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# Enhancement of the *in vitro* bioavailable carotenoid content of a citrus juice combining crossflow microfiltration and high-pressure treatments

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## ABSTRACT

High-pressure treatments combined with crossflow microfiltration were used to obtain citrus concentrates enriched in carotenoids. The aim of this study was to investigate the effect of this process combination on carotenoid bioaccessibility and uptake by intestinal Caco-2 cells. Two high-pressure processes, high hydrostatic pressure treatment (HHP) and ultra-high-pressure homogenization (UHPH) were compared to conventional pasteurization. Processing effects on carotenoid content and bioaccessibility, on physicochemical and structural characteristics of the product, on methylation degree of pectins and micelle size after *in vitro* digestion were assessed. UHPH at 400 MPa drastically enhanced carotenoid bioaccessibility compared to HHP and pasteurization. Moreover, carotenoid uptake by Caco-2 cells was significantly improved by UHPH underlining the importance of the micelle size after *in vitro* digestion and the degree of methylation of pectins in this uptake. Finally, the *in vitro* bioavailable carotenoid content of different concentrates was evaluated, taking into account carotenoid content, bioaccessibility and uptake. Combining crossflow microfiltration with UHPH increased by 4-6 fold the bioavailable carotenoid content in the final product. The process led to a concentrate of high nutritional quality compared to the original juice, raw or pasteurized concentrates.

**Keywords:** citrus based-product, carotenoid bioaccessibility, intestinal cells, High-pressure homogenization.

**HIGHLIGHTS**

- Neither pasteurization nor high pressure treatments altered carotenoid content
- UHPH led to the concentrate with the highest bioavailable carotenoid content (BCC)
- Carotenoid uptake is linked to micelle size and degree of methylation of pectins
- CFM/UHPH combination multiplied BCC in the concentrate by up to 6 compared to the juice

## Introduction

Citrus fruits are mainly processed into juices, which are consumed worldwide. The nutritional health benefit of the consumption of citrus fruits is now well established. Numerous epidemiological studies have reported that citrus consumption reduces the risks of lifestyle-related diseases, such as cancers, cardiovascular diseases, and diabetes (Ma, Zhang, Sugiura, & Kato, 2020). Citrus juices provide natural bioactive compounds such as vitamins (vitamin C, folate), dietary fibers (pectins), and minerals as well as phytomicronutrients, such as polyphenols and carotenoids. These liposoluble components are responsible for the attractive color of citrus fruits. Indeed, carotenoids contribute to provitamin A activity, as well as antioxidant (Rodriguez-Concepcion et al., 2018; Zou, Xi, Hu, Nie, & Zhou, 2016), antidiabetic and anti-cancer properties (Cancalon, 2016). To exert their beneficial effects on health, carotenoids have to be bioavailable. Carotenoid bioavailability corresponds to the absorbable fraction available for use and storage in the body. It includes various steps (absorption, metabolism and tissue distribution), the two most important being carotenoid bioaccessibility and their absorption by intestinal cells. Carotenoid bioaccessibility corresponds to the proportion of carotenoids released from the food matrix and transferred into mixed micelles that can then be absorbed by the human intestinal cells (Poulaert, Borel, Caporiccio, Gunata, & Dhuique-Mayer, 2012). The main barrier governing carotenoid bioaccessibility from fruit and vegetables is the food matrix microstructure associated with lipids or dietary fibers (Rodríguez-Roque et al., 2016; Verrijssen et al., 2014). Mechanical and thermal treatments could improve carotenoid bioaccessibility reducing particle size and helping the release of

carotenoids from cell microstructure (Gence, Servent, Poucheret, Hiol, & Dhuique-Mayer, 2018). In this regard, the same authors demonstrated that pro-vitamin A carotenoid bioaccessibility was greater from commercial citrus juice and concentrate than fresh juice and concentrate obtained by crossflow microfiltration. Moreover, the same authors showed that commercial citrus products were characterized by lower dietary fiber content, such as pectins, with smaller micelle size (micelles being then potentially absorbed by intestinal cells after *in vitro* digestion) compared to fresh products. Thus, processing decreased pectin content, which is conducive to higher carotenoid micellarization, by reducing micelle size, leading to better carotenoid bioaccessibility. In the same way, other studies on citrus juices showed that carotenoid bioaccessibility and bioavailability of β-carotene and β-cryptoxanthin were higher in pasteurized citrus juices than in fresh juices (J. Aschoff et al., 2015; J. K. Aschoff et al., 2015). These last results were supported by Netzel et al. (2011) and Aherne, Daly, Jiwan, O'Sullivan, and O'Brien (2010) who found that thermally processed/cooked carrot puree had higher carotene bioavailability than those untreated. More recently, concentrates formulated with 60/40 *C. clementina / C. paradisi* juices, obtained by crossflow microfiltration, were studied in order to optimize their nutritional properties such as sugar, pectin, carotenoid and flavonoid contents (Hammad, Dornier, Servent, Poucheret, & Dhuique-Mayer, 2021). Another study showed that an optimized process combining crossflow microfiltration with different operating conditions improved both carotenoid content and bioaccessibility, thus increasing the amount of bioaccessible carotenoids (di Corcia, Dhuique-Mayer, & Dornier, 2020).

In line with these last results, several other processes have been explored in order to increase carotenoid bioaccessibility in citrus juices such as high-pressure treatments including high hydrostatic pressure (HHP) and ultra-high-pressure homogenization (UHPH). Initially, these two processes were applied to reduce the number of microorganisms in food products, to stabilize them and to extend their shelf life. At the current time, there is an increasing interest for these innovative and friendly non-thermal stabilization technologies as an alternative to the conventional heat stabilization processes.

For the HHP process, pressure is equally applied in all directions of the food product whereas for UHPH, the pressure treatment is accompanied by high shear stress distribution (Augusto, Tribst, & Cristianini, 2018). Otherwise, in contrast to the high hydrostatic pressure treatment, ultra-high-pressure homogenization has a physical effect in addition to a thermal effect on the concentrate leading to different consequences on structural parameters and carotenoid release from food products. Impacts of UHPH were evaluated on several fruit juices, including orange (Betoret, Betoret, Carbonell, & Fito, 2009). This technology constitutes a promising alternative to produce high quality and healthy food products (Sentandreu et al., 2020). Recent studies showed that carotenoid bioaccessibility was greatly increased by high-pressure treatment (Sentandreu et al., 2020; Carla M. Stinco et al., 2020). Indeed, HHP could modify the rheological properties of products that enhance the release and micellar incorporation of carotenoids during *in vitro* digestion (Palmero et al., 2016). Finally, to the best of our knowledge, no studies have investigated the effect of high pressures on carotenoid bioaccessibility and absorption by intestinal cells at the same time. Furthermore, the combination between crossflow microfiltration and high-pressure processing has not been studied yet.

Thus, the aim of this work was to assess the impact of high pressures coupled to crossflow microfiltration on the bioaccessibility of carotenoids as well as on their uptake by Caco-2 intestinal cells from citrus concentrates. First, two high pressure treatments were carried out, HHP and UHPH, and their effects on carotenoid bioaccessibility of citrus concentrates were compared to those of conventional pasteurization. Then, the high-pressure process leading to the best carotenoid bioaccessibility was selected and their absorption by intestinal cells was also investigated using an *in vitro* digestion model coupled with the intestinal Caco-2 cell model. To go further, the *in vitro* bioavailable content of carotenoids was assessed.

# Materials and methods

## 1. Materials

Commercial and flash-pasteurized juices from clementine *(Citrus clementina* Hort. ex Tan.) and pink grapefruit (*Citrus paradisi* Macf.) were purchased in a local supermarket (Saint-Clément de Rivière, France). These 100% pure juices were kept at 4°C for one week until processing. A formulation with 60% clementine juice and 40% grapefruit juice was chosen in order to obtain a balanced carotenoid profile: β-cryptoxanthin (from clementine), lycopene (from grapefruit) and β-carotene (from both). 54 kg of juice were purchased for this study.

## 2. Processing

The whole process was detailed in **Figure 1**. Citrus juices underwent several treatments, the main one being crossflow microfiltration (CMF) described by di Corcia et al. (2020). First, citrus juices were pre-liquefied by pectinolytic enzymes in order to decrease their viscosity and to limit membrane fouling. The enzyme mixture, Ultrazym AFP, was commonly used for filtration and clarification of fruit juices (Sandri, Fontana et al. 2011, Bajpai 2012). Then, the concentration of carotenoids by crossflow microfiltration was carried out ending with a diafiltration step. A final stabilization step using pasteurization or a high-pressure treatment was added to stabilize the product eliminating the microorganisms retained in the concentrate.

### 2.1. Crossflow microfiltration

The crossflow microfiltration process was performed thanks to a TIA device as already described in detail by Polidori, Dhuique-Mayer, and Dornier (2018). Four tubular membranes (Tami industries, Nyons, France) in titanium oxide with an area of 55 $cm^2$ and an average pore size of 0.2 µm were installed in series. The products were filtered at a transmembrane pressure of 2.6 bar, a temperature of 30°C and a crossflow velocity of 5 $m \cdot s^{-1}$. Each trial was conducted increasing the mass reduction ratio (MRR) up to about 8. During the concentration step, retentate volume was maintained constant in the

system by adding the same mass of juice to the feeding tank as the mass of the extracted permeate. A diafiltration step followed the concentration step, using distilled water as a solvent, that allowed the retentate to be purified by removing the water-soluble compounds. The mass of distilled water added to the feed tank compensated the mass of permeate extracted up to reach a diamass ratio (DMR) of 1.

## 2.2. Stabilization by pasteurization and high pressure treatments

### 2.2.1. Pasteurization

Pasteurization of citrus concentrates was carried out with a pasteurization value $P_0$ of 100 min using a z-value of 10°C and 70°C as the temperature of reference (di Corcia et al., 2020). This treatment was in accordance with standard practices in the industry (Gates, 2012). Briefly, concentrates were distributed in several 15 mL glass tubes and put in a water bath at 80°C. The temperature of concentrates was measured every minute with a thermal probe placed into the tubes and $P_0$ value was calculated according to the standard Ball procedure. Once the desired $P_0$ value was reached (after 14 min), the tubes were quickly immersed in an ice water bath to cool down and stop the treatment. After treatment, samples were immediately stored at -18°C until analysis.

### 2.2.2. High pressure treatments

- *High hydrostatic pressure (HHP)*

HHP treatment was performed in a hydrostatic pressure device with 1 L capacity, and a maximum pressure of 400 MPa (ACB-Nantes, Gec-Alsthom L, 1L HP vessel, water as pressure transmitting fluid). Compression and decompression rates were 100 $MPa \cdot min^{-1}$. A computer program controlled pressure, time and temperature. Citrus concentrates were packed in several 30 mL flexible bags (made of polyvinylidene chloride) and treated at 400 MPa and 20°C for 15 to 60 min. During product pressurization, the adiabatic compression resulted in a temperature increase of 2-3°C every 100 MPa

(about 10°C at 400 MPa). This temperature increase is due to the heating of the water during compression (Cheftel & Culioli, 1997). After treatment, samples were immediately frozen and stored at -18°C until analysis.

- *Ultra-high-pressure homogenization (UHPH)*

UHPH was carried out with a FPG7575 homogenizer (Homogenizing Systems Ltd, Harlow, UK) which worked at pressures of up to 420 MPa with flow rates of up to 125 $L \cdot h^{-1}$. Temperature was controlled for the system inlet, in process and outlet post process. Concentrates were introduced into the circuit at 4-8°C, and were treated at 40, 100, 250 and 400 MPa. In some cases, at 400 MPa, the product was pre-treated at 40 MPa in order to limit the risk of clogging of the high-pressure valve. After passing the homogenizing valve, the concentrate temperature reached 20, 40, 60 and 100°C respectively according to the pressure applied. Maintained for 1 to 2 s at this temperature in the exit pipe, the concentrate was rapidly cooled down below 30°C thanks to a tubular heat exchanger fed with cold water at 10°C. After treatment, samples were immediately stored at -18°C until analysis.

## 3. Product characterization

### 3.1. Physico-chemical and structural parameters

Physico-chemical characteristics such as pH, titratable acidity TA, total soluble and suspended insoluble solids TSS & SIS, total dry matter TDM in the juice and concentrates were determined according to the methods described by di Corcia et al. (2020). This reference was also used for the measurements of the limit apparent viscosity for a 1000 $s^{-1}$ shear rate and the particle size distribution ($D_{50}$ and span obtained by Laser diffraction).

### 3.2. Degree of methylation of pectins

The measurement of the degree of methylation (DM) was based on the method used by De Roeck et al. (2009). The DM corresponds to the ratio between the molar amounts of methoxyl groups to the

molar amount of galacturonic acid in the alcohol-insoluble residue (AIR). Colorimetric methods were applied for methanol and galacturonic acid measurements using alcohol oxidase and m-hydroxydiphenyl, respectively.

### 3.3. Carotenoid analysis

#### 3.3.1. Carotenoid content

##### *3.3.1.1. Extraction*

Carotenoid extraction and HPLC analyses were carried out according to the method described by di Corcia et al. (2020) which was optimized and applied to citrus juices and concentrates. Briefly, 2 g of juice or 0.5 g of concentrate were mixed with 0.5 mL distilled water and 2 mL of ethanol with 1% pyrogallol and vortexed in a glass tube (20 mL). The tube was then placed in a water-bath at 70°C for 2-3 min, protected from light. Two mL of KOH 12 $mol.L^{-1}$ was added and the mixture was vortexed and set in a water bath at 70°C for 30 min. After cooling in an ice bath, 2 mL of distilled water was added to help the phase shift. The extraction was carried out twice with 5 mL of hexane. The hexane phase was pooled and evaporated under nitrogen in a water bath at 37°C. The dry extracts were dissolved in 500 µL of $CH_2Cl_2$ and 500 µL of methyl tert-butyl ether (MTBE)/methanol mixture (4:1, v/v) in an amber vial before injection in HPLC.

##### *3.3.1.2. HPLC analysis*

HPLC carotenoid analyses were performed according to a previous study (Poulaert et al. 2012) with an Agilent 1100 system equipped with a diode array detector and autosampler. The column used was a C30 YMC column (250 x 4.6 mm; 5 µm, YMC Europe GMBH, Germany). The injection volume was 20 µL, the flow rate was 1 $mL.min^{-1}$ and the analysis temperature was 25°C. The mobile phases were water (eluent A), methanol (eluent B) and MTBE (eluent C) following the gradient described by Gleize, Steib, André, and Reboul (2012). The absorbance was measured at 450 nm to identify β-cryptoxanthin (BCX),

β-carotene (BC) and 470 nm for lycopene (LYC). The different carotenoids were identified using a Chromatographic data and UV-Visible spectra (Agilent ChemStation Plus software).

### 3.3.2. Carotenoid bioaccessibility

#### *3.3.2.1. In vitro digestion*

The *in vitro* digestion model used in our study was initially developed by Reboul et al. (2006) especially for carotenoids. It had been validated against human studies and was considered to be a reliable model for carotenoid behavior during *in vitro* digestion (Etcheverry, Grusak, & Fleige, 2012). This model was adapted for citrus juices according to Dhuique-Mayer et al. (2007). Carotenoid extraction from digested samples was performed as previously described by Gence et al. (2018). Briefly, 30 g of juice or 5 g of concentrate was mixed in 32 mL of saline solution and homogenized in a shaking water bath for 10 min at 37°C. The pH was adjusted to 4.0 with 1 mol·$L^{-1}$ NaOH and 2 mL porcine pepsin (P6887 Sigma 3,200-4,500 units/mg protein) was added to mimic the gastric digestion step. The homogenate was incubated in the shaking water for 30 min at 37°C, was readjusted to pH 6.0 with 0.45 mol·$L^{-1}$ sodium bicarbonate and mixed with pancreatin (P1750 Sigma 4 x USP specifications), bile extract and cholesterol esterase (32 U·mg-1) to mimic the intestinal digestion step. Cholesterol esterase is needed to cleave esters of β-cryptoxanthin in order to be close to human metabolism (Breithaupt, Weller, Wolters, & Hahn, 2003). The homogenate was incubated again in the shaking water for 30 min at 37°C. Samples were then centrifuged (48 000 x g for 4 h at 10°C), the aqueous fraction was collected and 0.2 µm filtered with cellulose acetate membrane filters (Minisart, Syringe Filter, Sartorius).

#### *3.3.2.2. Carotenoid from micellar phase analysis*

Carotenoid extraction from filtered digested samples was performed as previously described by Dhuique-Mayer et al. (2007). Briefly, an aliquot of 10 mL of the micellar phase from a digested sample

was extracted 3 times with 10 mL of hexane and 5 mL of ethanol containing 100 µL of β-apo-8'-carotenal (at 24.4 $mg.L^{-1}$) as an internal standard. The collected hexanic phases were dried with anhydrous sodium sulphate. The pooled hexane extracts were evaporated and dissolved in 250 µL of $CH_2Cl_2$/250 µL of the MTBE/methanol (4:1, v/v) before HPLC analysis according to the analytic conditions described by di Corcia et al. (2020).Bioaccessibility of a carotenoid i, noted $\beta_i$, was calculated according to Equation 1 and expressed in percentage (%).

$$\beta_i = 100\frac{m_i}{m_{i0}} \qquad \text{Equation 1}$$

$m_i$: amount of carotenoid i in the micellar phase (mg)

$m_{i0}$: amount of carotenoid i in the initial sample of citrus juice or concentrate (mg)

3.3.3. Carotenoid uptake by intestinal cells (Caco-2)

*3.3.3.1. Cell culture*

The carotenoid uptake was measured using clone TC7 Caco-2 cells offered by U178 UNSERM (Villejuif, put a coma France). These TC7 clones have been widely used as an *in vitro* model for intestinal uptake measurements and their culture conditions were described by Gence et al. (2018). Cells were cultured for 18 days until reaching confluent differentiated cell monolayers, with a complete medium DMEM with 4.5 $g.L^{-1}$ of glucose, 2% of L-glutamine, 1% of non-essential amino acids, 1% of antibiotics (penicillin and streptomycin) and 20% of heat-inactivated fetal bovine serum. Once confluence was reached, cells were seeded at a density of 2.5 x $10^5$ per 25 $cm^2$ flask and then incubated at 37°C in a humidified atmosphere enriched with 5% $CO_2$. The culture medium was changed every 48 h.

*3.3.3.2. Experimentation*

After 18 days, cells had differentiated into enterocyte cells. Cells were washed twice with 2 mL of phosphate-buffered saline (PBS taurocholate) and then incubated with 7 mL of diluted (at 1:4 dilution) micelles from *in vitro* digestion of juice and concentrate for 2 h at 37°C. Cell monolayers were scraped

and collected in 2 mL PBS before being stored at -80°C under nitrogen. Carotenoid extraction and HPLC analysis were reported by Gies, Servent, Borel, and Dhuique-Mayer (2020).

### 3.4. Micelle size

Mixed micelle size of filtered digested citrus concentrates was also assessed. Size measurements were performed at room temperature (25°C) using a ζ-sizer (Nano-ZS90, Malvern Instruments Ltd., UK) with a He/Ne Laser ($\lambda$ = 633 nm) and 130° scattering angle. The sample was introduced into the cuvette and particle size distribution was recorded by dynamic light scattering (DLS).

### 3.5. Statistical analyses

Statistical analyses required XLSTAT software 2016 for means and standard deviations. Statistical significance used one-way analysis of variance with a post-hoc Fisher's test. A p-value < 0.05 was considered statistically significant.

# Results

## 1. Characterization of initial juice and raw concentrate

The initial juice, formulated with 60% clementine and 40% grapefruit juice and the raw concentrate from crossflow microfiltration at MRR above 8, were characterized (**Table 1**).

Regarding the physico-chemical parameters, the main differences between the juice and the raw concentrate was due, on one hand, to the total retention of the insoluble fraction by the membrane during the crossflow microfiltration. Because particle size was much higher than the average pore diameter of the membrane (0.2 µm), SIS content of the retentate was logically increased with the same order of magnitude than the reached FRM (di Corcia et al., 2020; Polidori et al., 2018; Servent, Abreu, Dhuique-Mayer, Belleville, & Dornier, 2020). On the other hand, the diafiltration step divided TSS, TA and TDM (mainly composed of soluble solids) by around 2. This value was in accordance with the DVR of 1 that was chosen considering the system as a perfect stirred reactor (Hammad et al., 2021). Indeed, because this type of membrane did not retain the solutes, such as sugars and organic acids, they were progressively removed from the retentate and carried away in the permeate. Moreover, crossflow microfiltration caused changes of particle size distribution and viscosity of the concentrate. $D_{50}$ was divided by 23 due to the mechanical disruption of particles by shear-stress in the system associated with the enzymatic treatment, as already shown by (Dahdouh et al., 2016; Gence et al., 2018). Viscosity was multiplied only by 1.6 during crossflow microfiltration probably because of the use of pectinolytic enzymes that limited the effect of the concentration of insoluble solids and of the decrease in particle size. These modifications of the physico-chemical and structural characteristics of the product modify of course the organoleptic quality of the product, texture, taste, aroma and its nutritional properties, carotenoid content and bioaccessibility (di Corcia et al., 2020; Hammad et al., 2021).

Otherwise, processing led to a decrease in the degree of methylation of pectins (**Table 2**) probably due to the use of a commercial mixture of pectinolytic enzymes which mainly had polygalacturonase and pectinelyase activities but may also had secondary activities such as pectin methylesterase. Regarding

the carotenoid content, a balanced profile of carotenoids was obtained from the 60% clementine / 40% grapefruit formulation. BCX and LYC were the main carotenoids found in the formulated citrus juice. Carotenoid content was logically multiplied 8 fold by microfiltration in accordance with the chosen MRR.

## 2. Effect of HHP treatment on structural parameters and carotenoid bioaccessibility of citrus concentrates

### *2.1. Structural parameters*

Even though there were statistical differences between raw concentrate, concentrates treated by pasteurization or by HHP at different durations, particle size, expressed as $D_{50}$, was only slightly modified by pasteurization and by the process duration of HHP (**Table 1**). Indeed, $D_{50}$ ranged between 29 and 38 µm. Thus, the pasteurization and high pressures had no effect on the particle size of the concentrates which had been largely reduced by high shear-stress during crossflow microfiltration. Some authors have studied the effect of HHP treatment on particle size of purees or juices (Westphal, Schwarzenbolz, & Böhm, 2018; Wibowo et al., 2019). A recent study showed that HHP treatment (400 MPa, 3 min) of apple juice slightly modified particle size (Wibowo et al., 2019).

The duration of the HHP treatment did not strongly modify viscosity which ranged from 3.9 to 5.5 mPa·s for concentrates. This last result was confirmed by those found by De Ancos, Rodrigo, Sánchez-Moreno, Pilar Cano, and Zacarías (2020) who demonstrated that high pressure only slightly modified the physico-chemical properties of orange juice, such as viscosity. In fact, this process has little impact on the rheological properties of the suspension because it does not significantly modify either the insoluble phase (particle size distribution, content, etc.) or the composition of the soluble phase.

### *2.2. Carotenoid content and bioaccessibility*

No difference of carotenoid content between raw, pasteurized and HHP treated concentrates was observed (**Table 1**). Thus, whatever the duration of the treatment, HHP did not affect carotenoid

content. HHP effects on carotenoid content were very variable according to the food matrix, the processing conditions (time, temperature) and the type of carotenoid studied. Incidentally, Sánchez-Moreno, de Ancos, Plaza, Elez-Martínez, and Cano (2009) demonstrated that high pressure treatment at 350 MPa from 2.5 to 15 min led to an increase of 20-43% in the amount of extractable carotenoids in orange juice whereas treatments between 50 and 200 MPa did not change carotenoid content. Other studies showed that HHP treatment caused no significant differences in carotene content in orange-lemon-carrot formulated juice (García, Butz, Bognàr, & Tauscher, 2001). That was also confirmed by Butz et al. (2003) who found no losses in α-carotene and lycopene in a tomato puree subjected to high pressure (600 MPa, 20°C, 60 min). According to Mahadevan and Karwe (2016), HHP affects only weak bonds (hydrophobic and hydrogen bonds) causing the unfolding of macromolecules such as protein chains. It did not affect covalent bonds. Thus, vitamins, bioactive compounds and pigments, such as carotenoids, logically remain unaltered. However, note that although the release of carotenoids from cell structures was facilitated by HHP treatment, the carotenoids would probably be more exposed to oxidation or isomerization that could lead to their degradation (Cano, Gómez-Maqueo, Fernández-López, Welti-Chanes, & García-Cayuela, 2019; Carla M Stinco et al., 2019). In our case, the absence of carotenoid degradation in the citrus concentrate could be explained by the fact that these compounds could be well protected by the food matrix.

However, high pressure did have an impact on carotenoid bioaccessibility compared to raw concentrate, notably from 15 min of treatment (**Figure 2**). Indeed, after 15 min, bioaccessibility was multiplied by 1.3 times for BCX, 1.4 for BC and doubled for LYC, leading to bioaccessibility of 33% for BCX, 24% for BC and 2% for LYC. These results were not correlated to particle size distribution. Nevertheless, the process could induce modifications in the smallest fraction of particle population (including organelles), that we could not observed with the granulometer we used.

Several studies on green beans, carrots, tomatoes and fruit juice-based beverages also highlighted the positive effect of high pressure on carotenoid bioaccessibility due to the disruption of cells and disaggregation of cell clusters (Knockaert, Lemmens, Van Buggenhout, Hendrickx, & Van Loey, 2012;

Rodríguez-Roque et al., 2016). In this regard, Cano et al. (2019) showed that persimmon juice subjected to high hydrostatic pressure (200 MPa, 25°C, 6 min) had carotenoids with higher bioaccessibility than in pasteurized juice. The plant cell disruption, induced by high pressure, facilitates the release and solubilization of lipophilic compounds resulting in an increase of carotenoid bioaccessibility (Barba et al., 2017).

Our results showed that pasteurization (80°C, 14 min) was equivalent to HHP treatment, whatever the treatment duration, in terms of carotenoid bioaccessibility, which is probably explained by differences in the matrix properties. In addition, the increase in treatment duration had no effect on carotenoid bioaccessibility, which remained unchanged when treatments longer than 15 minutes were applied. Therefore, 15 min of treatment was sufficient to release carotenoids from concentrate and to micellarize them. The high measurement uncertainties obtained for lycopene were probably related to its very low bioaccessibility, due to its high apolar structure and its accumulating as solid crystalline structures within the crystalline chromoplasts in the pink grapefruit vesicle cells (De Ancos, Rodrigo, Sánchez-Moreno, Cano, & Zacarías, 2020; Lado et al., 2015; R. Schweiggert & R. Carle, 2017). Indeed, contrary to BC and BCX, LYC is located in the center of lipid droplets which means it is released less easily from lipid droplets and therefore, less easily incorporated in to mixed micelles (Tyssandier, Lyan, & Borel, 2001). Moreover, fruits with high carotenoid content in crystalline chromoplasts had lower bioaccessibility than the others which accumulated carotenoid in globular chromoplasts (Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012). In order to better understand how the processing impacts the microstructure of chromoplastes it would be of great interest to go further thanks to microscopy tools.

## 3. Effect of UHPH treatment on structural and physico-chemical parameters and carotenoid bioaccessibility of citrus concentrates

### *3.1. Structural and physico-chemical parameters*

Regarding the particle size, even though there was a statistical difference between raw concentrate, pasteurized concentrate and concentrates treated at 40, 100, 250 MPa, results were very similar between them, i.e., a $D_{50}$ between 23 and 39 µm **(Table 1)**. Note this $D_{50}$ was in the same order of magnitude as the concentrates treated by HHP. In our case, there was no effect of homogenization pressure up to 250 MPa on particle size of concentrates. These results were not in accordance with Velázquez-Estrada, Hernández-Herrero, Guamis, and Roig-Sagués (2019) who showed a significant reduction of particle size when using UHPH from 100 MPa in orange juice. However, this work focused on juices whereas our study concerned concentrates. Therefore, the particle size reduction of our concentrates might be due to a greater effect of microfiltration, as opposed to UHPH up to 250 MPa, on particle size. However, at 400 MPa (pre-treated at 40 MPa), particle size was drastically increased and was multiplied by 7.6 compared to 100 MPa and reached 148.6 µm. Note that in contrast to the HHP treatment, UHPH induces many physical effects associated with a strong thermal effect on the concentrate. Indeed, turbulence, shear, cavitation or heating caused by homogenization could induce re-aggregation from small-homogenized particles, which could explain the increase in particle size at 400 MPa. This phenomenon has already been observed for emulsions such as mayonnaise or soy protein (Aganovic, Bindrich, & Heinz, 2018; Floury, Desrumaux, & Legrand, 2002). This same concentrate was also characterized by the lowest span **(Table 1)**. This low span characterized a very homogenous and narrower particle-size distribution.

No significant difference in viscosity between concentrates was observed (about 5.3 mPa.s) except for the one treated at the highest pressure, i.e., 400 MPa (pre-treated at 40 MPa) which had a viscosity that was slightly higher of 7.5 mPa.s. This last result was in accordance with those reported by Moelants et al. (2013) on carrot suspensions or which explain that UHPH improved the mechanical solubilisation of macromolecular bio-polymers, in particular pectins, leading to an increase in the viscosity of suspensions.

Regarding the pH and titratable acidity, no difference between raw concentrate, pasteurized concentrate and UHPH concentrate (400 MPa pre-treated at 40 MPa) was observed. For soluble and

insoluble fractions (TSS and SIS) and total dry matter, very slight fluctuations were noted between these 3 concentrates that could be considered negligible (**Table 2**). UHPH at 400 MPa maintained pH, TA, SIS and TDM of citrus concentrate, that was in agreement with results found by **Patrignani, Vannini, Kamdem, Lanciotti, and Guerzoni (2010).**

### *3.2. Carotenoid content and bioaccessibility*

Carotenoid content was similar between raw, pasteurized and UHPH treated concentrates whatever the pressure applied even if slight variations were observed **(Table 1)**. Although a high temperature increase occurs during UHPH, the holding time at the highest temperature was very short (1-2 s) resulting in products with less thermal damage, thus avoiding adverse effects on flavor and nutrients (Sentandreu et al., 2020). Thus, UHPH had no effect on carotenoid content in concentrates. This could be different for juices because the study by Carla M. Stinco et al. (2020) showed a decrease of carotenoid content by UHPH under pressure from 150 MPa for fresh orange juice. The authors explained these losses by cell disruption that led to carotenoid exposure to oxidation.

Carotenoid bioaccessibility was clearly increased by UHPH (**Figure 3**). For UHPH at 40 MPa, carotenoid bioaccessibility increased by 44% for BCX, 69% for BC and 36% for LYC compared to raw concentrate. Moreover, there was either no significant difference (BC and BCX) or only a slight difference (LYC) of carotenoid bioaccessibility between pasteurized concentrate and concentrates treated up to 250 MPa. Finally, an UHPH treatment at 40 MPa led to carotenoid bioaccessibility close to that obtained at 400 MPa by the HHP treatment.

Otherwise, the UHPH treatment at 400 MPa of concentrate with a pretreatment at 40 MPa drastically increased bioaccessibility by 2.8 for BCX, 3.2 for BC and 6.4 for LYC compared to raw concentrate reaching bioaccessibility of 70% for BCX, 48% for BC and 6% for LYC. Thus, the pre-treatment at 40 MPa followed by the treatment at 400 MPa led to the highest carotenoid bioaccessibility. Recent studies on carrot juices showed that when samples were homogenized after several passes, they had significantly higher carotenoid bioaccessibility than samples homogenized only once (X. Liu et al., 2019).

A previous study by Sentandreu et al. (2020) indicated a 5 fold increase in total carotenoid bioaccessibility in mandarin juices treated by UHPH at 150 MPa reaching a temperature of 68°C for 15 s. These same authors explained the enhanced carotenoid bioaccessibility in the homogenized-treated juices by a reduction of juice particle size, which could make the carotenoid-containing pulp

particles more accessible to the digestive enzymes, facilitating their release during digestion. However, in our case, the UHPH treatment was applied to concentrates and not to juices. The particle size of concentrates was already drastically reduced by CMF processing. Thus, there was no correlation between particle size and carotenoid bioaccessibility of citrus concentrates, that confirmed the results obtained by Gence et al. (2018) and di Corcia et al. (2020).

The impact of high pressure treatment on carotenoid bioaccessibility not only varied according to the food matrix and the carotenoid type (Panozzo et al., 2013), but also to the plastid and cell substructures (Palmero et al., 2016) and the formation of a fiber network (Colle, Van Buggenhout, Van Loey, & Hendrickx, 2010). Carotenoid release, thereby carotenoid bioaccessibility, could be governed by the carotenoid structure as well as by structural barriers of cells, including the chromoplast structure and the cell wall (Palmero et al., 2013). The pressure applied during UHPH could facilitate the release of carotenoids in a crystalline substructure from crystalline chromoplasts, enhancing their bioaccessibility by disrupting chromoplast organelles. However, the provitamin A carotenoids of citrus (BC and BCX) were easily released from chromoplasts because of the lipid dissolved forms in globular substructure chromoplasts (plastoglobuli). Carotenoids from these chromoplast structures generally found in some fruits were more bioaccessible than the solid crystalline carotenoid form in chromoplasts of carrots or tomatoes (Lado et al., 2015; Palmero et al., 2013; R. M. Schweiggert & R. Carle, 2017). Thus, cell walls and chromoplast substructure of citrus probably did not constitute the main barrier to the release of BC and BCX carotenoid.

Carotenoid bioaccessibility also depended on pectin concentration/structure and mainly on the degree of methylation (Gence et al., 2018). Pectins were concentrated 8-fold during crossflow microfiltration that conduced to pectin-richer concentrates compared to initial juice. In our study, the carotenoid bioaccessibility of concentrates with lower pectin DM was higher **(Table 2)**. Our results were hardly comparable to those of Gence et al. (2018) who found that carotenoid bioaccessibility increased with DM in a clementine concentrate. In our study, juice and therefore concentrates were formulated with 60% clementine and 40% grapefruit that led to a higher pectin content due to the addition of

grapefruit. Moreover, the UHPH concentrate was characterized by the highest viscosity and the lowest degree of methylation of pectins. The UHPH treatment of the concentrate may favor the modification and re-association of the pectin chains by weak bonds, leading to a higher viscosity. In this case, the pectin-pectin interactions could be increased at the expense of the interactions between pectins and bile salts, allowing the bile salts to be more available for lipid emulsification and carotenoid micellarization. In addition, the pectin molecules with low DM are also able to link together through ionic calcium bridges and so could be less able to interact with pectin-bile salts through H-bonds (Cervantes-Paz et al., 2016). Thus, reducing the possibilities of interactions with pectins, bile salts could be mobilized more easily for carotenoid micellarization leading to better bioaccessibility. Finally, the association of high pectin concentration with low DM are particularly favorable for carotenoid bioaccessibility, that was also confirmed by Cervantes-Paz et al. (2017). At this stage, none of the three treatments, i.e., pasteurization, HHP or UHPH, affected the carotenoid content of concentrates. Although pasteurization and HHP increased carotenoid bioaccessibility, UHPH at 400 MPa (pretreated at 40 MPa) appeared to be the treatment leading to the highest carotenoid bioaccessibility (**Figure 4**). Thus, this treatment was chosen and applied for the concentrates in the next part of the study.

## 4. Effect of UHPH treatment on carotenoid uptake by intestinal cells of citrus concentrates

### 4.1. Carotenoid uptake by Caco-2 cells

In order to complete carotenoid bioaccessibility study, micelles generated from *in vitro* digestion of the different citrus concentrates and initial juice were incubated on Caco-2 cells (Figure 5). The first interesting remark was that carotenoid accumulation by cells from micelles generated by initial juice was better compared to the concentrates (raw concentrate, pasteurized and 400 MPa UHPH, non-pretreated at 40 MPa in this part of the study due to low availability of product). Carotenoid uptake of the 3 concentrates was decreased by 33-48% for BCX and by 61-71% for BC **(Figure 5)**. Note that lycopene uptake was not detected. This result could be explained by the micelle size which was smaller

for the micelles obtained from the juice (3 nm) compared to those from the different concentrates (17-19 nm). Indeed, smaller micelles were generally absorbed better by intestinal cells, especially when carotenoid uptake occurs by a simple diffusion process (Yonekura & Nagao, 2007). Nevertheless, carotenoid uptake could also be mediated by transport proteins such as SRBI or CD36 (Reboul, 2013). Gence et al. (2018) also observed a similar phenomenon, suggesting that pectin from citrus concentrate could increase the micelle size. Moreover, the lipid composition of micelles can influence the intestinal carotenoid uptake. Indeed, Reboul and Borel (2011) reported that the nature of the lipids that constitute these micelles play a key role in protein-mediated transport. These authors explained that mixed micelles consisting of long phospholipid chains were less uptaken by intestinal cells than mixed micelles containing lysophospholipids. The presence of high pectin concentrations in the concentrates could change the structure of mixed micelles and modify their size and their intrinsic properties (shape, charge) (Cervantes-Paz et al., 2016; Verrijssen, Verkempinck, Christiaens, Van Loey, & Hendrickx, 2015).

Regarding only concentrates, carotenoid uptake by Caco-2 cells was significantly improved by UHPH treatment (400 MPa) but not by pasteurization. Indeed, compared to the raw concentrate, UHPH treatment at 400 MPa increased the percentage of BCX and BC uptake by about 30%. Note that carotenoid uptake differed between BC and BCX only for initial juice. BC seemed to be absorbed preferentially (the micelle BC/BCX ratio was inversed compared to that of cellular uptake). Previous studies generally reported a preferential uptake of BC for micelles from simulated digestion compared to xanthophylls, such as BCX or lutein (Dhuique-Mayer et al., 2007; During, Hussain, Morel, & Harrison, 2002; C.-S. Liu, Glahn, & Liu, 2004). The molecular composition and charge of the micelle added to the type of carotenoid (polarity and flexibility could partly explain different uptakes between carotenoids) may contribute to these differences (Reboul, 2019). Thus, the UHPH concentrate was the one with the best carotenoid bioaccessibility but also the best carotenoid uptake by intestinal cells.

### 4.2. Relationships between pectin DM, micelle size and carotenoid uptake

An attempt to establish relationships between the methylation degree of pectins, the micelle size and the carotenoid uptake was illustrated in **Figure 6**. The methylation degree of pectins from concentrates was 1.6-3 fold lower than juice. Focusing only on concentrates, the smaller the methylation degree of pectins and micelle size, the higher the carotenoid uptake. In addition, as previously observed, the UHPH concentrate presented both the lowest degree of methylation and micelle size compared to the pasteurized concentrate (no significant difference of micelle size between UHPH and pasteurized concentrates). A low DM, such as that of UHPH concentrate, seemed to improve carotenoid uptake. Gence et al. (2018) observed the same phenomenon: a higher DM in citrus concentrate impaired carotenoid uptake. The authors explained that pectins with high DM could interact with the structure of mixed micelles modifying their size and surface properties.

Even if the degree of methylation was lower for the UHPH concentrate (8%) than for the raw and pasteurized concentrates (16 and 14%), globally, all these values were considered as low DM for pectins. We observed that the more the concentrate was processed, the lower the DM. Indeed, although the UHPH alone did not cause the decrease of pectin DM (Shpigelman, Kyomugasho, Christiaens, Van Loey, and Hendrickx (2015), it was possible that UHPH induced enzyme activity of residual pectin-methylesterase that is responsible for the decrease of pectin DM. Otherwise, the different steps of our processing (enzymes, CMF, diafiltration, temperature during UHPH reaching 90-100 °C at 400 MPa) conducted in acidic medium led to pectin depolymerization through hydrolysis mechanisms and could favor the decrease of DM observed after UHPH (De Roeck et al., 2009; X. Liu et al., 2019). Finally, the UHPH concentrate was the one with the best carotenoid bioaccessibility but also the best carotenoid uptake by intestinal cells. In addition, as previously observed, this one was characterized by the lowest degree of methylation (8%) and by a low micelle size being the same as the pasteurized concentrate.

### *4.3. In vitro* bioavailable carotenoid content

To go further, the *in vitro* bioavailable content of carotenoids was assessed by multiplying carotenoid content by carotenoid bioaccessibility and uptake. The in vitro bioaccessible content of carotenoids has already been studied by multiplying the content by the bioaccessibility of carotenoids (Dhuique-Mayer et al., 2018). To go further in our study, the bioaccessible content of carotenoids was multiplied by their uptake by intestinal cells, allowing to estimate the in vitro bioavailable content of carotenoids.

This combination helped us to estimate the amount of carotenoids that can actually penetrate into the enterocytes after consuming 200 mL of product **(Table 3)**. Whatever the concentrates, the *in vitro* bioavailable carotenoid contents were better than in the juice (around 2-4 fold for BC and 3-6 fold for BCX). The UHPH concentrate (400 MPa) provided the highest bioavailable carotenoid content, followed by pasteurized and then by raw concentrate. The more the concentrates were processed, the higher the bioavailable carotenoid content, in the following order: UHPH concentrate > pasteurized concentrate > raw concentrate > juice.

## Conclusion

In this study, the impact of high pressures coupled to crossflow microfiltration on the *in vitro* bioavailable carotenoid content was assessed. Carotenoid content was multiplied 8-fold thanks to crossflow microfiltration and neither pasteurization nor high-pressure treatments (isostatic HHP and dynamic UHPH) altered it. A HHP treatment (400 MPa, 15 min) was sufficient to significantly increase by 33 to 109 % carotenoid bioaccessibility. In the same way, at equal pressure (400 MPa), the UHPH treatment increased carotenoid bioaccessibility by 2-3 fold compared to the HHP treatment. Pasteurization (80°C, 14 min) was equivalent to HHP treatment (400 MPa, 15 min) in terms of carotenoid bioaccessibility. The high carotenoid bioaccessibility obtained by UHPH seemed to be linked to pectin structure, concentration and methylation degree. The UHPH concentrate was characterized by high pectin concentration and a low degree of methylation. The combination of these two characteristics could reduce pectin-bile salt interactions allowing bile salt to be more available for carotenoid micellarization, leading to better carotenoid bioaccessibility. Among the different

concentrates, UHPH-treated concentrate was also characterized by the highest carotenoid uptake, linked to a low micelle size and low DM. Indeed, a relative small micelle size and a low DM favored carotenoid uptake by intestinal cells. The highest bioavailable carotenoid content was obtained by UHPH-treated concentrate with a 4-6 fold increase compared to the other concentrates and initial juice. Further *in vivo* studies must be conducted in order to validate the health-promoting properties of the carotenoid-enriched concentrates. Finally, it would be essential to investigate the carotenoid stability in that type of products during long-term storage.

# Nomenclature

BC: β-carotene

BCX: β-cryptoxanthin

CMF: crossflow microfiltration

$D_{50}$: median diameter below which 50% vol. of the particles are found (µm)

DM: degree of methylation of pectins

DMR: diamass ratio (mass of distilled water added during the diafiltration stage divided by the mass of retentate)

HHP: high hydrostatic pressure process

$J_p$: permeate flux ($kg \cdot h^{-1} \cdot m^{-2}$)

LYC: lycopene

MRR: mass reduction ratio (mass of the juice feeding divided by the mass of retentate)

$\text{Ø}_{pore}$: average pore diameter of the membrane (µm)

$P_0$: pasteurization value (min)

SIS: suspended insoluble solids ($g \cdot kg^{-1}$)

TA: titratable acidity ($g \cdot kg^{-1}$)

$T_{CMF}$: temperature of crossflow microfiltration (°C)

TDM: total dry matter ($g.kg^{-1}$)

$t_{liq}$: enzymatic liquefaction time (min)

$T_{liq}$: temperature of enzyme liquefaction (°C)

TmP: transmembrane pressure (bar)

TSS: total soluble solids ($g \cdot kg^{-1}$)

U: crossflow velocity ($m \cdot s^{-1}$)

UHPH: ultra-high pressure homogenization

## Declaration of competing interest

The authors of this work declare no conflict of interest.

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## Figure and table captions

**Figure 1.** Process flow diagram of the experimental setup (see abbreviation list for meaning).

**Figure 2.** Effect of high hydrostatic pressure (HHP) on carotenoid bioaccessibility of citrus concentrates (400 MPa for different durations 15-30-60 min). BCX: β-cryptoxanthin, BC: β-carotene, LYC: lycopene

**Figure 3.** Effect of ultra-high pressure homogenization (UHPH) on carotenoid bioaccessibility of citrus concentrates (40-100-250-400 MPa) (*Concentrate 400 MPa was pre-treated at 40 MPa). BCX: β-cryptoxanthin, BC: β-carotene, LYC: lycopene

**Figure 4.** Effect of the stabilization treatments on carotenoid bioaccessibility of citrus concentrates (*pre-treated at 40 MPa). BCX: β-cryptoxanthin, BC: β-carotene, LYC: lycopene, HHP: high hydrostatic pressure, UHPH: ultra-high pressure homogenization

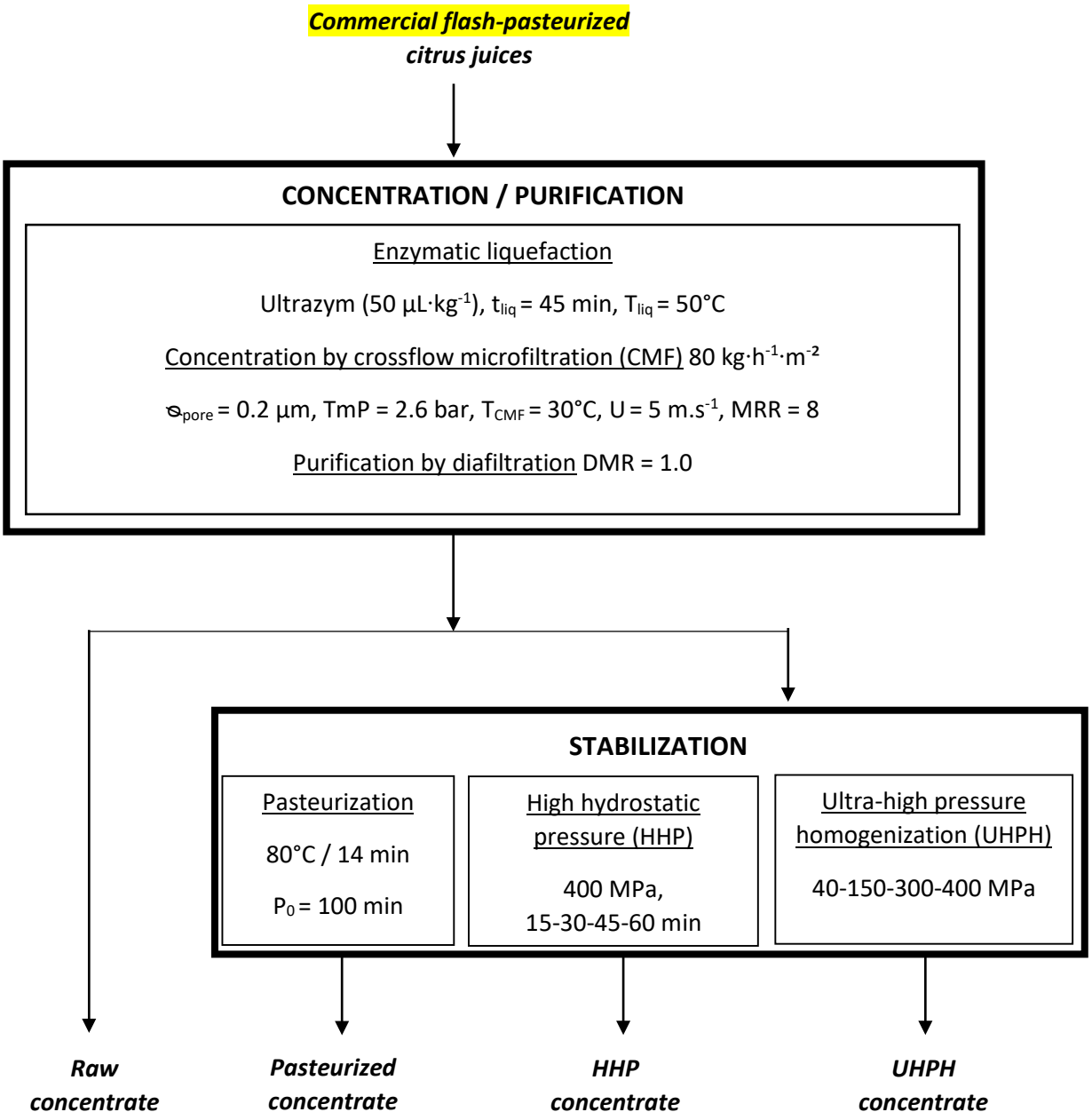
**Figure 5.** Percentage of carotenoid uptake by intestinal cells for citrus concentrates treated either by pasteurization or by UHPH and initial juice. BCX: β-cryptoxanthin, BC: β-carotene, UHPH: ultra-high pressure homogenization

**Figure 6.** Relationship between degree of methylation, micelle size and β-cryptoxanthin (BCX, the most representative carotenoid) uptake by intestinal cells. UHPH: ultra-high pressure homogenization

**Table 1.** Structural parameters and carotenoid content of initial citrus juice, raw, pasteurized, high hydrostatic pressure (HHP) and ultra-high pressure homogenization (UHPH) concentrates treated in different conditions.

**Table 2.** Methylation degree of pectins and physico-chemical parameters of initial citrus juice, raw concentrate, pasteurized concentrate and ultra-high pressure homogenization (UHPH) concentrate (400 MPa, pre-treated at 40 MPa).

**Table 3.** Estimation of *in vitro* bioavailable carotenoid content provided by 200 mL of juice, and raw, pasteurized and ultra-high pressure homogenization (UHPH) concentrates. BCX: β-cryptoxanthin, BC: β-carotene

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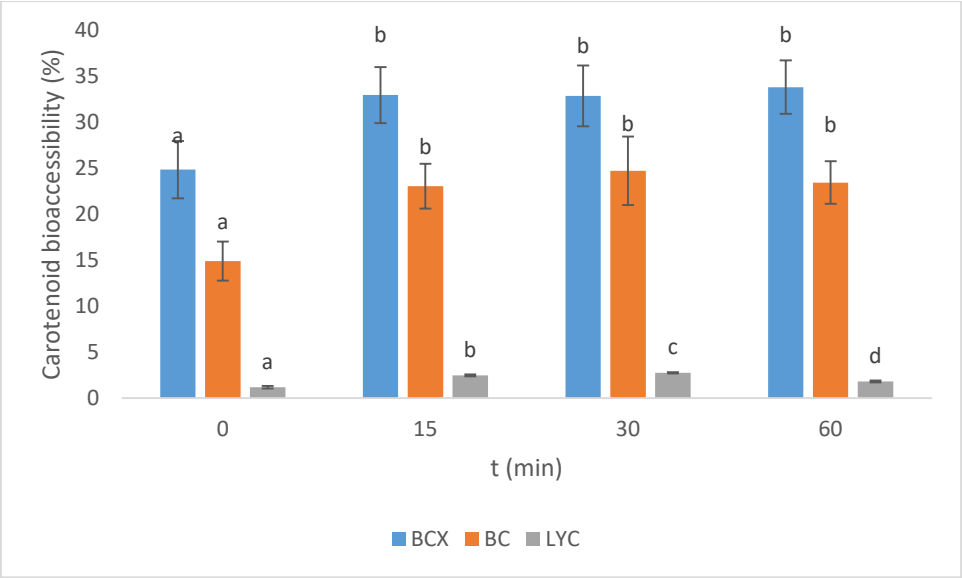


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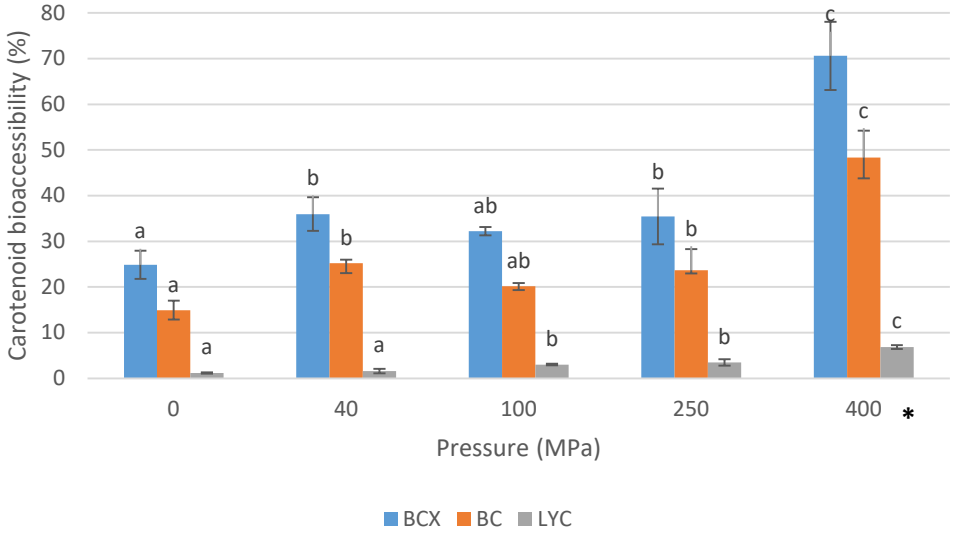


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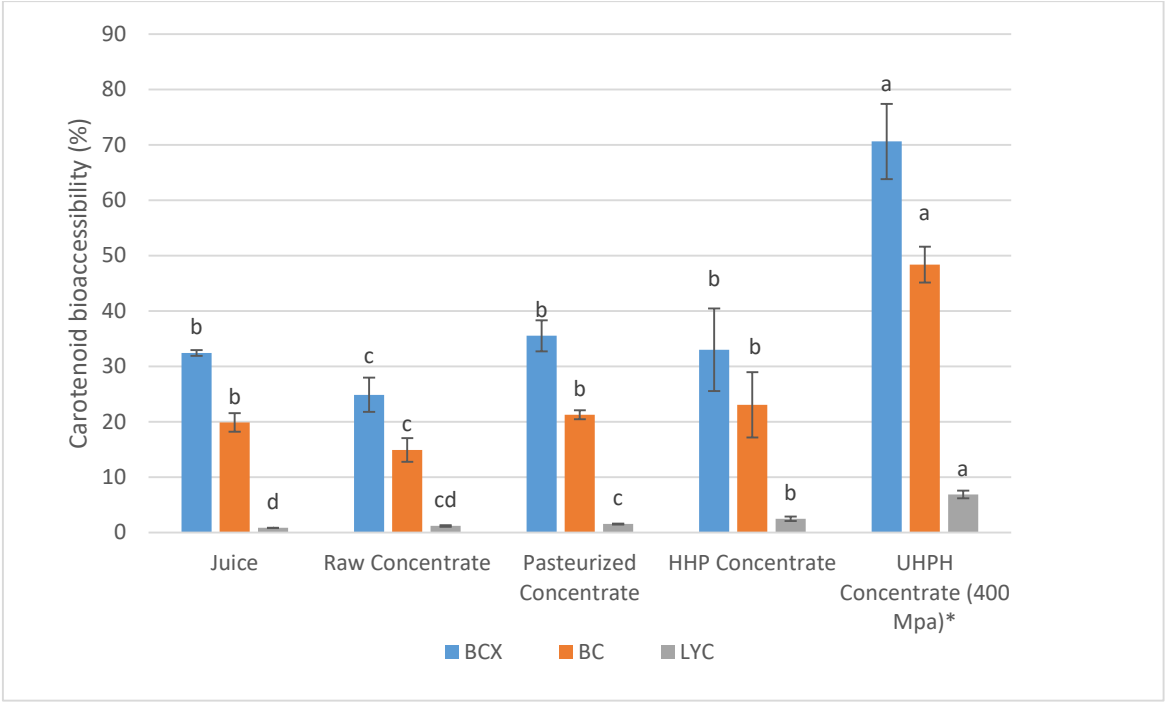


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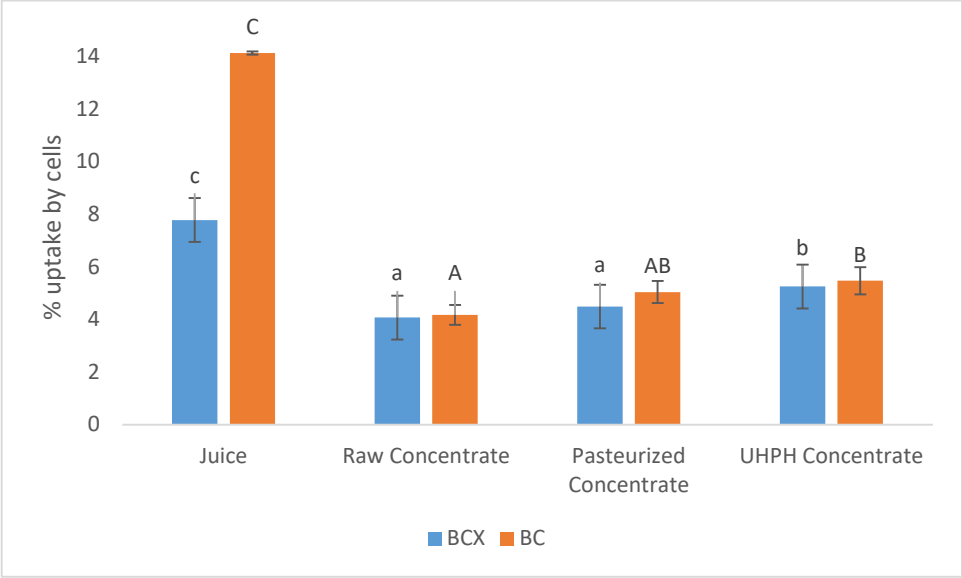


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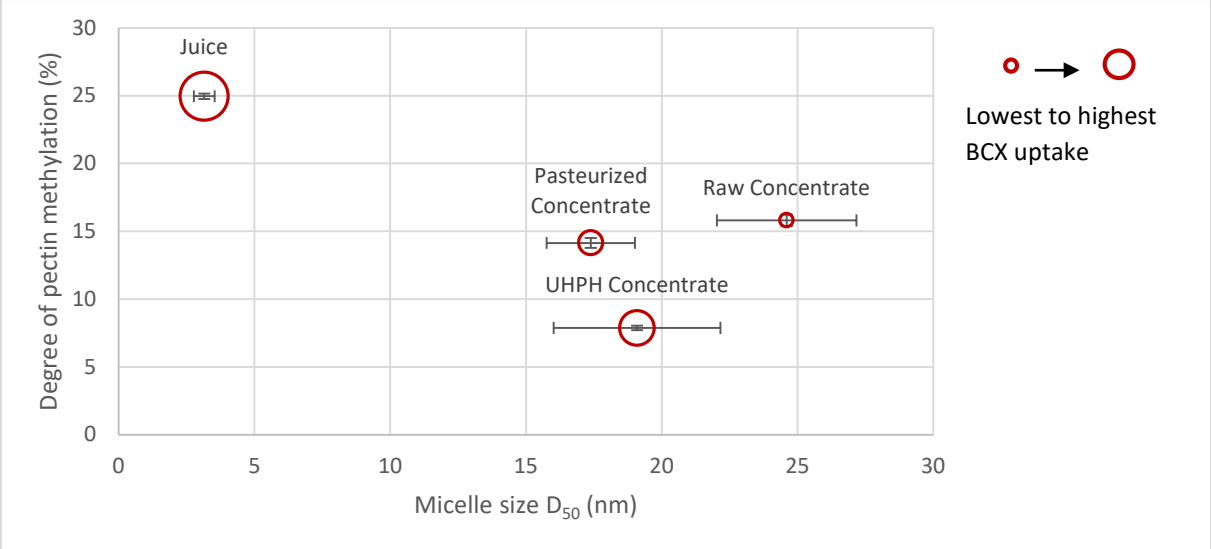


**Figure 6.** Relationship between degree of methylation, micelle size and β-cryptoxanthin (BCX, the most representative carotenoid) uptake by intestinal cells. UHPH: ultra-high pressure homogenization

**Table 1.** Structural parameters and carotenoid content of initial citrus juice, raw, pasteurized, high hydrostatic pressure (HHP) and ultra-high pressure homogenization (UHPH) concentrates treated in different conditions.

| | Juice | Raw concentrate | Pasteurized concentrate | HHP 400 MPa concentrates | | | | UHPH concentrates | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 15 min | 30 min | 45 min | 60 min | 40 MPa | 100 MPa | 250 MPa | 400 MPa, (pretreated at 40 MPa) |
| **$D_{50}$ (µm)** | 767.1 ± 59.1 a | 33.17 ± 0.67 e | 29.15 ± 0.51 f | 38.05 ± 0.60 c | 38.09 ± 2.09 c | 32.37 ± 0.61 e | 35.02 ± 0.75 d | 39.49 ±2.01 c | 34.89 ± 1.50 de | 23.84 ± 0.84 g | 148.56 ± 13.87 b |
| **Span** | 2.8 ± 0.2 | 6.03 ± 0.39 | 7.96 ± 0.61 | ND | ND | ND | ND | ND | ND | ND | 1.97 ± 0.25 |
| **Viscosity (mPa.s)** | 3.21 ± 0.29 f | 5.09 ± 0.44 bcd | 5.19 ± 0.13 bcd | 3.86 ± 0.59 ef | 5.48 ± 1.16 ab | 4.70 ± 0.24 cd | 3.66 ± 0.68 ef | 5.32 ± 0.07 bc | 5.39 ± 0.20 abc | 5.36 ± 0.03 bc | 7.49 ± 1.41 a |
| **BC ($mg.kg^{-1}$)*** | 1.93 ± 0.06 f | 14.77 ± 0.54 ab | 14.92 ± 0.31 a | 13.82 ± 0.74 bcde | 13.90 ± 0.55 bcd | ND | 13.74 ± 0.97 cde | 14.17 ± 0.36 abc | 12.96 ± 0.65 e | 13.64 ± 0.56 cde | 13.07 ± 0.63 de |
| **BCX ($mg.kg^{-1}$)*** | 3.28 ± 0.46 e | 25.17 ± 2.22 abc | 22.28 ± 0.14 d | 25.32 ± 1.37 abc | 25.77 ± 1.08 abc | ND | 24.38 ± 1.77 c | 26.16 ± 0.63 ab | 24.54 ± 0.79 bc | 26.38 ± 1.28 a | 25.37 ± 1.12 abc |
| **LYC ($mg.kg^{-1}$)*** | 5.17 ± 0.43 e | 42.30 ± 2.86 abc | 45.69 ± 0.33 a | 43.12 ± 2.25 ab | 40.95 ± 1.54 bcd | ND | 44.77 ± 2.15 a | 42.10 ± 0.91 bc | 40.02 ± 1.65 cd | 41.35 ± 1.97 bcd | 40.10 ± 2.02 cd |

Mean ± standard deviation from 3 or 9 repetitions in total*; BCX: β-cryptoxanthin, BC: β-carotene, LYC: lycopene ND: not determined; values within a line with different letters were significantly different at $p < 0.05$

**Table 2.** Methylation degree of pectins and physico-chemical parameters of initial citrus juice, raw concentrate, pasteurized concentrate and ultra-high pressure homogenization (UHPH) concentrate (400 MPa, pre-treated at 40 MPa).

| | **Juice** | **Raw concentrate** | **Pasteurized concentrate** | **UHPH concentrate 400 MPa** (pretreated at 40 MPa) |
|---|---|---|---|---|
| Degree of methylation (%) | 24.9 ± 0.2 a | 15.81 ± 0.43 b | 14.14 ± 0.36 c | 7.87 ± 0.17 d |
| pH | 3.45 ± 0.20 a | 3.49 ± 0.04 a | 3.52 ± 0.10 a | 3.48 ± 0.02 a |
| TA ($g.kg^{-1}$) | 9.80 ± 0.35 a | 4.46 ± 0.24 b | 4.40 ± 0.08 b | 4.24 ± 0.68 b |
| TSS ($g.kg^{-1}$) | 137.0 ± 1.7 a | 78.0 ± 1.0 c | 85.3 ± 2.3 b | 78.0 ± 1.7 c |
| SIS ($g.kg^{-1}$) | 2.50 ± 0.01 c | 23.6 ± 0.2 a | 22.8 ± 0.4 b | 23.2 ± 0.3 ab |
| TDM ($g.kg^{-1}$) | 113.7 ± 0.1 a | 66.6 ± 0.1 b | 66.2 ± 1.5 b | 64.3 ± 0.1 b |

Mean ± standard deviation in parenthesis, from 3 repetitions; values within a line with different letters were significantly different at $p < 0.05$

**Table 3.** Estimation of *in vitro* bioavailable carotenoid content provided by 200 mL of juice, and raw, pasteurized and ultra-high pressure homogenization (UHPH) concentrates. BCX: β-cryptoxanthin, BC: β-carotene

| **For 200 mL of product** | | Initial Juice | Raw concentrate | Pasteurized concentrate | UHPH concentrate (400 MPa) |
|---|---|---|---|---|---|
| Estimation of bioavailable carotenoids (µg) | BC | 10.2 ± 0.6 | 18.4 ±2.5 | 32.9 ± 2.3 | 37.3 ± 5.2 |
| | BCX | 15.5 ± 1.7 | 50.9 ± 7.1 | 71.0 ± 5.5 | 91.6 ± 11.6 |