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Bioaccessibility of phytoene and phytofluene is superior to other carotenoids from selected fruit and vegetable juices

Paula Mapelli-Brahm [b,1], Joana Corte-Real [b,c], Antonio J. Meléndez-Martínez [a,*], Torsten Bohn [b,c]

[a] Food Colour & Quality Lab., Dpt. of Nutrition & Food Science, Universidad de Sevilla, Seville, Spain
[b] Environmental Research and Innovation Dpt., Luxembourg Institute of Science and Technology, Belvaux, Luxembourg
[c] Population Health Dpt., Luxembourg Institute of Health, Strassen, Luxembourg

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ABSTRACT

Phytoene and phytofluene are major abundant dietary carotenoids largely ignored in the context of agro-food and health. The bioaccessibility of phytoene and phytofluene in tomato, carrot, blood orange (sanguinello cultivar), and apricot juices was analysed following simulated gastro-intestinal digestion with coffee cream as a lipid source, and compared with that of other main carotenoids from these matrices. The bioaccessibility of phytoene and phytofluene, and also total carotenoid bioaccessibility, followed the order: sanguinello > apricot > tomato > carrot. Phytoene was consistently the carotenoid with the highest bioaccessibility, up to 97%, generally followed by phytofluene. The higher bioaccessibility of these carotenoids could mainly be due to their marked difference in chemical structure and matrix distribution. For most juices, *cis*-isomers presented a higher bioaccessibility than their all-*trans* counterparts ($P < 0.05$). The dietary source that provided highest amounts of potentially absorbable phytoene/phytofluene was by far tomato juice (5 mg/250 mL juice).

1. Introduction

Many studies suggest that a diet rich in fruits and vegetables can decrease the risk of developing certain chronic diseases (World Cancer Research Fund & American Institute for Cancer, 2007). A convenient and efficient way to include fruits and vegetables in our daily diet is via juices. Juices are a rich source of nutritionally important compounds such as vitamins and minerals. Many juices are also a good source of carotenoids. Within the carotenoid family, there are compounds that have been largely ignored in the context of agro-food and health such as phytoene (PT) and phytofluene (PTF). Although the beneficial effect of these carotenoids on human health is still disputed, several reports have raised questions about their influence on human health (Kaulmann & Bohn, 2014; Meléndez-Martínez, Mapelli-Brahm, Benítez-González, & Stinco, 2015; Nishino et al., 2002). However, these colourless carotenoids are present in a wide variety of fruits and vegetables. According to one study, the daily per capita intake of phytoene and phytofluene combined has been estimated to represent 16% of the total dietary intake of carotenoids (Biehler et al., 2012). Interestingly, in the same study, the intake of phytoene (2.0 mg) appeared to be superior to that of carotenoids such as lycopene or lutein (1.8 and 1.5 mg, respectively). As the intake in the studied country (Luxembourg) is expected to be similar to that of other European countries, the results emphasize the importance of these carotenoids regarding carotenoid dietary intake in Europe. However, in order to estimate possible health benefits of these carotenoids, it is not only necessary to know their dietary intake, but also which fraction of this is truly available to the human body for supporting physiological functions i.e. their bioavailability.

Taking into account that carotenoids are lipophilic, they must be released from the food matrix and emulsified into lipid droplets

* Corresponding author at: Área de Nutrición y Bromatología, Universidad de Sevilla, Facultad de Farmacia, 41012 Sevilla, Spain.
E-mail address: ajmelendez@us.es (A.J. Meléndez-Martínez).
[1] Permanent address: Food Colour & Quality Lab., Dpt. of Nutrition & Food Science, Universidad de Sevilla, Seville, Spain.

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and then incorporated into mixed micelles prior to their uptake and absorption. These are considered key steps regarding the bioavailability of carotenoids and some of the factors that govern these steps can be evaluated in part by *in vitro* models (Alminger et al., 2014; Biehler & Bohn, 2010). Despite that these models are often static and do not account for host factors influencing carotenoid bioavailability such as transporters (Bohn et al., 2017), *in vitro* studies can be very useful to study the impact of the food matrix on carotenoid release and potential availability during the digestion process. These methods are cheaper, less time-consuming, simpler and more reproducible than *in vivo* models, due to controlled conditions, and elimination of inter-individual differences (e.g. genetic differences (Biehler & Bohn, 2010)), and are thus proposed for hypothesis building prior to confirmation by human studies.

The objective of the present work was to compare the bioaccessibility of phytoene and phytofluene with that of other major dietary carotenoids in fruit and vegetable juices with different carotenoid patterns, in order to assess the importance of phytoene/phytofluene regarding their contribution to total carotenoid intake and availability, as very little information on this topic is available. The effect of the *cis-trans* configuration of the carotenoids on their bioaccessibility was also evaluated. This study provides information on the concentration of the colourless carotenoids and their bioaccessible fraction from several fruit and vegetable juices, and contributes to the understanding of factors that can modulate the bioaccessibility of lipophilic secondary plant constituents.

2. Materials and methods

2.1. Description of the samples

Four frequently consumed juices, rich in both phytoene and phytofluene according to the literature (Biehler et al., 2012; Melén dez-Martínez et al., 2015) were chosen, taking into account their availability in Luxembourg. Thus, apricot (*Prunus armeniaca*) nectar, orange (*Citrus sinensis*), carrot (*Daucus carota*), and tomato (*Solanum lycopersicum*) juices were selected and purchased at a local supermarket (CORA S.A., Foetz, Luxembourg). The orange juice was prepared with a blood orange variety (sanguinello cultivar), hereafter referred to as "sanguinello juice". The producer and place of production were Rauch (Austria), Eckes Graninis (France), Tropicana (France), and Eden (Germany) for tomato, apricot, sanguinello and carrot juice, respectively. While the sanguinello and tomato juices were 100% fruit, the carrot juice and apricot nectar contained some lemon juice (percentage not specified on label). The apricot nectar contained apricot puree with a minimum level of 40% apricot fruit, and was termed apricot juice for simplicity. Tomato and sanguinello juices were processed by pasteurization and flash pasteurization, respectively. Tomato juice was made from concentrate (Table 1). The juices were continuously stored in a fridge until analysis (a couple of days).

2.2. Standards and reagents

Porcine bile extract, pepsin (porcine, ≥250 units/mg solid, measured as trichloroacetic acid-soluble products using hemoglobin as substrate), pancreatin (porcine, 4x United States Pharmacopeia specifications of amylase, lipase and protease) and the HPLC solvents, i.e. methanol (MeOH) and methyl *tert*-butyl ether (MTBE), were obtained from Sigma-Aldrich (Bornem, Belgium). 15-*cis*-phytoene, 9-*cis*-β-carotene, 15-*cis*-β-carotene, and 5-*cis*-lycopene were obtained from CaroteNature (Lupsingen, Switzerland); α-carotene, β-carotene, and lycopene were procured from Sigma-Aldrich; β-cryptoxanthin from Extrasynthèse (Lyon, France). Phytofluene was isolated from 0.1 g of an oleoresin of tomato (Lycored, Beer-Sheva, Israel) which was mixed with 1 mL of dichloromethane. Following ultrasonication (Ultrasons, JP Selecta, Barcelona, Spain) for 5 min the supernatant was filtered through a nylon membrane (13 mm × 0.45 μm) (Millipore Iberica S.A., Madrid, Spain) and transferred to a new 2 mL plastic tube. This solution was centrifuged at 18,000*g* for 5 min and at 4 °C. 30 μL of this solution were injected in a Hewlett-Packard HPLC 1100 system (Palo Alto, CA, USA), onto a C_{30} YMC semipreparative column (5 μm, 250 mm x 10.0 mm) (Wilmington, NC, USA). The column was kept at 30 °C, and the flow rate was 2 mL/min. The diode array detector was set at 350, 286 and 450 nm for the detection of phytofluene, phytoene, and the other carotenoids, respectively. The elution method was isocratic with 60% MeOH and 40% MTBE. The stop time was 30 min. The drain tube from the detector was left open and the fractions of interest were collected in 50 mL plastic tubes. The purity of the phytofluene standard was verified by HPLC. 18 MΩ water was obtained from a MilliQ water purification system from Millipore (Brussels, Belgium). All reagents were of analytical grade or higher.

2.3. Extraction of carotenoids from juices

Samples were kept on ice and under dim light during the whole analysis. All samples were extracted in triplicates prepared on the same day. The procedure was based on that of Biehler, Kaulmann, Hoffmann, Krause, and Bohn (2011), with some modifications. Four mL of juice was weighed into a 50-mL plastic tube and 9 mL of a mixture of hexane:acetone (1:1, v/v) was added. This solution was shaken and sonicated for 5 min (Elmasonic Ultrasonic Bath 37 kHz). Then, the sample was centrifuged (Heraeus multifuge X3R, Thermo Scientific) at 2500 *g* (5 min, 4 °C). The upper organic phase was conveyed to a new 50-mL tube. The aqueous phase was extracted three more times with 4.5 mL of hexane and 2 mL of saturated aqueous sodium chloride solution and once more with 4 mL diethyl ether, and all organic phases were combined in the second tube, to which 5 mL of saturated sodium chloride were then added. Following shaking, sonication and centrifugation, the supernatant was transferred to another 50-mL tube. The extraction was repeated once more with 4.5 mL of hexane and the upper organic phases were combined. A 10 mL aliquot was taken and evaporated to dryness under a stream of nitrogen, in a TurboVapLV® apparatus (Caliper Life Sciences Benelux, Teralfene, Belgium) and at 25 °C. The extracts were kept at −80 °C under a nitrogen atmosphere until HPLC analyses (for no longer than one week).

2.4. In vitro digestion

The method chosen for the *in vitro* gastro-intestinal digestion was based on an earlier protocol (Corte-Real, Richling, Hoffmann, & Bohn, 2014), and is in brief described below. Five replicates of each juice were analysed and all samples were digested on the same day. The extractions of the carotenoids from the micellar phase were carried out the day following the digestion.

2.4.1. Gastric phase

Four milliliters of each juice were weighed in a dark amber polypropylene tube of 50 mL. Two milliliters of coffee creamer (10% fat) were added as a lipid source in order to increase carotenoid bioaccessibility as done previously (Biehler et al., 2011), and the mixture was homogenized gently by hand. Fifteen milliliters of physiological saline and 2 mL of pepsin solution (40 mg/mL in 0.01 M HCl, prepared the day of usage) were added. The pH was then adjusted to 3 by adding HCl (0.1 M). To simulate the gastric passage, the samples were incubated for 1 h at 37 °C, in a water

Table 1
Nutritional composition of the food samples subjected to gastro-intestinal digestion, as provided on the food label.

Carotenoid	Composition of the samples				
	Apricot juice[b]	Sanguinello juice[c]	Tomato juice[d]	Carrot juice	Coffee cream[e]
Composition	water, apricot puree, sugar, lemon juice concentrate, ascorbic acid	blood orange juice (1600 μg vit. C)	tomato juice concentrate, salt, citric acid	carrot juice, lemon juice	diluted milk (10% fat)
Fibre[a]	–	0.8	–	0.3	–
Protein[a]	<0.5 g	0.6	–	0.7	3.1
Salt[a]	0.0	0.0	0.3	0.05	0.08
Carbohydrate (sugar)[a]	11.8 (11.6)	10 (10)	–	6 (5)	4 (4)
Fat (saturated)[a]	<0.2 (<0.1)	–	–	–	10 (6.9)

– Indicating no data available.
[a] In g/100 mL.
[b] 40% min. of fruit.
[c] Flash pasteurized.
[d] From concentrate, pasteurized.
[e] Ultra-high-temperature processing.

bath with integrated shaker (GFL 1083 from VEL, Leuven, Belgium) with reciprocating motion at 100 strokes per minute (spm).

2.4.2. Intestinal phase

The procedure was the same than the one previously reported (Corte-Real et al., 2014), except that 9 mL of the mixture of pancreatin and bile extract were added instead of 4.5 mL. At the end of the digestion an aliquot of 12 mL of digesta was centrifuged at 4825 *g* (20 °C, 1 h). Then, 6 mL of aqueous micellar phase was taken with a syringe and a hypodermic needle from the middle phase and was filtered through a 0.2 μm Nylon membrane filter (Acrodisc 13 mm Syringe Filters; PALL Life Sciences, Ann Harbor, MI). Four milliliters of the filtered aqueous micellar phase were taken, flushed with nitrogen and stored at −80 °C until carotenoid extraction.

2.4.3. Extraction of carotenoids from micellar phase

The protocol by Corte-Real et al. (2014) with the following modifications was carried out: 4 mL aliquot of micellar phase was extracted and 8 mL of hexane/acetone (1:1, v/v) was used in each extraction instead of 2 mL and 4 mL, respectively. The dried extract was kept at −80 °C under a nitrogen atmosphere until HPLC analysis.

2.5. HPLC analysis

Extracts of carotenoids from each matrix and the micellar phase were analysed during 50 min. on an Agilent 1260 Infinity Preparative HPLC. An Accucore™ C_{30} column (2.6 μm, 100 mm × 2.1 mm) (Thermo Fischer Scientific Inc.) was used. The column was kept at 30 °C; the flow rate was of 0.2 mL/min; the injection volume was 8 μL; the HPLC autosampler was cooled down to 4 °C. The diode array detector was set at 286 nm for the detection of phytoene, at 350 nm for phytofluene, at 450 nm for β-carotene, ζ-carotene, lutein, and α-carotene, at 455 nm for β-cryptoxanthin, and at 470 nm for lycopene. The elution gradient was as follows: 0 min: 99% MeOH + 1% MTBE; 35 min: 96% MeOH + 4% MTBE; 40 min: 70% MeOH + 30% MTBE; 41 min: 99% MeOH + 1% MTBE.

To identify carotenoids their peak height ratios III/II (expressed as %) and their retention times were compared with those of available standards. In addition, the characteristic hypsochromic shifts of the absorption maxima of the *cis*-isomers and the intensity of their *cis*-peaks were taken into account (Britton, 1995b) for their tentative identification. For identifying phytofluene isomers, these characteristics were compared to those obtained by Meléndez-Martínez, Stinco, Liu, and Wang (2013). Carotenoid quantification was achieved by external standard calibration. Calibration curves were obtained from several standard mixtures of different concentrations. Standard mixtures contained phytoene, phytofluene, β-carotene, lycopene, β-cryptoxanthin and α-carotene. The concentration of each individual carotenoid of the standard mixtures was determined spectrophotometrically. The standard of isolated phytofluene generated four peaks, all of which were quantified together as a single peak area. Samples and standard mixtures were dissolved in MeOH:TBME (70:30) before injection.

2.6. Calculations and statistical analysis

The content of each isomer of each carotenoid in both the original matrices and in the micellar phases was calculated as a percentage of all isomers of the respective carotenoid. Differences in isomer percentage between the original matrix and the micellar phase were also calculated.

Bioaccessibility was calculated as the percentage of the content of a carotenoid determined in the micellar aqueous fraction after centrifugation and filtration in relation to the respective initial content in the food matrices.

Values are reported as the mean ± standard deviation (n = 3–5). Statistical analysis was performed using SPSS 22 software (SPSS Inc., Chicago, IL). Normality and homoscedasticity criteria were evaluated by Shapiro-Wilk and Levene tests, respectively. Unless otherwise stated, all statistical analyses were performed using parametric methods, i.e. ANOVA followed by Bonferroni *post hoc* test for all pairwise comparisons. Differences were considered statistically significant for P values ≤ 0.05 (2-sided).

3. Results

3.1. Carotenoid profile and total carotenoid content in juices

The concentrations of major carotenoids, i.e. phytoene, phytofluene, β-carotene, α-carotene, β-cryptoxanthin and lycopene, were quantified (Table 2). In addition, ζ-carotene and lutein were identified in carrot juice, and ζ-carotene was also found in tomato juice, but they were not quantified because of low concentration and unavailability of standard, respectively. Typical chromatograms of the juice extracts and retention times and wavelengths of maximum absorption of the carotenoids are depicted in the Supplementary material.

In apricot and carrot juices, the predominant carotenoid was β-carotene, while in tomato and sanguinello juices it was phytoene. Significant differences in the concentration of phytoene and phytofluene were found between all samples. The highest concentrations of both carotenoids were found in tomato juice, followed by carrot

Table 2
Carotenoid concentration of the food samples, in mg/100 g juice.

Carotenoid	Juice			
	Apricot	Sanguinello	Tomato	Carrot
Phytoene	0.35 ± 0.01^{c}	0.05 ± 0.00^{d}	2.24 ± 0.05^{a}	0.94 ± 0.06^{b}
Phytofluene	0.15 ± 0.00^{c}	0.01 ± 0.00^{d}	0.86 ± 0.02^{a}	0.59 ± 0.04^{b}
β-Carotene	0.45 ± 0.01^{b}	ND	0.23 ± 0.00^{b}	3.77 ± 0.79^{a}
α-Carotene	0.01 ± 0.00^{b}	ND	0.02 ± 0.00^{b}	1.70 ± 0.42^{a}
β-Cryptoxanthin	0.01 ± 0.00^{b}	0.02 ± 0.00^{a}	ND	ND
Lycopene	0.05 ± 0.00^{b}	ND	0.99 ± 0.14^{a}	OV
Total	1.02 ± 0.02^{c}	0.09 ± 0.00^{c}	4.34 ± 0.09^{b}	7.01 ± 1.29^{a}

Values within a row with different lowercase letters indicate statistically significant differences ($P < 0.05$). ND, Not detected. OV, Overlapping with other signals. Values represent means ± SD of 3 replicates.

juice, while the lowest concentration was found in sanguinello juice. The sum of all major carotenoids in the carrot juice was significantly higher ($P < 0.05$) than the one of tomato juice, and the latter showed a higher total carotenoid concentration compared to sanguinello and apricot juices. However, it should be considered that the apricot juice only contained 40% fruit.

3.2. Isomer identification and cis/trans ratios

Several isomers were identified in the juices. Up to four isomers of phytofluene were found in tomato and carrot juices, three isomers were detected in apricot juice, and one was found in sanguinello juice. All-*trans*-phytofluene was detected in all samples except for sanguinello juice. All-*trans* and 15-*cis* isomers of phytoene were detected in all samples, except for sanguinello juice, in which only the 15-*cis* isomer was found. In all juices, the dominant isomer was 15-*cis*-phytoene. Its concentration accounted, for all juices, for over 80% of the total phytoene concentration (Table 3). Concerning β-carotene, three isomers, i.e. 9-*cis*, 15-*cis* and all-*trans*, were detected in apricot and carrot juices, while in tomato juice only 9-*cis* and all-*trans* isomers were found. In this case, the major isomer was the all-*trans* one in all juices, representing between 83% (apricot juice) and 88% (carrot juice) of the overall isomers (Table 3). Regarding lycopene, only one *cis* isomer and the all-*trans* isomer were detected in the apricot juice, while in the tomato juice the all-*trans* and three *cis* isomers were detected, one of them being 5-*cis*-lycopene. One *cis* isomer of lycopene was detected also in carrot juice but it could not be quantified as it overlapped with another carotenoid. The percentage of the all-*trans*-isomer of total lycopene content was higher in tomato (82%) than in apricot (48%) (Table 3).

All isomers of the different carotenoids presented in the original matrices were also found in the micellar phases, except for the all-*trans*-phytoene in carrot juice, which was not detected in the micellar phase. In almost all cases, the percentage of *cis* isomers of each carotenoid present initially in the juices was slightly lower than the percentage found in the micellar phase (Table 3).

3.3. In vitro bioaccessibility

3.3.1. Differences within the juices

The carotenoid with the greatest bioaccessibility in all juices was phytoene ($P < 0.05$ in tomato and carrot juices), followed by phytofluene in almost all matrices (Table 4). The biggest difference was found in tomato juice, with phytoene being up to 4 times more bioaccessible than lycopene.

The high bioaccessibility of β-cryptoxanthin is remarkable. In apricot, β-cryptoxanthin showed a significantly higher bioaccessibility than all other carotenoids except for phytoene and phytofluene (for which the difference was not statistically significant).

3.3.2. Differences across the juices

Taking into account all carotenoids analysed, and also their isomers, the total bioaccessibility was 94% for sanguinello, 59% for apricot, 39% for tomato, and 24% for carrot (Table 4); resulting in a 3.9-fold higher bioaccessibility when comparing sanguinello juice to carrot juice. Data showed normal distribution, but homoscedasticity was not achieved in all cases, also not following square root or log-transformation, thus nonparametric tests were employed (Welch test and *post hoc* with T2-Tamhane test). The only differences between both tests were found in the case of colourless carotenoids in tomato and apricot juices, in which no statistically significant differences between them were detected with the nonparametric tests.

A wide range of bioaccessibility was found across samples (Table 4); lowest bioaccessibility was found in the tomato juice for lycopene (15%), the highest was found in sanguinello for phytoene (97%). Also, substantial differences were seen regarding the bioaccessibility of the colourless carotenoids between the different matrices.

3.3.3. Bioaccessibility of the different isomers

In general, the *cis* isomers of the different carotenoids presented a higher bioaccessibility than their all-*trans* counterparts (Table 5). Only the all-*trans*-phytoene and the all-*trans*-phytofluene in tomato juice had a slightly higher bioaccessibility than their *cis* counterparts. However, *cis* and all-*trans* isomers of phytofluene had nearly equal bioaccessibility in all juices. Among the isomers of phytofluene, only the two predominant ones were analysed as the other two were present at very low concentrations, and were difficult to quantify.

3.4. Accessible carotenoid content

To determine the food source most rich in carotenoids, the total bioaccessible amount (the potentially absorbable fraction) of the respective carotenoid from each were compared, thus both the total initial amount present in the juice and bioaccessibility were considered (Fig. 1), also taking into account isomers. As can be observed, the source providing the highest quantities of bioaccessible phytoene and phytofluene was tomato juice, followed by carrot, apricot and sanguinello juice. The same order was followed when considering all major carotenoids determined. The tomato juice yielded a 25-fold higher amount of bioaccessible carotenoids than the sanguinello juice.

4. Discussion

The present study focused on the bioaccessibility of the two understudied colourless carotenoids phytoene and phytofluene. Phytoene contents were higher than those of phytofluene in all juices, a common trend in food samples (reviewed in Meléndez-Martínez et al., 2015). Phytoene was even present at higher

Table 3
Percentage of *cis* isomers for each carotenoid in relation to the respective total carotenoid content, for both the original matrix and the micellar phase following simulated gastro-intestinal digestion.

Carotenoid		Juice			
		Apricot (%)	Sanguinello (%)	Tomato (%)	Carrot (%)
Phytoene	Juice	87.93 ± 0.43	100.00 ± 0.00	80.27 ± 1.09	97.91 ± 0.04
	micellar phase	94.33 ± 1.53	100.00 ± 0.00	79.76 ± 1.07	100.00 ± 0.00
	Difference	6.40*	0.00	-0.51	2.09*
Lycopene	Juice	51.97 ± 0.81	ND	18.36 ± 2.79	100.00 ± 0.00
	micellar phase	57.59 ± 3.74	ND	26.32 ± 3.74	100.00 ± 0.00
	Difference	5.62*	-	7.96*	0.00
β-Carotene	Juice	17.19 ± 0.03	ND	13.34 ± 0.08	15.13 ± 3.32
	micellar phase	20.24 ± 0.62	ND	13.27 ± 0.39	18.86 ± 1.30
	Difference	3.05*	-	-0.07	3.56
Phytofluene	Juice	41.59 ± 0.18	100 ± 0.00	50.69 ± 0.15	51.46 ± 0.18
	micellar phase	41.85 ± 0.87	100 ± 0.00	50.19 ± 0.35	53.13 ± 0.44
	Difference	0.23	0.00	-0.50	1.67*

ND, Not detected. –: not applicable. Values represent means ± SD of 3–5 replicates.
* Statistically significant differences between juice and micellar phase ($P < 0.05$).

Table 4
Total carotenoid bioaccessibility and bioaccessibility of each carotenoid, expressed as percentage, following gastro-intestinal digestion of the juices. All carotenoid isomers were taken into account.

Carotenoid	Juice			
	Apricot	Sanguinello	Tomato	Carrot
Phytoene	89.34 ± 15.96^{Aa}	96.84 ± 1.65^{Aa}	61.73 ± 4.92^{Ba}	31.83 ± 1.74^{Ca}
Phytofluene	64.25 ± 12.67^{Bbc}	94.52 ± 1.86^{Aab}	50.12 ± 5.43^{Cb}	26.72 ± 1.55^{Db}
β-Carotene	49.40 ± 8.11^{Ace}	ND	46.25 ± 6.58^{Abc}	19.38 ± 2.27^{Bc}
α-Carotene	47.73 ± 3.97^{Acde}	ND	19.93 ± 2.40^{Bd}	16.27 ± 0.66^{Bc}
β-Cryptoxanthin	73.32 ± 11.44^{Bab}	91.89 ± 1.30^{Ab}	ND	ND
Lycopene	32.57 ± 4.03^{Ae}	ND	15.28 ± 2.81^{Bd}	OV
Total	59.44 ± 9.03^{Bbc}	94.42 ± 1.16^{Ac}	38.66 ± 3.88^{Cb}	23.55 ± 1.31^{De}

Values within a column and within a row with different lowercase and uppercase letters respectively indicate statistically significant differences ($P < 0.05$). ND, Not detected. OV, Overlapping with other signals. Values represent means ± SD of 5 replicates.

Table 5
Bioaccessibility (%) of each isomer of each carotenoid, in each juice following simulated gastro-intestinal digestion.

Carotenoid		Juice			
		Apricot	Sanguinello	Tomato	Carrot
Phytoene	All-*trans*	41.96 ± 6.80	ND	66.25 ± 2.91	0.00 ± 0.00
	15-*cis*	75.28 ± 14.20	96.84 ± 1.65	54.80 ± 5.06	32.04 ± 1.74
Lycopene	All-*trans*	29.09 ± 2.25	ND	13.87 ± 3.07	ND
	5-*cis*	ND	ND	19.39 ± 2.49	ND
	Other-*cis*	36.88 ± 6.47	ND	26.79 ± 2.58	ND
β-Carotene	All-*trans*	47.61 ± 5.42	ND	46.13 ± 6.71	18.44 ± 2.45
	9-*cis*	57.03 ± 7.88	ND	47.08 ± 5.87	21.59 ± 1.83
	15-*cis*	59.23 ± 6.19	ND	ND	32.47 ± 1.67
Phytofluene	All-*trans*	64.10 ± 13.44	ND	50.66 ± 5.80	25.82 ± 1.72
	Other-*cis*	64.87 ± 11.73	90.40 ± 9.41	50.59 ± 5.31	27.63 ± 1.41

ND, Not detected. Values represent means ± SD of 5 replicates.

concentration than lycopene in the tomato juice. This is less common, though there are tomato varieties richer in phytoene than lycopene (Ishida & Chapman, 2012; Jeffery, Turner, & King, 2012).

All analysed carotenoids were previously found in human plasma (Meléndez-Martínez et al., 2013). To the best of our knowledge, only two studies (Jeffery, Turner, et al., 2012; Rodrigo, Cilla, Reyes, & Zacarías, 2015) reported bioaccessibility for phytoene, and only one study for phytofluene (Rodrigo et al., 2015). The results of these two studies are in line with those of the present investigation. It should be pointed out that the processing of juices greatly impacts the bioaccessibility of carotenoids (Stinco et al., 2012).

A remarkable finding is the rather high bioaccessibility for most carotenoids of the studied juices. Rodrigo et al., reported values between 1.8 and 17.7% in different citrus juices (Rodrigo et al., 2015). In this study, both phytoene and phytofluene were analysed, and one of the primary reasons for the higher bioaccessibility found in our work could have been the addition of coffee creamer, likely fostering the formation of mixed micelles required for solubilisation (Granado-Lorencio, Herrero-Barbudo, Blanco-Navarro, Pérez-Sacristán, & Olmedilla-Alonso, 2009; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). On the other hand, the matrices they analysed were different from our matrices and the digestion procedure was also different.

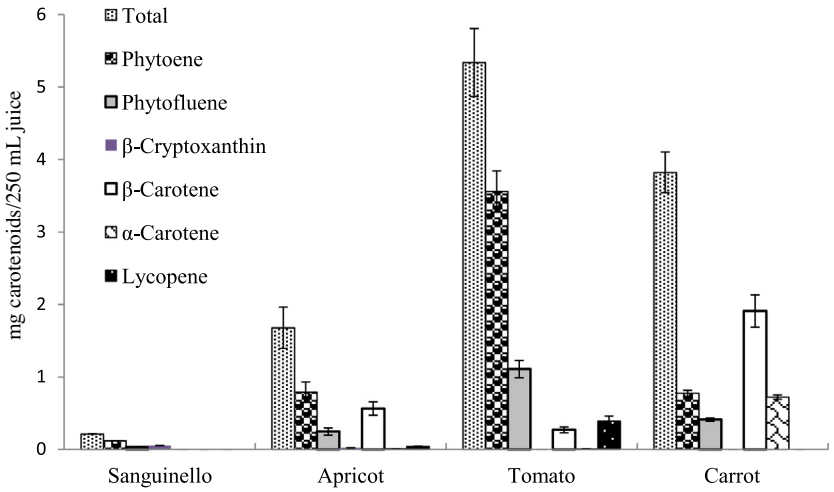

Fig. 1. Total *in vitro* bioaccessible content and *in vitro* bioaccessible content of each carotenoid for the investigated juices, expressed for a portion of juice (mg carotenoids/250 mL juice).

The large differences found in the total bioaccessibility between matrices, and the observation that every carotenoid followed the same trend across different matrices, highlights the importance of matrix-related factors on carotenoid bioaccessibility. However, it is important to note that only phytoene and phytofluene were present in all juices and that lycopene, α-carotene, β-carotene and β-cryptoxanthin were present only in some juices. The matrix-related factors include 1) thickness and type of the cell wall; 2) the organization and location of carotenoids in chromoplasts; and 3) the presence of other compounds that prevent or aid the release of carotenoids from the matrices (Borel, 2003; Jeffery, Holzenburg, & King, 2012; Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012). Among those factors, the composition and thickness of the cell wall appears to constitute one of the main structural barriers influencing carotenoid bioaccessibility (Palmero, Panozzo, Simatupang, Hendrickx, & Van Loey, 2014). Although this factor is more important in raw foods, this may also have some effect on the juices as some cells may remain intact even after juice processing (Gupta, Kopec, Schwartz, & Balasubramaniam, 2011). It has been shown that carrot have a smaller and more fibrous cell walls compared to tomato (Jeffery, Holzenburg, et al., 2012), which could be one of the reasons why tomato juice exhibited a higher total bioaccessibility than carrot juice. Moreover, it can also be hypothesized that the distribution of phytoene in the matrix such as in plastoglobules and in membranes of subcellular organelles (Lado et al., 2015; Nogueira, Mora, Enfissi, Bramley, & Fraser, 2013) contributes to its rapid release and high bioaccessibility compared to that of other carotenoids such as lycopene in tomato and β-carotene in carrot, which are present in a solid crystalline deposition form, reducing the solubility and bioaccessibility (Schweiggert et al., 2012; Zhou, Gugger, & Erdman, 1996). No information is present regarding the possible deposition form of phytofluene.

Though they were rather present at low concentration, differences in the amount of fat, sugars (increasing viscosity, (Dogaru et al., 2014)), and dietary fibre (entrapping carotenoids and decreasing their bioaccessibility), between the juices (Table 1) (Zhou et al., 1996), could have contributed to different carotenoid bioaccessibilities. Finally, various juice processing steps could also affect the release of carotenoids from the matrix. Tomato and sanguinello juices underwent a process of pasteurization and flash pasteurization, respectively. As indicated in a recent study (Aschoff, Rolke, et al., 2015) these processes can improve carotenoid bioaccessibility. Tomato juice was also made from concentrate, but whether this preparation impinges on carotenoid bioaccessibility is not known.

The bioaccessibility of different phytoene and phytofluene isomers was analysed for the first time. The more linear shape of the all-*trans* isomers makes them more likely to aggregate and crystallize than their *cis* counterparts, explaining why all-*trans* isomers have, in general, a lower bioaccessibility and bioavailability compared to their *cis* analogues (Britton, 1995; Failla, Chitchumroonchokchai, & Ishida, 2008; Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Pérez-Sacristán, et al., 2007; Ross et al., 2011; Stahl & Sies, 1996). In almost all cases, the percentage of *cis* isomers of each carotenoid that was present initially in the juices was slightly lower than that found in the micellar phase (Table 3), which could be either attributed to their greater bioaccessibility compared to their all-*trans* isomer counterparts (Table 5), or due to an additional *trans* → *cis* isomerization during digestion. These 2 aspects could not be distinguished in the present investigation. The higher bioaccessibility of the *cis* isomers was however, not remarkable in the case of phytofluene (Table 5).

Interestingly, phytoene and phytofluene had a higher bioaccessibility in all matrices (considering all isomers) (Table 4), which may be explained by the higher content of *cis* isomers of these carotenoids in the original matrix compared to other carotenoids (Table 3). Phytoene was found in all samples essentially as 15-*cis*-phytoene, and phytofluene as a mixture of *cis* isomers. However, the remainder of the carotenoids was found mainly in their all-*trans* form. The 15-*cis*-phytoene form is considered the predominant isomer in most carotenogenic organisms (Than, Bramley, Davies, & Rees, 1972) and there is evidence that some *cis* isomers of phytoene and phytofluene are more stable than their all-*trans* forms (Meléndez-Martínez, Paulino, Stinco, Mapelli-Brahm, & Wang, 2014).

Among the carotenoids analysed without terminal rings, phytoene was in all juices more bioaccessible than phytofluene, and both much more than lycopene (Table 4). These differences may be due to some extent to the differences in the number of conjugated double bonds (cdb). The higher number of cdb of lycopene (11 cdb) makes this carotenoid more linear and rigid than phytofluene (5 cdb) and phytoene (3 cdb). This increases the possibility of aggregation of molecules, which in turn can reduce bioaccessibility (Meléndez-Martínez et al., 2014). The difference found between the bioaccessibility of phytoene and phytofluene has

not been observed in *in vivo* studies in which the bioavailability of both carotenoids appeared comparable (Aust, Stahl, Sies, Tronnier, & Heinrich, 2005; Paetau et al., 1998). This fact could be due to a lower absorption efficiency of phytoene or to a greater degradation during its absorption. However, phytoene is known to be of greater stability than phytofluene (Nara, Hayashi, Kotake, Miyashita, & Nagao, 2009).

In addition to shape, hydrophilicy plays an important role. It has been observed that the lower the hydrophobicity of a carotenoid, the higher its transfer into the mixed micelles and the greater its bioaccessibility and bioavailability (Borel, 2003; Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Blanco-Navarro, et al., 2007; Sy et al., 2012; Tyssandier, Lyan, & Borel, 2001). In the present study, we observed an inverse relationship between hydrophobicity and bioaccessibility for all carotenoids, except for phytoene and phytofluene, indicating that the shape of the molecule may play a more important role than the hydrophobicity. While the order of hydrophobicity follows phytoene (log *P*: 13.38) > phytofluene (13.02) > lycopene (11.93) > α-carotene (11.17) > β-carotene (11.12) > β-cryptoxanthin (9.74), the order in bioaccessibility was slightly different (phytoene > phytofluene > β-cryptoxanthin > β-carotene > α-carotene > lycopene) in all juices analysed (Table 4).

Finally, there were marked differences in the concentrations of carotenoids in some sources, and this could also have had an impact on their bioaccessibility (Borel, 2003), such as a lower tendency to aggregate. Thus, the concentration of colourless carotenoids was higher in tomato juice, followed by carrot, apricot and sanguinello juices, while their bioaccessibility followed the opposite order, with the exception of the transposition of carrot and tomato (Tables 2 and 4).

To judge the contribution of each juice to the total daily intake of phytoene and phytofluene it is necessary to know the daily intake of each juice. The most approximate information on this was found in the work of Biehler et al. (2012), which however distinguished only between the consumption of vegetable and fruit juices. By using these data, the juice contributing most to the total daily intake of the colourless carotenoids is the tomato juice, providing 1.6 mg phytoene/day and 0.5 mg phytofluene/day, representing 79% and 70% respectively of the total intake of these carotenoids (Biehler et al., 2012). On the other hand, the juice with the lowest contribution was carrot juice, only providing 0.02 and 0.01 mg/day of phytoene and phytofluene, respectively. These results are strongly influenced by large differences between the daily intake of vegetable juices (6.9 g/day) and fruit juices (114.1 g/day). In addition, as stated before, the bioaccessible contents that would be obtained in this work if coffee creamer had not been added would surely be lower than those obtained. Nevertheless, it should be stipulated that adding dairy products to fruit and vegetable juices is a common practice in the food industry. In addition to fat, milk contains proteins, vitamins and minerals (World Cancer Research Fund & American Institute for Cancer, 2007) in a way that gives added nutritional value to the juices in which it is incorporated.

5. Conclusions

This study has shown that colourless carotenoids are found in significant amounts in various juices and that they may contain several isomers of phytofluene. Phytoene proved to be more bioaccessible than phytofluene, and both were in general more bioaccessible than other carotenoids present, i.e. β-cryptoxanthin, β-carotene, α-carotene, and lycopene. It can therefore be concluded that the investigated juices and nectar are good sources of phytoene and phytofluene, and constitute good alternatives to raw fruits and vegetables.

Although it is generally accepted that, there is an inverse relationship between hydrophobicity and bioaccessibility of carotenoids, this is not observed in the case of colourless carotenoids. This is one of the major findings of our study and indicates that their markedly different shape, as compared to other major dietary carotenoids, is a major factor governing their incorporation into mixed-micelles, perhaps especially for the *cis*-isomers. Due to their importance in the diet and their possible health benefits, further studies on this topic are warranted. These can produce new and valuable insights with potential applications for the industry such as in the area of functional foods.

Conflict of interest

All authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.074.

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