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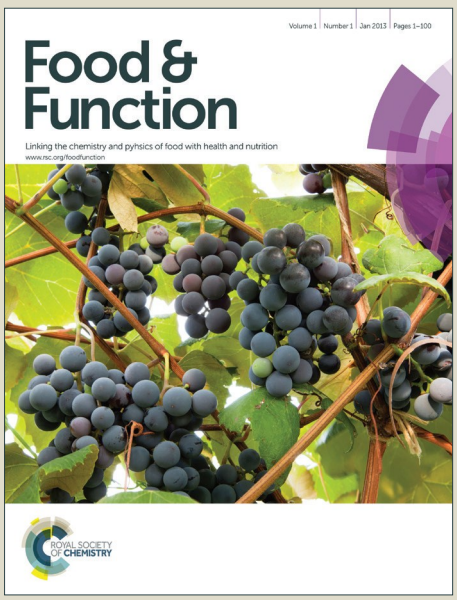

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Biaccessibility of provitamin A carotenoids from fruits: application of a standardised static *in vitro* digestion method.

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Provitamin A carotenoids (β-carotene, α-carotene, β-cryptoxanthin) contribute to the dietary intake of vitamin A and are associated with decreased risk of many chronic diseases. Besides their contents in foods, their bioaccessibility is of great interest since it represents the amount that will be absorbed in the gut. The aims of this study were to adapt, for the first time, the *in vitro* digestion model suitable for food, proposed in a consensus paper by Minekus *et al.* (2014), to assess the bioaccessibility of carotenoids from the fruits that are the major contributors to the intake of β-cryptoxanthin in Spain (orange, tangerine, red pepper, peach, watermelon, and persimmon) and loquat. The highest β-cryptoxanthin content and the lowest bioaccessibility was found in mandarin and loquat (13331.6 and 929.2 μg/100g respectively), whereas the highest contents of β-carotene and α-carotene were recorded in red pepper (1135.3 and 90.4 μg/100g repectively). The bioaccessibility of β-cryptoxanthin was similar to that of β-carotene (0.02-9.8% and 1-9.1 %, respectively) and was higher than that of β-carotene in red pepper, watermelon and peach. α-Carotene bioaccessibility ranged between 0% and 4.6%. We discuss the critical factors for comparing our data: the form of the food being analyzed (raw/cooked/previously frozen, in the presence or absence of oil/fat) and the protocol for bioaccessibility assessment. Different food processing techniques may increase carotenoid bioaccessibility compared to raw food. However, given the difficulties encountered when comparing the results of studies on bioaccessibility, it seems logical to propose the application of the previously mentioned standardized *in vitro* protocol.

Introduction

Carotenoids are plant pigments with an important role in nutrition since, for most of the population, some of these compounds (β-carotene, α-carotene, β-cryptoxanthin) are the major contributors to their dietary intake of vitamin A[1, 2] an essential nutrient for normal growth and development, eyesight and immune response. Carotenoid intake is also associated with decreased risk of many chronic diseases[2, 3]. The human body does not synthesize carotenoids and therefore depends on diet as their source.

Besides the contents of carotenoids in foods, their bioavailability is of great interest since it represents the amount of carotenoids that will be absorbed in the gut and will be available for use or storage by the body.[4]

This information is of growing interest to establish effective nutrition intervention programs. Although bioavailability varies depending on the type of carotenoid and food matrix[2, 5] in the calculation of the contribution of carotenoids with vitamin A activity to the intake of vitamin A, it is assumed, based on theoretical yield, that the bioavailabilities of α-carotene and β-cryptoxanthin are equal and that both contribute in the same proportion to vitamin A intake, each being half the bioconversion factor of β-carotene in the formulas used to express vitamin A activity (retinol activity equivalents [RAE] or retinol equivalents [RE]).[6] However, β-cryptoxanthin, the most important provitamin A xanthophyll in the human diet, mainly supplied by fruits of red/orange color, as other xanthophylls, seems to be more efficiently absorbed and converted into retinol than the carotenes (i.e., α-carotene).[1, 7]

Carotenoid bioaccessibility and bioavailability are generally quite low in raw fruits and vegetables, as a consequence of the lipophilic nature of these compounds and the complex structural organization in which they are embedded. They need to be released from the cellular matrix and incorporated into the lipid fraction during digestion before being absorbable.[8, 9] The bioavailability of carotenoids depends on many factors, which can be intrinsic (e.g., physiological state, homeostatic control, sex), environmental or dietary (e.g., chemical state of the components in the intestine, amount ingested, interactions with other components of the diet). Bioavailability is assessed using *in vivo* or *in vitro* (digestion, cellular, *in silico*) models. *In vitro* models provide information on the pre-absorption phase of bioavailability and, subsequently, on the fraction of bioactive compounds released from the food during digestion and made available for absorption (bioaccessibility). The *in vitro* models were developed as a simple, low cost and reproducible tool for studies of stability, digestive micellization and intestinal transport, as well as to predict the bioavailability of the food components. Although the information they afford is incomplete, as it does not include data on host-related factors, a prerequisite for carotenoid bioavailability is the bioaccessibility in the gut.[2, 9, 10]

In vitro methods attempt to mimic physiological conditions *in vivo*, simulating digestion processes, and typically

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include the oral, gastric and small intestinal phases, and, occasionally, large intestinal fermentation. The majority of the methods reported in the literature are static and, although they all take into account the presence of digestive enzymes, pH, digestion time and salt concentrations, among other factors, the models vary widely in the use of those parameters, making it quite difficult to compare results and deduce overall findings from the information that can be gathered.[9, 11] A standardized digestion method was needed, and a general method to be applied for various endpoints has recently been proposed in a consensus paper by Minekus *et al.* [11].

The aims of this study were to adapt the in vitro method proposed by Minekus *et al.* to assess the bioaccessibility of provitamin A carotenoids (β-cryptoxanthin, β-carotene and α-carotene), particularly that of β-cryptoxanthin, from the fruits that are the major contributors to the intake of β-cryptoxanthin in Spain.

Material and methods

Foods

The plant foods analyzed are the major contributors to the dietary intake of β-cryptoxanthin in the Spanish population that also contain β-carotene[12]: orange (Citrus sinensis, L. Lane Late navel variety), tangerine (Citrus reticulate, L. Tango variety), red pepper (Capsicum annuum, L.), peach (Prunus persica, L.), watermelon (Citrullus vulgaris, Schered), and persimmon (Diospyros kaki). Loquat (Eriobotrya japonica) is a fruit of seasonal consumption and was analyzed because of its high β-cryptoxanthin and β-carotene content.[13]

Reagents and standards

The following reagents were used to prepare the simulated digestion fluids: KCl (Merck, Darmstadt, Germany), $MgCl_2$ $(H_2O)_6$ and $(NH_4)_2CO_3$ (Sigma- Aldrich, Missouri, USA), KH_2PO_4, $NaHCO_3$, Na Cl, HCl and $CaCl_2$ $(H_2O)_2$ (Panreac, Barcelona, Spain).

α-amilase from porcine pancreas, pepsin from porcine gastric mucosa, cholesterol esterase from porcine pancreas, cholesteryl linoleate 98%, bile from bovine and ovine, maltose monohydrate from potato, pancreatin from porcine pancreas , TAME (N-alpha-p-tosyl-L-arginine methyl ester hydrochloride), hemoglobin from bovine blood, ammonium carbonate, albumin from bovine serum, sodium taurodeoxycholate hydrate 96%, sodium potassium tartrate and tributyrin 97%, triethylamine, celite, petroleum ether, methyltert-butyl ether, 3,5- dinitrosalicylic acid, trichloroacetic acid, all of them were purchased from Sigma- Aldrich (Missouri, USA). Kit (TBA) total bile acids was supplied by Spinreact (Girona,Spain).
Methanol, ethanol, diethyl ether, anhydrous sodium sulfate, sodium chloride, pyrogallic acid potassium hydroxide, isopropanol and acetone were supplied by Panreac (Barcelona, Spain).

β-carotene and α-carotene were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and β-cryptoxanthin from CaroteNature GmbH (Ostermundigen, Switzerland).

Sample preparation

We analyzed fresh produce, purchased at a local market. Starting with 0.5 to 1.0 kg of each food, the nonedible portion was removed and the edible portion was homogenized using a kitchen blender. The analysis of the carotenoid content and *in vitro* digestion were done immediately thereafter, always on the same day. Three 5-g samples were weighed for *in vitro* digestion and three 10-25-g samples for carotenoid analysis.

In vitro digestion method

This method was based on the standardized static *in vitro* digestion model suitable for food, proposed in a consensus paper, within the COST Infogest network[11], with several modifications to enable us to assess carotenoid bioaccessibility.

The *in vitro* digestion method included the oral, gastric and small intestinal phases followed by separation of the supernatant. Reagents and phases were those proposed by Minekus *et al.*[11] Simulated digestion fluids were used as follows: for the oral phase, a simulated salivary fluid (pH 7) (SSF); in the gastric phase, a simulated gastric fluid (pH 3) (SGF); and in the duodenal phase, a simulated intestinal fluid (pH 7) (SIF). Enzyme solutions were prepared using those fluids as solvents. Within the options offered by this protocol, we introduced certain modifications in order to adapt it to the assessment of provitamin A carotenoids.

As fruits and vegetables contain starch, we included the oral phase in the model, using α-amylase. In the gastric phase, pepsin was added. In the duodenal phase, the consensus protocol suggests the possibility of using pancreatin or individual enzymes; we chose pancreatin as it is the enzyme most widely employed in the studies published to date and because it is less costly than using individual enzymes. In this phase, cholesterol esterase was added, as it has been reported to be important in the assessment of the bioaccessibility of xanthophylls, present mainly in ester forms, as it acts to hydrolyze them.[14] Finally, we added bile salts in the form of bile extract for the same reasons we used pancreatin.

Briefly, the protocol was as follows: 5 grams of homogenized sample were weighed in triplicate. In the oral phase, the following reagents were added at 37ºc: 3.5 mL of SSF, 0.5 mL of α-amylase (1500 U/mL), and 25 µL of a $CaCl_2$ solution (0.3 M); finally, distilled water was added to a final volume of 10 mL, and the sample was introduced into a shaker bath at 37ºC for 2 minutes. Upon completion of that phase, the sample was placed in an ice bath and the following reagents were added, at 37ºc, to initiate the gastric phase: 7.5 mL of SGF, 1.6 mL of pepsin solution (25 000 U/mL), and 5 µL of a $CaCl_2$ solution (0.3 M); pH was adjusted to 3±0.2 using 6 M HCl, and distilled water was added to a final volume of 20 mL. The sample was again placed in the shaker bath at 37ºc for 2 hours. pH was not readjusted throughout phases because our laboratory is not equipped with an automatic titrator and manual readjustment requires too much time, during which samples are not under the adequate conditions. After the 2 hours, the sample was again placed in an ice bath and the duodenal phase initiated by adding the following reagents: 10 mL of SIF (in the consensus protocol,[11] 11 mL are added, but in our modification, 1 mL of SIF was used to dissolve the cholesterol esterase), 1 mL of cholesterol esterase solution (3.08 U/mL), 5 mL of pancreatin solution (800 U/mL of trypsin activity), 2.5 mL of bile salt solution (160 mM bile salts) and 40 µL of $CaCl_2$ solution (0.3 M). At this point, pH was adjusted to 7±0.2 using 1 M NaOH, and distilled water was added to a final volume of 40 mL. Samples were placed in the shaker bath at 37ºc

for another 2 hours, after which they were cooled (to 10-15ºc) in an ice bath or water. Finally, the supernatant was collected after centrifuging at 10 000 g for 10 minutes, and frozen (for a maximum of 1 week) until extraction.

The *in vitro* digestion method applied in this study indicates that the enzymes must be incorporated according to their enzyme activity.[11] This activity, as well as the bile salt concentration of the bile extract, should be assessed on the basis of previously described protocols.[15] The procedures followed in the present study are described below.

Enzymatic assay of α-amylase

This assay was based on the following definition of enzyme activity unit: one α-amylase unit liberates 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20ºc. The protocol is described elsewhere.[15] Briefly, a 1% starch solution in phosphate buffer was incubated for 3 minutes, under the conditions described above, with 3 different α-amylase concentrations (between 0.33 and 0.50 µ/mL in the test tube prior to enzyme inactivation) in order to produce 3 different concentrations of maltose. Maltose was reacted with sodium potassium tartrate and 3,5-dinitrosalicylic acid (color reagent). This mixture was subjected to 15 minutes at boiling temperature during which it took on color, which was measured by spectrophotometry at 540 nm and maltose was quantified against a standard curve with concentrations ranging between 0.001% and 0.033%.

Enzymatic assay of pepsin

The enzyme activity unit was defined as follows: one unit will produce an increase in absorbance of 0.001/min at λ=280 nm at pH 2 and 37ºc, measured as trichloroacetic acid (TCA)-soluble products. The protocol is described elsewhere.[15] Briefly, different amounts of pepsin were added to the substrate solution (2% hemoglobin). The protocol suggests the use, for instance, of concentrations between 0.8 µg/mL and 5 µg/mL, but in order to ensure linearity in the measurement of absorbance, enzyme was added to reach concentrations between 3.3 and 7.5 µg/mL in the reaction cuvette. The mixture was incubated for exactly 10 minutes at 37ºc. Pepsin acted on the hemoglobin and TCA-soluble aromatic amino acids (tyrosine) was released. Then, 5% TCA was added to each sample to stop the reaction. Hemoglobin was precipitated by centrifugation at 6000 g x 30' at 4ºc and absorbance of the supernatant was measured. The increase in absorbance of the sample over the blank under the conditions described above was calculated.

Enzymatic assay of pancreatin (trypsin activity)

According to the protocol,[15] the amount of pancreatin added to the *in vitro* digestion sample should be determined on the basis of the trypsin activity. Although it also indicates that the amylase and lipase activity of pancreatin should be measured, this was not done for economic reasons.

The trypsin activity assay is based on the following enzyme activity unit: 1 trypsin unit hydrolyzed 1 µmol of TAME (p-toluene-sulfonyl-L-arginine methyl ester) per minute at 25ºC, pH 8.1. This hydrolysis released p-toluene-sulfonyl-L-arginine, which absorbs light at λ=247 nm under the conditions described. To determine the µmol of TAME hydrolyzed per minute, absorbance was measured every 29 seconds during 10 minutes. This assay was performed with a concentration of the enzyme in the in the cuvette in the cuvette ranging from 0.3 to 0.6 µg/mL.

Enzymatic assay of cholesterol esterase

As the use of cholesterol esterase is not mentioned in the consensus protocol.[15] in order to measure its enzyme activity, we chose a widely used enzymatic assay,[16] and for the purpose of simplifying the assay and lowering its cost, we used a kit for clinical use for the determination of the free cholesterol concentration in blood, that was based on the same fundamentals and used the same reagents, several of which were contained in a single solution. The fundamentals were as follows: 1 unit of cholesterol esterase caused the formation of 1 micromole of hydrogen peroxide (0.5 micromoles of quinoneimine dye) per minute under the conditions described below. The approach was based on the fact that when a cholesterol ester came into contact with the enzyme, there was a release of free cholesterol, the concentration of which was measured with the kit as follows: it was oxidized by the action of the cholesterol oxidase, producing hydrogen peroxide, which reacted with 4-aminoantipyrine and phenol to yield quinoneimine dye and producing an increase in absorbance, which was measured by spectrophotometry at 500 nm at 37ºC every 30 seconds. All these reagents were contained in a solution provided with the kit (R reagent).

The cholesterol ester (in this case, cholesterol linoleate) was dissolved in isopropanol, after which it was diluted in a 1% hot triton X100 solution to obtain a concentration of the ester of 0.39 mg/mL. This was allowed to cool and sodium cholate was added. The enzyme was dissolved in 2% BSA and a 1:10 dilution was prepared in phosphate buffer at pH 7 to obtain a concentration of 0.085 mg/mL.

Cholesterol linoleate was added to the spectrophotometric cuvette (to reach a concentration in the cuvette of 0.052 mg/mL), followed by addition of R reagent and buffer for dilution. We then waited the time necessary for the previously free cholesterol in the ester solution to react with the R reagent, after which 0.1 mL of enzyme were added (of buffer in the blank), and measurements were made every 30 seconds. The enzyme activity was calculated on the basis of the slope of the initial linear portion of the absorbance curve.

Assay for bile salt concentration in the bile extract

To measure the bile salt concentration in the bile extract, we used an assay based on the following reaction: in the presence of NAD, 3 α-hydroxysteroid dehydrogenase oxidized bile acids, leading to the formation of thio-NADH, the concentration of which was determined by the increase in absorbance at 405 nm. For this assay, we employed a kit for clinical use that provided all the reagents necessary for measurement in serum samples. For this reason, as the solvent for bile extract, we utilized a mixture of sera in which the bile salt concentration was previously determined. The assay was performed in accordance with the instructions provided with the kit. Briefly, we measured the increase in absorbance at 405 nm at 37ºc between seconds 60 and 120, after having combined the reagents (serum bile extract with concentrations in the reaction cuvette of between 0.05 and 0.26 mg/mL, and kit reagents). The concentration was obtained by comparing said increase with that of a calibrator of known concentration subjected to the same procedure.

Extraction, saponification and HPLC analysis of carotenoids

Extraction was performed according to the procedure of de Rosso.[17] Briefly, sample homogenate (10-25 g depending on the color) was ground with celite in a mortar and extracted with acetone; this was repeated until the extract was colorless. It was then transferred to a mixture of diethyl ether/petroleum ether (1:1) in a separatory funnel and the acetone was eliminated with water. Extraction was repeated until the aqueous extract was colorless. Possible remains of water were eliminated with anhydrous sodium sulfate and the sample was taken to dryness in a rotary evaporator. It was reconstituted in the injection solvent Methyl tert-butyl ether/Methanol (MeOH/MTBE) (70:30), and passed through a 0.45-µm filter. Each sample was injected once or twice, thus yielding between 3 and 6 chromatograms for each food.

The carotenoids present in the extract after *in vitro* digestion were extracted in duplicate, with diethyl ether. The aqueous and gas phases were separated with 10% NaCl and centrifugation at 10 000 g at 4ºc for 10 min. Then, once the complete elimination of water had been ensured by addition of anhydrous sodium sulfate, the extract was taken to dryness in a rotary evaporator, it was reconstituted in MeOH/MTBE (70:30), and passed through a 0.45-µm filter. Each sample was injected 2 or 3 times, thus yielding between 6 and 9 chromatograms for each food. Chromatogram obtained before (A and B: extract and saponified extract, respectively) and after the *in vitro* digestion (C) of loquat is shown in figure 1.

To hydrolyze carotenoid esters and clear the carotenoid extract of interfering substances, such as chlorophylls and unwanted lipids, the extraction of carotenoids (from raw foods) was followed by alkaline saponification of the extract according to the procedure of Granado *et al.*,[18] with slight modifications. Small aliquots were taken of the extracts of each food (three initial weightings) and were saponified (1 of them in duplicate) as follows. A small aliquot was taken of the extract and was mixed with 0.1 M pyrogallic acid in ethanol and with KOH in 30% methanol, and was subjected to ultrasonication for 7 min. The extract was then washed with water and extracted with a 1:1 mixture of diethyl ether : petroleum ether, with vortexing and centrifugation, and, finally, the supernatant was desiccated/dried in a nitrogen atmosphere, and was reconstituted in MeOH/MTBE (70:30) to be injected onto High-Performance Liquid Chromatography (HPLC). Saponification was carried out only in the undigested food samples, since a test run in the extract of digested loquat, a food with a high β-cryptoxanthin content, showed that the irremediable loss of carotenoids derived from saponification resulted in the nearly total absence of carotenoids in the sample.

The HPLC analysis was performed using a system consisting of a model 600 pump, a Rheodyne injector and a 2998 photodiode array (PDA) detector (Waters, Milford, MA, USA). The system included a C30 YMC column (5 µm, 250 × 4.6 mm i.d.) (Waters, Wilmington, MA) with a guard column (Aquapore ODS type RP-18) using as mobile phase a linear gradient of MeOH with 0.1% triethylamine (TEA)/MTBE from 95:5 to 70:30 in 30 min, to 50:50 in 20 min, and maintaining this proportion for 35 min. The flow rate was 0.9 mL/min. All chromatograms were processed using Empower 2 software (Waters, Milford, MA, USA). Identification was carried out by comparing retention times with those of authentic standards and on-line UV-VIS spectra. Considering that the amounts of cis isomers were close to the limit of quantification and, in most samples, they were absent, the tentative identification was carried out based on comparison of elution order and the peaks in the absorbance spectrum with the literature.[17] Single calibration sample was used for each carotenoid before the preparation of the standard curve to assess retention times, peak shapes and sensibility. The repeatability of the carotenoid concentration was checked by means of six repeated injections of standards within the same day and one injection each day samples were running. Blanck was injected as the first and last sample each day and after sample injection with a high concentration of any compounds. The precision was evaluated (using two curves concentration) by the relative coefficient of variability which on average was lower than 10% for each carotenoid.

Quantification was performed using standard curves for β-cryptoxanthin, α-carotene and β-carotene. Due to the variability in the carotenoid contents among the foods analyzed and between the raw foods and the extract after *in vitro* digestion, two curves were used for each carotenoid. All three carotenoids had the same concentration at each point of the curve. The values in the higher curve ranged between 1 µg/mL and 12 µg/mL with R^2=0.992 for α-carotene, R^2=0.992 for β-carotene and R^2=0.991 for β-cryptoxanthin. Those of the lower curve ranged between 0.05 µg/mL and 1 µg/mL and R^2 was 0.995, 0.995, 0.997 for α-carotene, β-carotene and β-cryptoxanthin, respectively. Cis-isomers were quantified against the corresponding all-trans carotenoid.

Statistical analysis

Carotenoid concentrations are expressed as mean and standard deviation (SD) of 4 -6 analyses corresponding to three weights from each food. The percent of bioaccessibility was calculated versus the mean concentration of the carotenoid content in each raw food. A non-parametric test (Mann-Whitney U) was applied to test for differences between the carotenoid concentrations among foods and, supernatant concentrations (in vitro digestion) and bioaccessibility percentages. Statistical analysis was performed using the SPSS v.21 software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Table 1 shows the β-cryptoxanthin, α-carotene and β-carotene content in each food and in the supernatant after *in vitro*

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Table 1. Carotenoid content in food (µg/100g) and in supernatant (SN) (µg/100g food) and percent bioaccessibility (BA) of carotenoids after in vitro digestion expressed as mean ±SD.

	β-cryptoxanthin			all-*trans*-α-carotene			9-*cis*-α-carotene			all-*trans*-β-carotene			9-*cis*-β-carotene			13-*cis*-β-carotene		
	Food	SN	BA	Food	SN	BA	Food	SN	BA	Food	SN	BA	Food	SN	BA	Food	SN	BA
Loquat	929.19 ^{abcde} ± 104.27	0.19^{a} ± 0.04	0.02 ^{a} ± 0.00	34.79 ± 2.34	0.00 ± 0.00	0.00 ^{a} ± 0.00				613.16 ± 53.95	0.64^{ab} ± 0.03	0.10 ^{a} ± 0.01	14.91 ± 4.47	0.00 ± 0.00	0.00 ± 0.00			
Mandarin	1331.63 ^{abcde} ± 372.73	3.77 ^{ab} ± 1.23	0.28 ^{b} ± 0.09	12.35 ± 1.54	0.00 ± 0.00	0.00 ^{b} ± 0.00				547.04 ± 44.75	8.25 ^{b} ± 1.26	1.51 ^{b} ± 0.23						
Orange	84.67 ^{abde} ± 1.34	0.90 ^{ab} ± 0.34	1.07 ^{a} ± 0.40	16.12 ± 1.39	0.14 ± 0.09	0.87 ^{a} ± 0.53				24.20 ± 6.13	0.44 ^{ab} ± 0.10	1.80 ^{ab} ± 0.40						
Peach	59.01 ^{bcde} ± 50.33	1.97 ^{ab} ± 1.15	3.35 ^{ab} ± 1.94							35.62 ± 5.26	0.55 ^{c} ± 0.23	1.55 ^{ab} ± 0.63	5.19 ± 1.89	0.00 ± 0.00	0.00 ± 0.00			
Pepper (red)	282.58 ^{acde} ± 17.47	17.84 ^{ab} ± 7.78	6.31 ^{a} ± 2.75	90.42 ± 4.86	3.21 ± 1.40	3.55 ^{ab} ± 1.55	297.51 ± 20.66	6.05 ± 2.71	2.04 ± 0.91	1135.26 ± 140.64	39.33 ^{a} ± 15.11	3.46 ^{a} ± 1.33	86.83 ± 16.13	0.00 ± 0.00	0.00 ± 0.00	50.04 ± 7.21	4.85 ± 1.70	9.70 ± 3.40
Persimmon	215.58 ^{abc} ± 71.36	1.72 ^{b} ± 0.29	0.80 ^{a} ± 0.13	6.29 ± 3.78	0.29	4.61 ^{ab}				38.93 ± 4.19	3.53 ^{abc} ± 5.09	9.08 ^{ab} ± 13.07	14.56 ± 8.09	0.38	2.58			
Watermelon	1.22 ^{abde} ± 0.53	0.12 ^{ab} ± 0.01	9.84 ^{ab} ± 1.16							57.63 ± 4.77	3.52 ^{ab} ± 1.51	6.10 ^{a} ± 2.62						

Different superscript letters mean significant differences of content or bioaccessibility between foods.

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digestion, as well as their bioaccessibility, defined as the percentage of the initial content in the food, and expressed in µg/100 g of food (mean ± SD). Carotenes were quantified in nonsaponified samples and β-cryptoxanthin in saponified extracts. These carotenoids were present in all-*trans* form and as *cis*-isomers in red pepper (9-*cis*-α-carotene and 9-*cis*- and 13-*cis*-β-carotene), as well as in persimmon, loquat and peach (9-*cis*-β-carotene). The highest β-cryptoxanthin content was found in mandarin and loquat, whereas the highest contents of β-carotene and α-carotene were recorded in red pepper. When the amounts of these carotenoids were expressed as percentages of the total provitamin A carotenoids in the foods analyzed, the percentage of β-cryptoxanthin was highest in persimmon and those of β-carotene and α-carotene were highest in watermelon and orange, respectively.

Other authors have reported higher, lower, and similar β-carotene contents in red peppers[5, 19-23] and higher in persimmon[24], and similar levels of β-cryptoxanthin have been found in both fruits[5, 20, 23, 25]. All three provitamin A carotenoids are present in oranges, but the reported contents differ widely among studies[26-31], especially for β-cryptoxanthin[5, 28-30, 32]. Mandarin has the same provitamin A carotenoid profile as orange, but the contents of β-carotene and β-cryptoxanthin in the present study were higher than those shown in other reports[32]. In watermelon, we found a content of β-carotene similar[28, 29] and higher[33] to that reported in other studies, and that of β-cryptoxanthin was similar [34], probably because it was analyzed at the start of the watermelon season. Other authors have reported higher and lower content of β-carotene in loquat [5, 35] and lower content of β-cryptoxanthin than in this study[5, 35]. Peach contained β-cryptoxanthin and all-*trans* β-carotene in concentrations similar to those found in other studies[28, 29], and α-carotene and 9-*cis* β-carotene were identified.

The wide disparity in carotenoid profiles reported in different studies is due to a number of factors related to the food (variety, climate, geographic location, season, ripening stage at harvest, part of the plant analyzed, agricultural practices, and postharvest handling, processing and storage, among others), [2, 36] and to the analytical procedure, such as the instrumental technique employed (HPLC, spectrophotometry, etc.) and whether or not it includes saponification[2, 36], and how the results are expressed.[37]

***In vitro* digestion**

Given the importance of knowing the fraction of carotenoids that is available for absorption, we assessed not only the content, but the bioaccessibility, as well. For this purpose, we used the recently published consensus protocol designed to harmonize and validate *in vitro* digestion models and facilitate the comparison of results from different studies.[11] The main feature of the protocol is the incorporation of enzymes on the basis of their activity, which must be determined in each of the reagents employed using assays described in an appended document, as well as a procedure for assessing the bile salt concentration in the bile extract,[15] since the findings for enzyme activity differ depending on the protocol used to measure them.

Among the provitamin A carotenoids, the bioaccessibility of β-cryptoxanthin was higher than that of β-carotene in red pepper, watermelon and peach. β-cryptoxanthin showed a percent bioaccessibility ranged between 0.02% and 9.84% (Table 1). Our results are similar to those reported by other authors for orange[32], red pepper[21, 22] and loquat[5], and lower for mandarin[32] and red pepper [23]. In this regard, the food matrix appears to influence bioaccessibility, as different findings have been reported by authors using the same protocols for the same carotenoid, but in different food matrices.[5] In the present study, the lowest bioaccessibility of β-cryptoxanthin was found in mandarin and loquat, foods having the highest content of this carotenoid.

There are few data on the carotenoid bioaccessibility in fruits; most of them refer only to β-carotene and very few to α-carotene and β-cryptoxanthin. The bioaccessibility of β-carotene in the present study was similar to that of β-cryptoxanthin and ranged between 0.1% and 9.08% (Table 1), and is similar to that found in other studies analyzing foods that were not cooked and foods with no added oil / fat **(**Table 2**)**, important variables that make it difficult to compare different studies. For example, some authors have reported a β-carotene bioaccessibility of around 0% in a typical salad[38-40], which increased to up to 20% when a fat source was added[40]. In orange-fleshed sweet potato, an important source of vitamin A in many countries, similar percentages were described with and without fat addition (from 0.6% to 3%)[41, 42]. In other studies (Table 2) analyzing green pepper[23], cassava[43, 44], banana[45], wild garlic, baby leaf salad, orange, yellow and red peppers[46], carrot, tomato,[47] broccoli, spinach, lettuce, tomato paste, carrot, red pepper, kiwi, pineapple and loquat[5] and, again, orange-fleshed sweet potato[48, 49], higher bioaccessibility values are reported and the potential influencing factors are commented on below.

Regarding α-carotene, the percentage after the digestion assay ranged between 0% and 4.61%, depending on the food (Table 2), with values similar to those obtained by others in salads[38-40] and orange-fleshed sweet potato[42], but lower than some found for yellow and red pepper[46], carrot[5, 46, 47], and banana[45] (Table 2) .

Food and analytical factors influencing the assessment of the bioaccessibility

The bioaccessibility of carotenoids in foods is known to be influenced by aspects related to the food (variety, ripening stage, preparation of the food, etc.) and by analytical factors (extraction and saponification methods, *in vitro* digestion protocol, etc.).[2] Among those factors, we comment on those we found to be critical for comparing the data of the present study: the form of the food being analyzed (raw / cooked / previously frozen, in the presence or absence of oil / fat) and the protocol for bioaccessibility assessment.

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Table 2. Content, bioaccessibility and protocol reported in the cited studies performed in individual foods (with the exception of fat addition) in which the results are expressed as percentages of the total content in the food.

			Differences in protocol								Differences in handling				
Food	Content in food (µg/100 g)	Bioaccessibility	Duration and pH	Enzymes						Frozen	Heated	Fat added	Protocol based on	Ref.
				AA	Pe.	Pa.	BS	CE	Li.					
Banana	α-Car 6 - 440 β-Car 7 - 862	α-Car 14 - 41% β-Car 10.2 - 32%	OP:10min; GP:0.5h-pH4; DP: 0.5h-pH6	=	>	<	<	-	-	yes	yes	yes	Reboul, et al., 2006	45
Broccoli	β-Car 794	β-Car 21.6%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	no	yes	no	Oomen, et al., 2003	5
Broccoli	β-Car 812	β-Car 18%	OP:5min; GP:1h-pH1.1; DP:2h-pH7.8	>	>	>	>	<	-	no	yes	no	Oomen, et al., 2003	37
Carrot	Graph difficult to understand	α-Car 25% β-Car 12%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46
Carrot	α-Car 2280 β-Car 3870	α-Car 38% β-Car 37%	GP:0.5h-pH4 & 0.5h-pH2; DP:2h-pH6.9	-	>	<	<	-	-	yes	yes	yes	Garrett et al., 1999	47
Carrot	α-Car 1294 β-Car 3230	α-Car 72% β-Car 76.5%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	no	yes	no	Oomen, et al., 2003	5
Cassava	β-Car 23 - 899	β-Car 9 -50%	OP:10min; GP:1h-pH2; DP:2h-pH7.5	>	<	< ó = ‡	<	-	used	yes	yes	yes	Thakkar et al., 2007	43
Cassava	β-Car 0 - 700	β-Car 12 - 30%	OP:10min; GP:1h-pH 2.5; DP:2h-pH6.5	>	>	<	=	-	used	yes	yes	no	Thakkar et al., 2007	44

Cont. table 2. Content, bioaccessibility and protocol reported in the cited studies performed in individual foods (with the exception of fat addition) in which the results are expressed as percentages of the total content in the food.

Garlic (wild)	Graph difficult to understand	β-Car 21%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46
Kiwi	β-Car 31	β-Car 56.9%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	no	no	no	Oomen, et al., 2003	5
Lettuce	β-Car 96	β-Car 51%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	no	no	no	Oomen, et al., 2003	5
Loquat	β-CX 469 β-Car 222	β-CX 9.6% β-Car 17.6%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	NS	NS	no	Oomen, et al., 2003	5
Maize (flour and porridge)	(maize flour) β-CX ND - 280 β-Car 4 - 900	(Maize porridge) β-CX 28 - 30% β-Car 16.7%	OP:10 min; GP:1h-pH2.5; DP:2h-pH6.5	>	>	<	=	NS	used	yes	yes	no	Thakkar et al., 2007	50
Mandarin	β-CX 378	β-CX 20.4%	GP:2h-pH2; DP:2h-pH6.9	-	>	>	=	-	-	yes	no	no	Cilla et al., 2012	32
Mango (different ripening stages)	β-CX 10 - 30 β-Car 1070 - 3940	β-Car 4.5 - 7%	GP:1h-pH3; DP:2h-pH7	-	>	=	>	-	-	no	no	no	Garrett et al., 1999	51
Orange	β-CX 0	β-CX 42.3%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	no	no	no	Oomen, et al., 2003	5
Orange (Cara Cara)	β-CX 21 β-Car 17	β-CX 9.7% β-Car 5.9%	GP:2h-pH2; DP:2h-pH6.9	-	>	>	=	-	-	yes	no	no	Cilla et al., 2012	32
Orange (Navel)	β-CX 57	β-CX 7.1%	GP:2h-pH2; DP:2h-pH6.9	-	>	>	=	-	-	yes	no	no	Cilla et al., 2012	32
Pepper (green, jalapeño)	β-Car 310 α-Car 20	β-Car 28.5%	GP:1h-pH2; DP: 2h-pH7	-	>	=	>	-	used	no	no	no	Garrett et al., 1999	23
Pepper (orange)	Graph difficult to understand	β-Car 57%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46

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Table 2. Content, bioaccessibility and protocol reported in the cited studies performed in individual foods (with the exception of fat addition) in which the results are expressed as percentages of the total content in the food.

Pepper (red jalapeño)	β-CX 280 β-Car 2110	β-CX 44.5% β-Car 7.2%	GP:1h-pH2; DP: 2h-pH7	-	>	=	>	-	used	no	no	no	Garrett et al., 1999	23
Pepper (red)	Graph difficult to understand	α-Car 27% β-Car 30%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46
Pepper (red)	β-CX 314 β-Car 618	β-CX 98.1% β-Car 70.6%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	NS	NS	no	Oomen, et al., 2003	5
Pepper (red, 17 different genotypes from 3 species)	β-CX 1295 - 17327 β-Car 568 - 5749	β-CX 2.4 - 16.5% β-Car 0.4 - 13.1%	GP:1h-pH2; DP:2.5h-pH7.4	NS	NS	NS	§§	NS	-	no	no	no	Garrett et al., 1999	21
Pepper (red, bell pepper)	β-CX 2484 β-Car 5635	β-CX 6.2% β-Car 33.1%	GP:1h-pH2; DP:2.5h-pH7.4	-	>	>	§§	-	-	no	no	no	Garrett et al., 1999	22
Pepper (yellow)	Graph difficult to understand	α-Car 45% β-Car 36%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46
Pineapple	β-CX 26 β-Car 6	β-CX 46.2% β-Car 98.7%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	no	no	no	Oomen, et al., 2003	5
Rocket	Graph difficult to understand	β-Car 14%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46
Salad	α-Car 1650 β-Car 3250	α-Car 1.4% β-Car 0%	GP:1h-pH2; DP:2h-pH7.5	-	>	>	>	-	-	yes	no	no	Garrett et al., 1999	40
Salad	NS	α-Car 12 - 14% β-Car 8 - 11%	OP:10min; GP:1h-pH2.5; DP:2h-pH6.5	>	>	<	=	-	used	NS	no	yes	Thakkar et al., 2007	39
Salad	α-Car 1170 β-Car 3830	α-Car 2 % β-Car 2.8%	GP:1h-pH3; DP: 2h-pH7	-	>	<	=	-	used	yes	no	no	Garrett et al., 1999	38
Salad (baby leaf)	Graph difficult to understand	β-Car 22%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46

Table 2. Content, bioaccessibility and protocol reported in the cited studies performed in individual foods (with the exception of fat addition) in which the results are expressed as percentages of the total content in the food.

Sorghum	β-CX 5 - 45 α-Car 4 - 75 β-Car 70 -1070	β-Car 2 - 8.2%	OP:10min; GP:1h-pH2.5; DP:2h-pH6.5	>	<	<	<	-	used	yes	yes	yes	Kean, et al., 2011	52
Spinach	Graph difficult to understand	β-Car 12%	OP:15min; GP:1h-pH1; DP:2h-pH7.8	<	<	<	<	-	used	NS	no	no	Oomen, et al., 2003	46
Spinach	β-Car 662	β-Car 25.5%	OP:5min; GP:1h-pH1.1; DP:2 h-pH7.8	>	>	>	>	<	-	yes	yes	no	Oomen, et al., 2003	5
Sweet potato (orange-fleshed)	β-Car 5706 - 9294	β-Car 23.7 - 40.8%	OP:15min-pH6.7; GP:0.5h-pH4 & 0.5h-pH2; DP:2h-pH6.9	>	>	<	=	-	-	yes	yes	no	Hedren et al, 2002.	48
Sweet potato (orange-fleshed)	β-Car 17890 - 23290 (dry weight)	β-Car 28.8 - 76%	OP:15min-pH6.7; GP:0.5h-pH4 & 0.5h-pH2; DP:2h-pH6.9	>	>	<	=	-	-	yes	yes	yes	Hedren et al, 2002.	49
Sweet potato (orange-fleshed)	β-Car 11210 - 28080	β-Car 0.6 - 3%	OP:10min; GP:1h-pH2.5; DP:2h-pH6.5	>	<	=	>	NS	used	yes	yes	yes	Thakkar et al., 2007	41
Sweet potato (orange-fleshed, 3 varieties)	α-Car 0 - 27 β-Car 1 - 11440	α-Car ND β-Car 4.3 - 8%	OP:10min; GP:1h-pH2.5; DP:2h-pH6.5	-§	>§	<§	<§	-	-	yes	yes	yes	Thakkar et al., 2007	42
Tomato	β-Car 140	β-Car 62%	GP:0.5h-pH4 & 0.5h-pH2; DP:2h-pH6.9	-	>	<	<	-	-	yes	yes	yes	Garrett et al., 1999	47
Tomato (paste)	Composition includes raw tomato	β-Car 100.0%	OP:5min; GP:1h-pH1.1; DP:2h-pH7.8	>	>	>	>	<	-	NS	NS	no	Oomen, et al., 2003	5
Watermelon	β-Car 109	β-Car 30.2%	GP:1h-pH2; DP:2h-pH6.9	-	=	<	§§	-	used	no	no	yes	Garrett et al., 1999	33

AA = α-amylase, Pe.= Pepsine, Pa.= Pancreatine; BS= Bile Salts, CE= cholesterol esterase, Li.= Lipase
β-CX= β-Cryptoxanthin, β-Car=β-Carotene, α-Car=α-Carotene
OP=Oral phase, GP= Gastric phase, DP= Duodenal Phase
NS= Not specified; ND= Not determined.
>, <, = : higher, lower or equal enzyme amount respect the sample weight compared with the amount used in the present study (see text in page 13).
- : enzyme not used.

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‡Depending on if they use 0.3 or 1.5g of sample.
§ Amount of sample not indicated.
§§ Individual bile salts.

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A) Sample preparation: Thermal processing facilitates cell wall disruption, followed by the release of the carotenoids from the food matrix and increased bioaccessibility, as has been reported in cassava[43, 50], banana[45], orange-fleshed sweet potato[48, 49], corn[53], carrot and tomato[47], and broccoli[37]. In the present study, foods were analyzed in raw form because that is how they are consumed (with the exception of peppers, which are consumed raw or cooked), and although differences could be expected with respect to heat-processed foods, similarities were found for sweet potato[42], sorghum[52] and orange-fleshed sweet potato[41].

Freezing foods prior to analysis also affects bioaccessibility as it facilitates the release of carotenoids and access to the enzymes present in the food. Thus, high bioaccessibility from samples that were frozen prior to the digestion process is reported in cassava[43, 50], banana[45], orange-fleshed sweet potato[48, 49], corn[53] and carrot[47]. However, as occurred with heat-processing, some studies involving frozen-stored samples did not obtain high bioaccessibility values in sweet potato[42], salad[38, 40], sorghum[52] or orange-fleshed sweet potato[41]. Although, frozen storage of samples is useful because it facilitates laboratory analysis of numerous samples, standardizing sample collection procedures, etc., it is not useful from the nutritional point of view in fruits, as the circumstances in which they are consumed after freezing are limited.

Finally, the addition of a fat and/or oil to *in vitro* digestion is critical, as can be observed in studies with and without fat addition[21, 45, 48], as the bioaccessibility of β-carotene increased from 0% to 20% when fat was added to the digestion process[40]. Fat produces a lipophilic environment that facilitates the transfer of the carotenoids from the food matrix to lipid droplets during the gastric phase. Moreover, the products of lipid hydrolysis modify the physicochemical characteristics of the micelles, a circumstance that may increase the uptake of carotenoids. It also increased the secretion of pancreatin and of bile salts, although since a static method was used, the amounts of enzymes were predetermined in the method[38]. The addition of fat may be the reason for increased bioaccessibility in studies involving watermelon, to which full-fat yogurt was added[33], cassava[43], banana[45], orange-fleshed sweet potato[49] and carrot[47], although, as was pointed out above with respect to heat and freezing, there are exceptions, and low bioaccessibility has also been reported for foods with added fat: sweet potato[41, 42], sorghum[52] and salad[39]. Fruit is not often consumed together with a source of fat, as this combination is not a common culinary offering. However, fruit is often eaten for dessert after a meal that contains fat, a practice that should have a positive effect on bioaccessibility. [51]

B) Analytical method: On comparing *in vitro* digestion methods, the lack of homogeneity among them is striking, and applies to differences not only between digestion protocols, but between ways of expressing the results, as well. The main aspects to be considered in the protocol are the phases of digestion to be simulated, their duration, pH, and number and amounts of enzymes added. As can be seen in Table 2, one of these protocols omits the oral phase[54], but all of them include the two main phases, gastric and duodenal, and the studies in which they are employed differ little in terms of pH (pH 1 - 2.5) and duration (1-2 h), with the exception of those following the protocol proposed by Reboul[45] and the combination of those of Garrett and Hedrén[49]. The widest variability is observed in the amounts of enzymes added, as there are nearly no coincidences across studies, there being cases in which the amount was 200-fold higher[22] or lower[43]. Table 2 indicates whether the amount of each enzyme in proportion to the grams of sample was higher, lower or equal to that used in the present study; however, it should be kept in mind that in the protocol we followed, the enzymes were added depending to their activity, rather than simply according to weight. When we compared our results with those of other studies in which the durations, pH and concentrations were similar or different, we found similarities and differences. However, exactly the same method used in this study was applied In a previous study by our group (with the only modification of not applying cholesterol esterase because no xanthophyll was present), for assessing the bioaccesibility of β-carotene form sweet potato flower both raw and subjected two different heat treatments, and bioaccessibility percentages ranged from 15,1% to 42,8%. Maybe the fact of start off from a sample previously dried, grinded up and frozen, increased the bioaccessibility in addition to the effect of heat treatments in those samples in which they were applied.[55]

The results of the studies on bioaccessibility carried out to date differ widely due to differences in the food matrix, in food handling (freezing, cooking, fat addition) and/or in the *in vitro* digestion model. Thus, the analysis of carotenoids calls for the design and widespread use of a standardized method of *in vitro* digestion to limit the number of variables that influence the disparity among the findings. It is important not only to standardize the protocol, but to provide complete information on the food analyzed (variety, ripening stage, plant parts, etc.) and on its prior handling (processing times and temperatures, freezing, etc.).

Finally, we must stress the importance of expressing the results in such a way as to enable their comparison across studies, as the differences in their presentation sometimes make this impossible. For example, in some studies, they are expressed as content in the supernatant[32] and, in others, as content in the micellar phase[39], whereas other authors report measurements in the duodenal phase without specifying whether before or after centrifugation[14]. In other reports, the amounts present in the micellar phase or in the supernatant are expressed as a percentage of a total; in the majority of cases, this total is the content in the food, but in others, it is the content at the end of the duodenal phase, and in still others, it is the supernatant, and the results are expressed as the percentage transferred from these phases to the micelles[56]. When the study involves xanthophylls, there is another especially relevant datum—whether or not the initial or final samples were subjected to saponification—that is not always expressly indicated.

Conclusions

In spite of the numerous variables that influence bioaccessibility and the extremely wide discrepancies in the reported data, the bioaccessibility found in this study and in many others is surprisingly low. With this level of availability, it seems difficult to explain the serum concentrations of these compounds reached in food dietary intervention studies [7, 57]. On the other hand, these data question the lesser contribution to vitamin A intake attributed to β-cryptoxanthin, and are supported by those from another report[1, 38], in which its bioaccessibility was higher than that of β-carotene in nearly half of the fruits analyzed.

Our findings argue in favor of using standardized and validated procedures in the analysis of the bioaccessibility of provitamin A carotenoids. This would enable the proper assessment of the intake of vitamin A, which could serve as an aid in public health interventions and possibly in the development of functional foods targeting individuals with low vitamin A intake.

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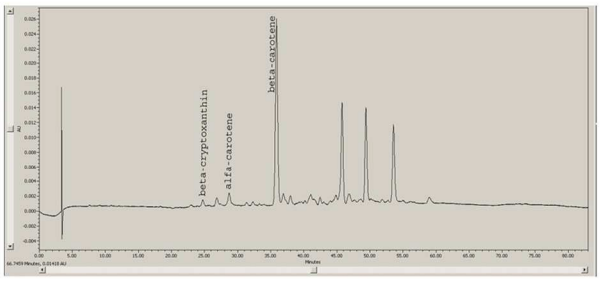
a)



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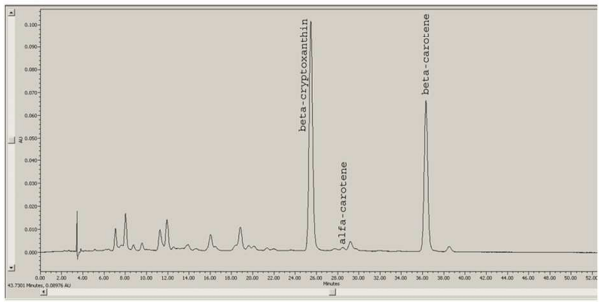

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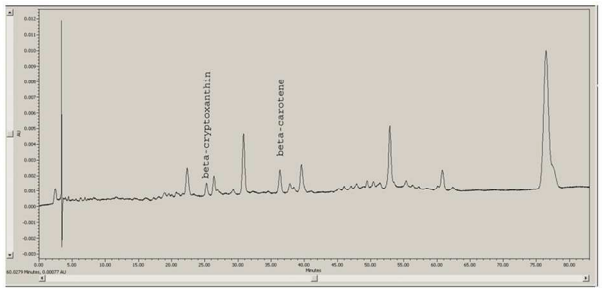

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