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In vitro bioaccessibility as a tool to test improved cultivars performance with higher levels of β-carotene from foods

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ABSTRACT

Increasing β-carotene content from food is not a guarantee of combat micronutrients deficiencies, such as hidden hunger, because there are many aspects involved in absorption and metabolization by human body. Therefore, *in vitro* bioaccessibility studies have been used as a prediction tool of understanding food matrix factors that may cause release to its absorption. These studies are conducted by applying *in vitro*" digestion methods which expose lineage /micronutrient to human physiological conditions, by mimicking oral, gastric and intestinal digestion human. This work aimed to implement 'in vitro" digestion methodology as tool to determinate 'in vitro" bioaccessibility from improved cultivars with higher levels of β-carotene. Analyses involved enzymes as α-amylase, pepsin, bile, pancreatin, lipase and mucin; and inorganic compounds such as KCl, KSCN, NaH_2PO_4, Na_3PO_4, NaOH, NaCl, $CaCl_2$, HCl, $NaHCO_3$. Physiological variations were reproduced by the heating bath shaker with orbital gyrus (37ºC) and centrifugation (5000g, 45 min). Quantification and determination of the carotenoids profile were performed by high performance liquid chromatography (HPLC) with YCM® C_{30} Carotenoid S-3 4.6 x 250mm column and UV-Vis spectrometer. Therefore, this methodology proved to be faster and cheaper, inasmuch as *in vivo* studies are more costly, complex and require more time.

KEYWORDS

Digesta;
Micellar;
Carotenoids;
HPLC;
β-carotene;
Crops;
Biofortification.

INTRODUCTION

About three million people suffer effects of vitamin A deficiency, such as xerophthalmia. Each year, 500 million people get blind due vitamin A limitation. This micronutrient deficiency is a serious problem in over 60 countries[1]. Its prevalence is particularly higher in regions such Asia, Africa and Latin America. Globally, about 60% of vitamin A source come from content provitamin A foods. This number increases to 80% in developing countries[2]. To minimize this deficiency in Brazil, Embrapa research centers, in partnership with other universities, have developed projects to select and produce crops with higher β-carotene levels. This selec-

tion goes beyond the field, since "*In vitro*" bioaccessibility studies have been used as an evaluation tool to determination of promising lines with higher β-carotene contents. These studies indicate a crop that may provide a better pro-vitamin A absorption. Brazil's biofortification program has been studying eight different crops at the same time, such as pumpkin, rice, sweet potatoes, beans, cowpeas, cassava, maize, and wheat[3].

β-carotene (BC) is one of the most abundant carotenoid in the human diet and more effective as vitamin A precursor. Bioavailability of vitamin A from foods and food formulations is dependent of several factors, including food matrix[4], where carotenoid is incorporated (species), type of molecular bonding, type of carotenoids consumed in the meal, processing, fat content, and is related to absorption and bioconversion. Related work of preformed vitamin A and provitamin A indicate about 70% to 90% of absorption efficiency for the first compared with 20% to 50% for provitamins[5] after ingestion of a rich meal with these compounds.

Traditionally, for decades, bioavailability studies of carotenoids in humans have been done using plasma assay. These data were essential, since through them we knew five major circulating carotenoids: β-carotene, α-carotene, lutein, lycopene and β-cryptoxanthin. However, this method is flawed, because it does not indicate actual accessible quantities absorbed and metabolized. In the last decade, several models of "*in vitro*" digestion (bioaccessibility) have been developed in order to elucidate first steps of carotenoids bioefficacy. It attempts to quantify carotenoids that are released from the food matrix during digestion and transferred to the micelles (efficiency of micellization), in other words, the amount of carotenoids transferred from the digested fraction (digested food) to the micellar fraction (aqueous). This understanding is important, since they are early indicators of bioavailability[6].

Most "*in vitro*" tests are based on gastro-intestinal physiology and mimic human digestion conditions. Natural saliva, gastric juice and duodenal solutions are changed by artificial ones to simulate environment of each digestive compartments. All these solutions contain enzymes, amino acids, organic salts, inorganic acid and hydrochloric acid[7]. Three areas of human digestive system are important to design digestion methodology: mouth, stomach and small intestine. The mouth is a key compartment of the whole process of human digestion, however, food remained presented for a short period of time, and often is not included "*in vitro"* methods. In the stomach, food is subjected to pepsin at pH 2 for several minutes (usually 8 minutes) to several hours (3 h), and in the small intestine (duodenum, jejunum and ileum) is subject to the action of intestinal juice, consisting enzymes such pepsin, amylase, pancreatin, bile salts and other salts, such as bicarbonate. As physical processes occur "*in vivo*", were not reproduced (cutting, mixing, hydration, changes in weather conditions, peristalsis), this "*in vitro*" model was defined as a static or biochemist. The dynamic models mimic physical processes "*in vivo*", and consider new variables, such as changes on viscosity of the digesta, particle size reduction, diffusion, and partitioning of nutrients[8].

Thus, the objective of the study was to implement an in vitro digestion methodology of β-carotene using different foods and later to determinate the efficiency of micellization in vegetables, especially in sweet potato with higher β-carotene content as a tool to calculate itsin vitro bioaccessibility.

EXPERIMENTAL

Several matrices, including: papaya, orange-fleshed sweet potatoes (three cultivars of Biofort's project), yellow cassava and carrot were used to implement the methodology. All matrices were obtained from local market (Rio de Janeiro, Brazil), except orange fleshed sweet potato and yellow cassava which were cultivated at Embrapa Vegetables, Brasilia, Brazil and sent to Embrapa Food Technology, Rio de Janeiro, RJ, Brazil.

All reagents and enzymes used "*in vitro*" digestion: KCl, $NaPO_4$, NaOH, Urea, NaCl, anhydrous $CaCl_2$, KSCN, NaH_2PO_4, Pepsin 1:10,000, $NaHCO_3$, α-amylase type VI-B mucin type II, Porcine bile extract, Porcine Pancreatin, Lipase type II pig pancreas were purchased from Sigma-Aldrich®- Brazil and HCl Suprapur was purchased from Merk ®Brazil. Also micellar extraction reagents were purchased from Tedia®Brazil: sodium chloride PA grade, sodium sulfate-pesticide grade, anhydrous sodium sulphate. All reagents from chromatography analysis were HPLC grade, including acetone, acetonitrile, petroleum ether,

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methanol and tert-butyl and were purchased from Tedia ® Brazil. Reagents used during carotenoids extraction were purchased from: Celite 545 from Tedia ® Brazil, Magnesium hydroxide PA from Merck®-Brazil, anhydrous sodium sulfate and sodium chloride PA from Quimex®, ultrapure water (18.2MΩ) from Milli-Q® and Nitrogen level 4.6 from White Martins®.

All samples were cut by hand and suffered quartering. Two opposite parts were fragmented and crushed in a mill (Mod A11 IKA®). All samples were performed three independent times (n=3), for undigested and digested analysis. Those used to select sample amount, were prepared six times.

Method adaptation

(a) Selecting sample amount

Pilot tests were conducted several times to determine maximum quantity of started sample that ensured enough material containing sufficient carotenoid level for precise analysis in order to assess stability and micellarization. Two[2], 5 and 10 g of food were tested.

(b) Samples preparation

Three forms of sample preparation were tested: 1st - *in natura* samples were cut, homogeneizated and weighted directly in tubes reaction, at the analysis moment ; 2nd – samples were weighed and stored in individually containers, one day before analysis, 3rd - *in natura* samples were cut and homogenizated into a flask and stored in freezer (- 8ºC) until analysis moment. Then, they were thawed and weighed up.

(c) Samples preservation

Orange fleshed sweet potatoes roots were peeled cut by hand and suffered quartering. Two opposite parts were fragmented and crushed in a mill. Afterwards the pasta was stored in glass bottle, covered with metallic paper and stored into a freezer (-18ºC) for 24 hours. Then it was introduced into a lyophilizer and after 24 hours cicle, a dry material was obtained. So, it was again crushed in a mill, a slim powder was obtained.

(d) Selecting oil type

In natura carrots were peeled, cut by hand and suffered quartering. Two opposite parts were fragmented and crushed in a mill. Five[5] g of carrots were homogenizated and tested. Three types of oil were selected (sunflower, soybean and canola) and samples containing 10% (v/w) of three oils (two of each them) were extracted[9]. Also two undigested samples without oil were extracted for comparison.

(e) Selecting oil concentration

In natura sweet potatoes were peeled, cut by hand and suffered quartering. Two opposite parts were fragmented and crushed in a mill. Two[2] g of samples were homogenizated and tested. Three undigested samples without oil were extracted, three digested samples containing 2% (v/w) of canola oil, three digested samples containing 5% (v/w) of canola oil and three digested samples containing 10% (v/w) of canola oil were also extracted.

"I*n vitro*" digestion solutions

(a) Salive stock solution

To prepare 50 mL of stock solution aliquots of 1 mL, 1 mL, 1 mL, 1 mL, 0.17 mL, 0.18 mL, 0.8 mL were mixed, respectively, of the following solutions: KCl (89.6 g/L), KSCN (20g/L), Na_3PO_4 (88.8g/L), NaH_2PO_4 (57g/L), NaCl (175.3g/L), NaOH (40g/L) and urea (25g/L) and fattened with ultrapure water (Milli-Q ®, Millipore). Then 14.5 mg of α-amylase and 5 mg of mucin were added and mixed[6]. All solutions were prepared 24 h before analysis.

(b) Solution A

To prepare 500 mL of solution, 0,3468 g of $CaCl_2$, 3,5210g of NaCl and1904g of KCl were mixed and dissolved simultaneously with ultrapure water to reach 6mM CaCl, 120 mM NaCl and 5mM KCl solution.

(c) Pepsin stock solution

1 g of pepsin was mixed and dissolved in a 25 mL volumetric flask with 100mM HCl solution to obtain a final concentration of 40 mg/mL 100mM HCl.

(d) Bile stock solution

1 g of bile was mixed and dissolved in a 25 mL volumetric flask with 100 mM $NaHCO_3$ to obtain a final concentration of 40 mg/mL 100mM 100 mM $NaHCO_3$.

(e) Pancreatin- lipase stock solution

250 mg g of pancreatin and 125 mg of lipase were mixed and dissolved simultaneously in a 25 mL volumetric flask with 100 mM $NaHCO_3$ to obtain a final

concentration of 10 mg + 5 mg lipase/mL 100 mM $NaHCO_3$.

Carotenoids extraction from vegetables samples, before *in vitro* digestion

All procedure was performed as described by Rodriguez-Amaya *et al*.[10], and optimized by Pacheco[11] with limited light and controlled temperature to minimize degradation and isomerization of carotenoids. All analysis were performed in duplicate.

In vitro digestion of vegetables after implementation

The protocol described by Garrett *et al*.[12] and Failla & Chitchumroonchokchai[13] for simulation of human digestion was followed after small adaptations. All digestion steps were performed sequentially from oral phase to micellar fraction. The digestion starts by introducing 7 mL of saliva solution to 5-10 g of vegetables, then blanketed with nitrogen, sealed, mixed and transferred to 37ºC shaking water bath for 10 minutes at 60 rpm. After 10 minutes, the mixture were removed from water bath and placed on ice immediately. Gastric digestion begins subsequently when solution A was added to the mixture to increase volume of 30 mL and well mixed and The pH value was checked and adjusted to the appropriate interval (pH 2.5 ± 0.1) using 1mL of 1M HCl. At this time, 2mL pepsin stock solution (final concentration of pepsin is 2mg/mL) was added and volume was increased to 40 mL using solution A and incubated in 37ºC shaking water bath for 1 h at 60 rpm and then placed in ice. Moving forward during small intestinal digestion, pH range (6.0± 0.2) was adjusted using 1,4 mL of 1M $NaHCO_3$ and 3 mL of Bile extract stock solution (final concentration 2.4 mg/mL) were added and mixed well, then 2 mL of pancreatin-lipase stock solution were added, mixed well. Again 1 mL of 1M NaOH was used to adjust pH range (6.5±0.1) and final volume was increased to 50mL with solution A (Final concentration of bile, pancreatin and lipase are 2.4, 0.4 and 0.2 mg/mL, respectively). Plus the tubes were then blanketed and closed under N_2 and again incubated at 37 °C, 60 rpm orbital shaking, for 2 h. Finally, the tubes were removed, placed in ice and a 10 mL aliquot of the digesta was transferred to a ultracentrifuge tube (Ultracentrifuge Sorval® Stratos) and rotated for another 45 minutes to separate micelle fraction (Figure 1).

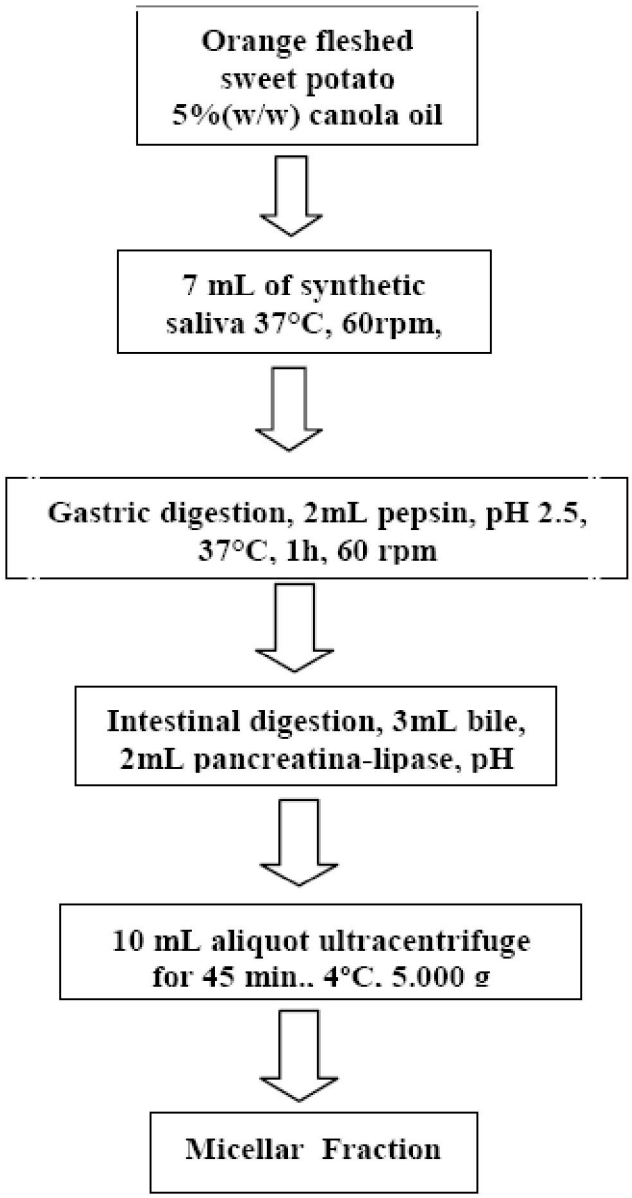

Figure 1 : Schematic representation of simulated *in vitro* digestion model to assess relative bioaccessibility. Some adaptation were made specially on micellar fraction extraction

Carotenoid extraction from micellar fraction

Micellar fraction was collected from centrifuge tubes using a clean plastic 5 mL tip from an automatic Brand pipete. 10 mL aliquot was introduced in a 500 mL separatory funnel, containing 30 mL of petroleum ether. Then, 200 mL of aqueous NaCl 10% (w/v) solution were added and agitated. After resting, fractions were separated and aqueous phase was discarded. Organic phase was washed twice, using 200 mL Na_2SO_4 (2%) solution[14], and extract was passed through a funnel to

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an ambar volumetric flask, containing 10 g of anhydrous sodium sulfate. Further, the extract was concentrated in a rotary evaporator, suspended in petroleum ether and transferred to a 10 mL and the volume was made up by petroleum ether[15].

Instrumental analyses: total carotenoids content and profile

Total carotenoids content of the samples were determined by spectrophotometry at 450 nm (Mod UV-1800, SHIMADZU), using petroleum ether as blank. Carotenoids profile was determinate by taking a 2mL aliquot and transferred to a amber vial, dried under a N_2 atmosphere, ressuspended in 100 µL acetone, using a vortex during 10 seconds, and taken for analysis by HPLC[10].

Identification, quantification and determination of carotenoids profile were performed by HPLC, using a Waters® liquid chromatograph, a 33ºC column oven, photodiode array detector (PDA 996- Waters®), a Empower Waters® Software, a C_{30} YCM® S-3 Carotenoid Column (4.6 mm × 250 mm; Waters). Carotenoids separation was obtained by a gradient elution of methanol and methyl tert-butyl ether gradient. Using flow rate of 0.8 mL/ min, injection volume of 15µl and run time of 28 min[10]. Quantitative analysis was performed through external standardization with a calibration curve of seven standards, including all-*trans*-β-carotene isolated in the laboratory. All carotenoids were identified by comparing their retention times with carotenoids' standards and by evaluating UV/Vis absorption spectra. All standards were obtained by natural sources with purities greater than 97%.

Efficiency of micellarization

Efficiency of micellarization or *in vitro* bioaccessibility of total carotenoids and β-carotene was calculated based on the levels of carotenoids in the digested samples compared to their respective levels in undigested samples. Calculation was expressed by using above equation, previously described by Oomen *et al.*[7].

$$\text{Efficiency of Micellarization (EM)} = \frac{(\beta\text{-carotene Micellar Fraction})}{(\beta\text{-carotene from matrix})} \times 100$$

DISCUSSIONS AND RESULTS

The models introduced by Garret *et al.*[12] and Failla *et al.*[13] were used as a starting point for the implementation of our *in vitro* digestion model. This model is static gastrointestinal models, since dynamic models mimic *in vivo* physical processes so that they take into account new variables, such as changes on viscosity of the digesta, particle size reduction, diffusion and partitioning[7].

Pilot tests were conducted several times to determine maximum quantity of started sample that ensured enough material containing sufficient quantity of carotenoid for precise analysis to assess stability and micellarization. Two[2], 5 and 10 g of food were tested, and after all, it was concluded that amounts between 5 and 10g were necessary for most of the studied vegetables, although it is necessary to conduce an individual evaluation. Matrix preparation was tested using three different type of treatment, and the more appropriated one was when *in natura* samples were cut, homogenized and weighted directly in tubes reaction, at the analysis moment, since freezing process caused cell matrix disruption, which artificially increased carotenoids bioavailability. The use of individual containers resulted in excessive sample loss, since this set remained the same. This phase represented a critical step, since it corresponds to the chewing stage, whose aim was to simulate mastication and to expose food matrix to digestive enzymes action.

All salts and enzymatic solutions preparation represented a big challenge because it required a lot of detail in its execution. Saliva, gastric juice, pancreatic juice, lipase and bile solution were prepared and used in a maximum of one day[7]. Saliva and saline solutions were mixed, according to the volumes described in the same article and raised to 50 mL with milli-Q water. All implementation methodology started by quantifying each stage of digestion using papaya samples with no oil. This objective was to verify carotenoids transfer from matrix to aqueous phase after each step. *In vitro* digestion was performed six times with the same sample and two were quantified after oral phase, two after gastric phase and two after intestinal phase (data not shown). The results, however, showed no presence of carotenoids in extracts of each stage, which was an indicative of component absence. So, it was not possible to quantify carotenoids in aqueous phase. Indeed, this impossibility is due to oil absence along the array,

as papaya is not a rich lipids food. Another problem commonly found in the fruits analysis was due a later stage saponification before and after digestion, since these matrixes contain oxygenated carotenoids in esters form and it was necessary to hydrolyze carotenoids before their release, to be extracted and quantified by HPLC. Moreover, to study fruits it would require cholesterol ester lipase to the mixture for simulated small intestinal digestion, because it contains xanthophylls esters[16]. This would make analysis more complex and increasing the number of critical steps. As β-carotene is the array focus of BIOFORT biofortification project, this work was concentrated in carotenes compounds, so papaya matrix was discarded as a study matrix.

Physiologic bile production is stimulated by dietary fat intake. So, the consumption of fat with a meal containing carotenoids increases the efficiency of absorption, and previously authors[17] suggested at least 3 to 5 grams of fat intake per meal. The micelles formation is dependent, among other factors, of the presence of oil in the intestine, and consequently their co-ingestion with carotenoids. It can be said that this step is crucial and the most important dietary factor for their availability for absorption. Results of three types of vegetable oils: canola, soybean and sunflower showed association with type of fatty acid composition and number of unsaturations. So, canola oil showed greater ability to emulsify carotenoids, because total carotenoids values were 35µg/g, while other two oils had lower values 25 and 21µg/g, respectively (Figure 2), as also previously described[18]. Canola oil, in theory, contain fatty acids such as oleic acid (C18: 1 - 53-70%), linoleic acid (C18: 2 - 15-30%) and linolenic acid (C18:3 -5-13%), which are long chain carbon compounds, allowing a greater interaction between carotenoids and fatty acids, producing higher bioaccessibility.

As described by Huo *et al*[18], not more than 2.5% oil would be necessary to promote β-carotene emulsification and transference to micellar fraction. Thus, we decided to test three concentrations 2, 5 and 10% (w/w) and results showed that there were no changes in carotenoids emulsification and micellization when concentrations varied (Figure 3).

Thus, we decided to reduce canola oil concentration of 10% to 5% (w / w), for economic and practices reasons. The effect of oil vegetable presence caused

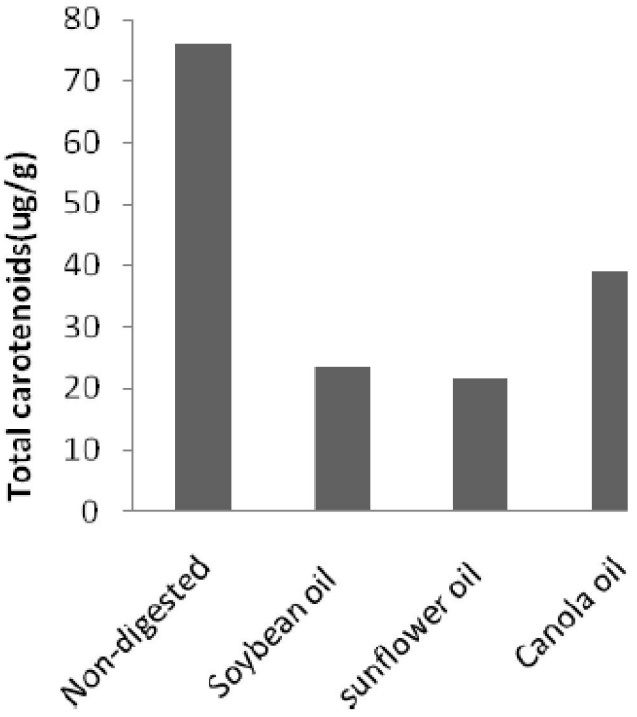

Figure 2 : Quantity of total carotenoids in non-digested samples and partitioned into micelle fraction during simulated *in vitro* digestion with 5% (w/w) soybean, sunflower and canola oils in carrots. Data are means from three independent (n=3) *in vitro* digestion of each cultivar

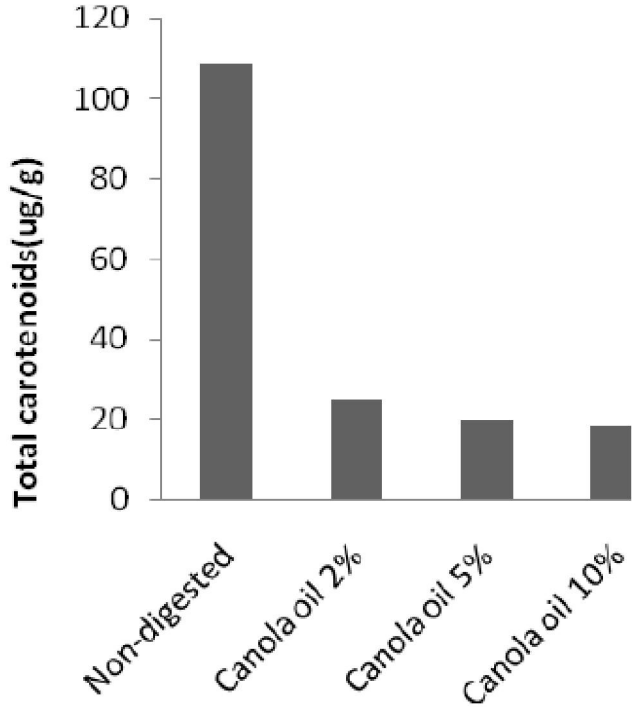

Figure 3 : Quantity of total carotenoids partitioned into micelle fraction during simulated *in vitro* digestion with three different concentration of canola oils: 2%, 5% and 10% (w/w) in carrots. Data are means from three independent (n=3) *in vitro* digestion of each cultivar

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different forms of incorporation, according to the class of carotenoids. Xanthophylls are located in outer regions of the micelles, while β-carotene is located more internally[19]. As some vegetables are harvested in specific time of the year, we opted to Lyophilize fresh samples as a preservation method in order to increase self-life. This method was not acceptable since results showed that total carotenoids values tended to start much higher than *in natura* samples, as also previously described[18]. Lyophilized process makes carotenoids more available, since breaks cell´s walls, letting inside content out (Figure 4). Furthermore, this was not the natural way of food intake.

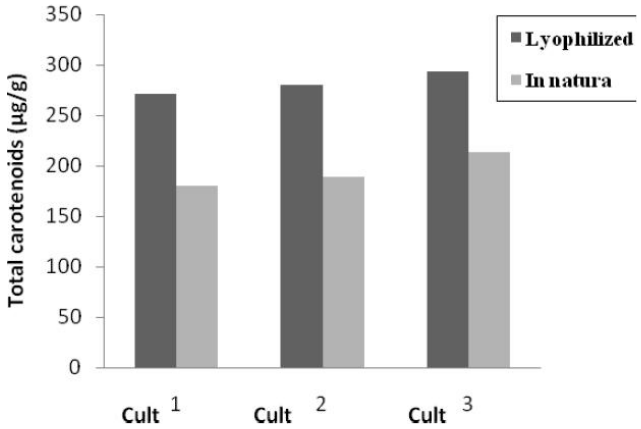

Figure 4: Comparison of total carotenoid from three cultivars (1, 2 and 3) of orange fleshed sweet potatoes partitioned into micelle fraction during simulated *in vitro* digestion using *in natura* and lyophilized samples. Data are means from three independent (n=3) *in vitro* digestion of each cultivar

Also, despite of the correct storage (metallic package, under light protection) and temperature (- 18 ° C), after two weeks all samples lost their orange color, so we decided to quit this method. Also we evaluated 1M HCl, 1M Na_2HCO_3 and 1 M NaOH volumes required to adjust pH values of each stage, since different matrices provide different environment analysis conditions (TABLE 1). These different volumes demonstrated that standardization of exact required amounts is not possible, unless preliminary tests for each array were made. Time of analysis and pH adjustment was faster as possible in order to minimize carotenoids degradation and isomerization. Also, the whole analytical procedure was performed under controlled temperature (25º C) and limited light. PH adjusting represented a critical stage of the process, since each type of enzyme has its optimum pH activity. Micellar fraction extraction demanded deepest investigation, since methods previously described were applied to solid and semi-solid samples[11].

TABLE 1: Total carotenoids content of non-digested and digested (µg/g) and efficiency of micellarization (EM%) of simulated digestion from orange fleshed sweet potato in presence of 5% (w/w) canola. Data are means for three independent digestion of each sample (*n*=3)

Orange Fleshed sweet potato	Total carotenoids (µg/g)	Total carotenoids Micellar Fraction (µg/g)	Efficiency of micellarization (%)
Cult 1	179,44	13,40	7,5
Cult 2	188,84	36,62	19,4
Cult 3	213,62	70,70	33,1

As reported by Fernandez-Garcia and colleagues[8] and after adaptations, micellar fraction extraction could be accomplished by introducing 5 mL aliquot to a separatory funnel, addition of 50 mL diethyl ether or petroleum ether (depending on the carotenoid) and 200 mL of 10% NaCl (w/v) and agitation. Aqueous phase was waived and the organic phase was washed twice using 200 mL of Na_2SO_4 2% (w/v). Type of organic solvent was chosen, from diethyl ether to petroleum ether, according to greater polar character, since β-carotene is more lipophilic. We also introduced an organic phase passage by sodium sulphate, in order to remove all traces of water and after we took the extract to a rotary evaporator vacuum to concentration.

TABLE 2: Quantity of HCl, Na_2HCO_3 and NaOH a 1 M volume required to adjustment pH of each phase of simulated digestion from four different matrixes

Vegetables	HCl 1 M volume (µL)	Na_2HCO_3 1M Voleme (µL)	Volume de NaOH 1 M (pH 6,5) (µL)
Papaya	110	NN	NN
Carrot	1800	800	NN
Cassava	1200	1600	30
Sweet popato	1000	1400	1000

NN: Not necessary

As we determined efficiency of micellization of total carotenoids from the three cultivars of orange sweet potatoes[20], results showed that cultivar 2 indicated higher carotenoid transfer from matrix to the micelles (TABLE 2). Moreover, cultivar 1, despite of having initial high values of total carotenoids before *in vitro* digestion, showed the lowest value of bioaccessibility.

Also, cultivar 3 had 12% lower initial concentration than cultivar 2, but showed 59% lower efficiency of micellization (TABLE 2). Thus, these data indicated that ingestion of equivalent amounts of the three cultivars, possibly provided greater bioavailability from cultivar 1. Chromatogram profile of cultivar 3 is represented to compare carotenoid profile before and after *in vitro* digestion and to show that the method is well applied to β-carotene compound (Figure 5).

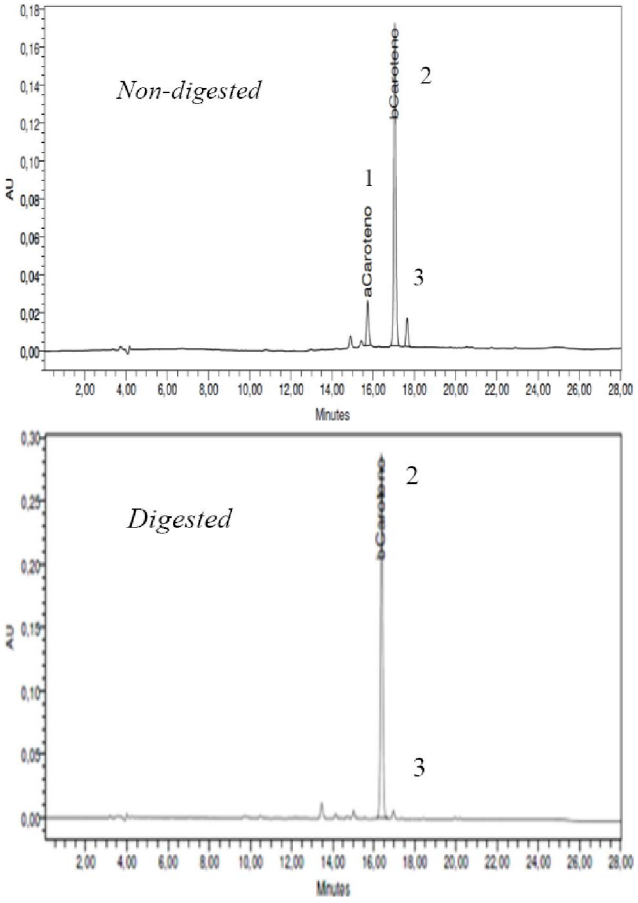

Figure 5: A representative chromatogram of carotenoids extracted from cultivar 3 of orange fleshed sweet potato before and after *in vitro* digestion. Identitication of chromatographic peaks: (1) α-carotene, (2) all-*trans*-β-carotene, (3) and 9-*cis*-β-carotene

CONCLUSIONS

So, this methodology provides great interest since it uses available materials and reagents and allows evaluation of many samples simultaneously. Also it represents an initial tool to predict bioavailability and proved to be faster and cheaper, inasmuch as *in vivo* studies are more costly, complex and require more time. Determination of bioaccessibility provides valuable information to select proper amount of food matrixes or even the better matrix, able to ensure an effective nutrition. However, some aspects, such as absence of microorganisms in digestive tract should be improved, as well as *in vivo* tests, to compare results and to validate the methodology.

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