls than carotenes in vegetables due to their degree of polarity (Petry and Mercadante, 2017; Dube *et al.* 2018). Also, β -carotene, lycopene, α -carotene and lutein micellarization are affected differently by addition of unsaturated fat (higher micellarization) or saturated fat (lower micellarization) (Mashurabad *et al.* 2017). Therefore, in the case of preferable incorporation of some carotenoids, the spectrophotometric method used to determine total carotenoids will misread interpretation of data, that is why we also used liquid chromatography to follow singly β -carotene and lycopene (Figure 4). There is no significative differences regarding efficiency of encapsulation of lycopene in pitanga due to treatment or surfactant used. Degradation of lycopene was light and it was significative only for WPI T-U 4'-8'. The same was observed regarding absolute amount of encapsulated Lycopene. Total and encapsulated amounts of β -carotene from buriti decreased for T80 T-U 4'-8' microemulsion. By general observation of Figure 3 and 4 it is possible to note that lycopene and β -carotene tendency of degradation is related to intensification of the processing independently of fruit or surfactant used.

Pitanga (T80, Lyc)

Pitanga (WPI, Lyc)

Buriti (T80, βC) Buriti (WPI, βC)

Figure 4 – Effect of treatment and surfactant on pitanga's lycopene (Lyc) and buriti's β -carotene (βC) contents and its encapsulation for each fruit. Results are determined by HPLC-DAD. Acronyms meaning treatments HSH-US (T-U) and time of processing (min-min). Different letters for the same series represents significant differences between treatments ($p < 0.05$).

Carotenes are very sensitive molecules and can easily degrade due to, light, heat, energy input, presence of oxygen and acidity (Rodriguez-Amaya 2010). Since preliminary study of processing the whole pulp showed great reduction for total carotenoids (Figure 1), it was expected great reduction of β -carotene and lycopene due to microemulsion preparation. However the significant impact of processing microemulsion was not severe (Figure 4). We attribute this to the protection effect of surfactant, as discussed above. Accordingly, Hejri *et al.* (2013) demonstrated that surfactants have a protective effect in the light stability of β -carotene in microemulsion during storage and observed that this protection is surfactant dependent. They also suggested that surfactants may provide another effects besides photoprotection against β -carotene breakdown in food products. Lycopene microemulsions made with eight different surfactants, including the T80, were processed by diverse methods (Freeze–thaw cycles, pasteurization and short exposure to UV irradiation) and had none effect on lycopene concentration while higher-heat shorter-time processing, and sterilization had a 25% reduction on lycopene content (Amiri-Rigi and Abasi, 2017). These results show the importance about carotenoid stability during microemulsion formulations and how carotenes microemulsion present a relative high stability to processing.

Usually lycopene extraction, recovery and bioaccessibility are very low, mainly due to its crystalloid form and high hydrophobicity. But micro and nano emulsions have been used to increase lycopene extraction and bioaccessibility for commercial purposes. Amiri-rigi *et al.* (2016) reached 36% of lycopene extraction from tomato waste using microemulsions made with saponin and glycerol as surfactant and co-surfactant. Li *et al.* (2017) increased the carotenoids bioaccessibility from tomatoes up to 5 times by mixing them with microemulsions (2% olive oil, 1% WPI) and the authors attributes this effect to the ability of the oil droplets go through the cellular walls and solubilize the carotenoids.

5.3.4. Carotenoid recovery after dynamic simulation of the digestion in the gastrointestinal system

Degradation and losses of β -carotene, lutein and lycopene during static *in vitro* digestions, in the presence or not of digestive enzymes as well as dietary pro-oxidants, were minutely studied in the work of Kopec *et al.* (2017). They reported a decrease in the remaining β -carotene, lutein and lycopene of approximately 40%, 40% and 20% *after in vitro* digestion with digestive enzymes without pro-oxidants, respectively. The degradation reached up to 80% of β -carotene *in vitro* digested with enzymes and presence of metmyoglobin (Kopec *et al.*

2017). Our results of total carotenoids, β -carotene and lycopene recovery from whole pulp and fruit microemulsions after digestion in dynamic gastrointestinal system are presented in the Figure 5. Whereas our samples presented high instability of carotenoids during the digestion (ranging 8.7% - 53 % of total recovery, Figure 5) along with high variation of data, we choose to give statistics indicating highly significative ($p < 0,01$, ***) and significative ($p < 0,05$, **).

Final recovery of total carotenoids from microemulsions was higher than whole pulps. Pitanga microemulsion also presented a higher recovery of total carotenoids in stomach, *i.e.* after 90 min of digestion. Recovery was calculated individually for stomach and duodenum while final recovery is related to all digestive steps (stomach, duodenum, jejunum and ileum). Therefore, bigger losses of total carotenoids in buriti, β -carotene and lycopene in pitanga happened during jejunum and ileum phases. β -carotene from buriti had very low recovery, especially in the stomach that was 3.1% and 5.4%, respectively for pulp and microemulsion. Blanquet-Diot *et al.* (2009) reported recoveries for β -carotene from yellow and red tomatoes digested in the TNO gastrointestinal tract model (TIM) of ~ 20% in the stomach and ~ 6.5 % in the duodenum.

Figure 5 – Total carotenoids (TC), β -carotene (β c) and lycopene (Lyc) recovery after dynamic gastrointestinal digestion. Recovery of stomach and duodenum were determined from an aliquot collected at 90 min and 120 min respectively. Final recovery is the fraction between the amounts found in jejunal filtrate, ileum filtrate and the digesta residue (unfiltered) with the initial sample. Statistics are differences between whole pulp and microemulsion (*** = $p < 0.01$; ** = $p < 0.05$).

Since the oil droplets size is an important factor in microemulsions, we tentatively tried to measure the particle size of microemulsions by light scattering diffraction (DLS), however reliable data could not be obtained for the lipid droplets in the presence of fruit matrix. The various size of fruit fibers, fragments and agglomerates (Figure 2) dominated the light-scattering signal. For this reason, the DLS was used only during the digestions to follow samples charge (ζ -potential) over the stomach, duodenum, jejunal and ileal filtrates as well as the residual digesta (figure 6). ζ -potential followed the expected during the digestion in the gastrointestinal simulator, keeping up with the pH from acidity in the stomach to alkalinity in the intestinal phases (Pinheiro *et al.* 2013). Initial whole pulp and microemulsion diluted 1:1000 v/v presented negative ζ -potential (ranged from -15 mV to -10 mV) significantly increasing in the stomach (-13 mV to 4.8 mV, $p < 0.05$) and then decreasing in the duodenum (-34 mV to -21 mV, $p < 0.05$). Initially, pitanga pulp and microemulsion differed between them and from buriti samples, that is explained by pitanga pulp and microemulsion pH (1.7 and 3.5 respectively), while buriti maintained the 3.2 pH. There were no differences between samples inside the digestion system, excepting for pitanga pulp in the stomach that can be attributed to high acidity of the initial sample. The charge variation can help to explain the degradation observed for the carotenoids since as far from 0, the ζ -potential indicates intensity of reactions that have potential to occurs (Pinheiro *et al.* 2013).

Figure 6 - ζ -potential values of pitanga and buriti whole pulps and microemulsions (T80 4'-4') as they passed through the dynamic gastrointestinal system. Initial samples were diluted 1:1000 (v/v), while chyme and digesta were diluted 1:10 (v/v). Statistics are mentioned in the text discussion.

The results obtained are not only due to β -carotene degradation, but also to substantial losses that happen inside the gastrointestinal systems. It was observed that after the whole digestion the stomacher bags where the digestion occurs changed to light yellow color (for buriti) and light red (for pitanga), especially in the stomach phase. Carotenes easily interacts with plastic materials due to their hydrophobicity, mainly when they are dispersed in aqueous solution (Rodriguez-Amaya 2001). For some buriti samples, the light yellow color appeared as a line coincident to the digesta surface, that is an evidence samples oil after released migrated to surface in the stomach thus allowing carotene contact with the stomacher bag. In the case of pitanga, small red fibers were observed when cleaning the hollow-fibre device, indicating material losses entrapped in the system. These losses contribute to the low recovery found, and probably caused and underestimation of the bioaccessibility results despite the agreement of our findings with others (Kopec *et al.* 2017; Blanquet-Diot *et al.* 2009). Thus, the recovery of carotenoids after dynamic gastrointestinal digestions may be caused not only by carotenoids degradation – *i. e.* oxidation, isomerization and breakdown due to pH, oxygen, enzymes and pro-oxidants – but also by limitations of the system applied – such carotenoid adherence at stomacher bags, entrapment of undigested fibers and photodegradation, since the unit reactors are made with transparent glass. Our data, corroborating with literature (Kopec *et al.* 2017; Blanquet-Diot *et al.* 2009), implicates that researchers need to increase the attention on the main rule of carotenoid stability during human digestion and its simulation (*in vitro* digestions and gastrointestinal systems).

5.3.5. Carotenoid bioaccessibility determined by the dynamic gastrointestinal system

Our hypothesis was that the direct processing of the whole pulp, surfactant and oil together would transfer the carotenoids, specially β -carotene and lycopene, to the oil droplets and increase their bioaccessibility. The carotenoids encapsulation into the oil droplets is demonstrated in a parallel work (unpublished) as well as the microemulsion microstructure exposed. The present paper confirms that is possible to increase β -carotene and lycopene bioaccessibility ($p < 0.01$), and results are exposed in the Figure 7. The microemulsion processing and formulation chosen was able to improve final bioaccessibility of total carotenoids from buriti, β -carotene from pitanga and buriti, and of lycopene from pitanga (Figure 7). The final bioaccessibility is the sum of jejunal and ileal bioaccessibility. The microemulsion of pitanga presented better bioaccessibility of β -carotene and lycopene in the jejunum, while microemulsion of buriti had higher bioaccessibility of β -carotene in the ileum

(Figure 7). General bioaccessibility is higher in the Jejunum ($p < 0,05$). Whole pulp was better than microemulsion in total carotenoids and β -carotene bioaccessibility from pitanga only in the ileal phase.

Carotenoids bioaccessibility are very dependent of the fruit matrix, specially their deposition form in chromoplasts, and can variate widely. β -carotene bioaccessibility reported in Schweiggert *et al.* (2012) measured by the static *in vitro* digestion was approximately 0.5% in carrots, 3% in tomatoes, 5% in papaya and 10% in mango. Lycopene in the same work was $\sim 0.3\%$ for tomato and papaya, and significantly increased to $\sim 0.7\%$ for tomato by the addition of 2.5% of sunflower oil (Schweiggert *et al.* 2012). Beyond the simply oil addition, excipient microemulsions were able to increase carotenoid bioavailability from vegetables, like total carotenoids from yellow peppers (Liu *et al.* 2015), total carotenoids (Li *et al.* 2017) and lycopene (Salvia-Trujillo and McClements, 2016) from tomatoes, and α -carotene and β -carotene from carrots (Zhang *et al.* 2016). For example, for the excipient emulsions made with corn oil and WPI as surfactant mixed with raw carrots, the final extent of α -carotene and β -carotene bioaccessibility was approximately 0.9, 0.8, 13 and 26 % respectively for microemulsions made at 0, 2, 4 and 8 % of corn oil (Zhang *et al.* 2016). Note that these mentioned reports assessed the carotenoid bioaccessibility by a static model of *in vitro* digestion. Also the direct preparation of microemulsion using the whole pulp is a innovation did by our group. Therefore it can explain some discrepancies in data from literature when compared to our results.

Figure 7 – Total carotenoids (TC), β -carotene (β C) and lycopene (Lyc) bioaccessibility after dynamic gastrointestinal digestion. Bioaccessibility is the fraction between the amounts found in jejunal filtrate and ileal filtrate in relation to the amount in the initial sample. Statistics are differences between whole pulp and microemulsion (*** = $p < 0.01$; ** = $p < 0.05$).

Despite the positive conclusions regarding microemulsion data, some of our results are lower than previous works found in the literature. We attribute this discrepancy primarily to differences of static/dynamic *in vitro* digestion models applied, and also to the low stability and carotenoids losses inside the gastrointestinal system. Previous research using the same dynamic gastrointestinal system equipment that is applied in the present work found ~ 15% bioaccessibility of β -carotene from lipid nanoparticles of cupuaçu butter (*Theobroma grandiflorum*) (Gomes *et al.* 2017). The authors demonstrated that their lipid nanoparticles were highly stable during storage and digestion, being that nanoparticles were very resistant to stomach acidity, releasing β -carotene only in the duodenum. So that, they identified lower losses of β -carotene inside the system (*e.g.*, residues adhered to the dynamic digestion model walls). The carotenoids degradation and losses may happen preferentially with the already released carotenoids from the cellular structures or from microemulsion microcapsules. There is only a few works that evaluated bioaccessibility of carotenoids microemulsions by the dynamic gastrointestinal model. Van Loo-Bouwman *et al.* (2014) reported 30 and 53 % of β -carotene bioaccessibility from a mixed diet and an oil diet, respectively, measured by the TIM-1 equipment, but they did not compared results with other studies using the dynamic gastrointestinal models. Bioaccessibility of egg's xanthophylls, zeaxanthin and lutein, were measured by the TIM equipment, that presented a range of 20 – 40 % of these compound found in the ileal and jejunal filtrates (Nimalaratne *et al.* 2015). Note that carotenoids recovery in the both studies were very high (69 – 105 %) (Van Loo-Bouwman *et al.* 2014; Nimalaratne *et al.* 2015). In summary, pitanga and buriti microemulsion processing and formulation established in the present work were able to increase carotenoids bioaccessibility. The improvement was higher for pitanga's microemulsion, specially lycopene, that was 4 times higher than the whole pulp.

5.4. Conclusion

The treatments and microemulsion formulation in this work were able to: 1) release carotenoids from pitanga and buriti fruit matrices; 2) encapsulate the released carotenoids into oil droplets from fruit microemulsions; 3) protect the carotenoids against degradation during processing; 4) provide higher recovery of total carotenoids from microemulsion than from whole pulp after passing through a dynamic gastrointestinal system; 5) and increase β -carotene and lycopene bioaccessibility in the microemulsions. The limitations are related to the sensitivity of the carotenoids to the extensive work – *i. e.* many steps from whole pulp take of,

drying and transportation, microemulsion fabrication, five hours being digested, extractions in organic solvents and finally the HPLC analysis. Nevertheless, reliable data were produced to conclude regarding carotenoid behavior during the pitanga and buriti microemulsions processing and its bioaccessibility. For the best of our knowledge, this is the only work where microemulsion is produced by directly processing surfactant, oil and fruit pulp rich in carotenoid that encapsulate their carotenoid and increase their bioaccessibility, specifically lycopene and β -carotene. The overall bioaccessibility of carotenoids in complex systems including microemulsion still need much research. Moreover, still lack research applying dynamic gastrointestinal models for studying carotenoids bioaccessibility in complex matrices. On this way, our work contributes to the field of structure designing food matrices for increasing carotenoid bioaccessibility.

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6 FINAL CONSIDERATIONS

This research had an intention to improve the knowledge of non-conventional tropical fruits, especially the ones that are native from Brazilian biomes. Regarding this objective this thesis provide many data that can stimulate the interests of researchers, industry, food technologist, nutritionists and consumers on the studied fruits, specially pitanga and buriti. Further studies should be conducted to boost the sustainable use of Brazilian native fruits.

The structure design approach showed to be an effective tool in the development of fruit microemulsion with increased carotenoid bioaccessibility. However there is a vast field to be explored at this topic in order to address more positive properties to final products. Along with this approach, microscopy strategies emerges with great potential for studying food structure in relation to chemical and nutritional impact.

The microemulsion produced had their structure revealed, what evidence the key role played by carbohydrates and surfactant interactions and their influence on bioaccessibility. On this way, despite the microemulsion that was developed increase stability and bioaccessibility of carotenoids from pitanga and buriti, a more detailed and extensive work is needed to explore and explain the potentials and constraints of fruit microemulsified products.

The methods used in the present thesis for inferring the carotenoids behavior during the human digestion are until now among the best techniques available – in vitro digestion, human intestinal cells cultures and dynamic simulators of digestive systems. However, these techniques still have many limitations that may have influenced the assumptions concluded, despite our efforts and care of data interpretation.

Pitanga and buriti fruits have great commercial potential as raw materials for new products. They may introduce to consumers new textures, flavors and options among healthy food. The present research had shown only one possibility of product that takes advantage from pitanga's and buriti's carotenoid profiles, contents and fruit matrices. Many other options of processing and formulations could be tested and applied with the main highlight: these fruits are very rich sources of diverse carotenoids with recognized properties for improving consumers health.