RESEARCH ARTICLE

# Effects of physicochemical properties of carotenoids on their bioaccessibility, intestinal cell uptake, and blood and tissue concentrations

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Received: January 19, 2012
Revised: May 15, 2012
Accepted: May 23, 2012

**Scope:** Carotenoid bioavailability is affected by numerous factors. Our aim was to assess the involvement of known carotenoid physicochemical properties (e.g., hydrophobicity, van der Waals volume, ...) on the transport of the main dietary carotenoids ($\beta$-carotene, lycopene, lutein, and astaxanthin, from their food matrix to their main storage tissues.

**Methods and results:** We used four complementary models: synthetic mixed micelles, an in vitro digestion procedure, Caco-2 cell monolayers, and a gavage experiment in rats. The efficiency with which pure carotenoids were incorporated into synthetic mixed micelles was related to their melting points ($r = 0.99$, $p = 0.015$). The efficiency with which pure carotenoids were transferred from dietary triglycerides into mixed micelles was related to carotenoid hydrophobicity ($r = -1$, $p = 0.005$). There was no relationship between the carotenoid physicochemical properties studied and their uptake efficiency by Caco-2. The postprandial plasma carotenoid response to carotenoid gavage was related to carotenoid hydrophobicity ($r = -0.99$, $p = 0.006$). Carotenoid adipose tissue response was not related to the carotenoid physicochemical properties studied.

**Conclusion:** Thus, carotenoid hydrophobicity is important for bioaccessibility and postprandial blood response of carotenoids. In contrast, the carotenoid physicochemical properties studied are apparently not strong determinants of carotenoid uptake by enterocytes and adipose tissue.

**Keywords:**
Bioavailability / Caco-2 / In vitro digestion model / Micelles / Rat

## 1 Introduction

The carotenoids are lipophilic pigments not biosynthesized by humans. About 40 of the 700–750 naturally occurring carotenoids are present in the human diet and are found in human blood and tissues. $\beta$-Carotene, α-carotene, and lycopene are the main carotenes (hydrocarbon carotenoids), while lutein, zeaxanthin, $\beta$-cryptoxanthin, and astaxanthin are the main xanthophylls (carotenoids with oxygen functions). Epidemiological studies suggest that the consumption of carotenoid-rich foods is beneficial to human health. The potential benefits of carotenoids for health [1, 2] were initially attributed to their antioxidant activity [3]. However, other putative protective mechanisms have also been reported, e.g., their effects on signaling pathways and gene expression [4], secretion of pro-inflammatory cytokines [5], cell proliferation and differentiation [1] and blue light damage to the eye [6].

The potential beneficial effects of carotenoids on health are probably linked to their blood and tissue concentrations, which are in turn related to their absorption efficiency and metabolism. The fraction of the dose entering the systemic circulation to participate in physiological functions (or stored for later use) is termed bioavailability. Carotenoid bioavailability depends on several critical steps. The first is the release of carotenoids from the food matrix, generally a fruit or vegetable matrix, and their transfer into mixed micelles during digestion. The percentage of the ingested dose transferred

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into mixed micelles is termed bioaccessibility. The second step is uptake by intestinal cells, recently shown to be facilitated by membrane proteins identified as cholesterol transporters with broad substrate specificities [7]. After absorption and during the postprandial period, carotenoids are incorporated into chylomicrons and transported to the liver. The liver is the main storage organ for carotenoids, and it also controls the distribution of carotenoids to other tissues. Carotenoids are secreted by the liver in VLDL and are subsequently recovered in other lipoproteins, i.e., LDL and HDL [8]. Carotenoids are found in several tissues [9–11], but the mechanisms by which they are distributed to tissues are not accurately known, although lipoprotein receptors and membrane proteins [12] are assumed to be involved.

Carotenoid bioavailability is assumed to be influenced by a complex set of factors represented by the acronym SLAMENGHI [13]. Among these factors, the food matrix is assumed to play a major role. However, as all the studies on carotenoid bioavailability have used carotenoids in food matrices or in supplements, and no study has ever compared the bioavailability of pure carotenoids, we do not know whether the observed differences in bioaccessibility, absorption efficiency, and tissue distribution of different carotenoids are due to intrinsic physicochemical properties of carotenoids, matrix effects, or both.

The aim of this study was thus to assess the involvement of known physicochemical properties of carotenoids, e.g., hydrophobicity, molar volume, surface tension, density, etc., on their relative absorption and their accumulation in the blood and main storage tissues, namely the liver [10] and adipose tissue [14]. To this end, we compared efficiency of incorporation into synthetic micelles, bioaccessibility (of both pure carotenoid incorporated in triglycerides and carotenoids naturally incorporated in a food matrix), efficiency of uptake by intestinal cells and tissue distribution of two carotenes ($\beta$-carotene and lycopene) and two xanthophylls (lutein and astaxanthin), to determine whether the values obtained were correlated with known physicochemical properties of carotenoids.

## 2 Materials and methods

### 2.1 Supplies and chemicals

Foods without antioxidants, acidifiers, or preservatives were purchased from a local supermarket: canned steamed carrots processed with sugar, salt, and water (D'Aucy Compagnie Générale de Conserve, Vannes, France), canned peeled Roma tomatoes processed in their juice (own brand, Auchan distributor Villeneuve d'Ascq, France), canned chopped spinach boiled and processed with salt and water (D'Aucy), and salted vacuum-packed Norwegian smoked salmon, (own brand, Auchan distributor). (All-*E*)-$\beta$-carotene (>95%) and (all-*E*)-lutein (>98%) were kindly provided by DSM LTD (Basel, Switzerland). (All-*E*)-lycopene (>90%) was isolated from tomato oleoresin and supplied by Conesa Conservas Vegetales de Extremadura SA (Villafranco del Guadiana–Badajoz, Spain). (All-*E*)-astaxanthin (>98%), salts ($NaHCO_3$, NaCl, KCl, $CaCl_2.2H_2O$, and $K_2HPO_4$), mucin, α-amylase, pepsin, porcine pancreatin, porcine bile extract, 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine), monoolein, free cholesterol, oleic acid, sodium taurocholate, pyrogallol (used as antioxidant preservative), and apo-8′-carotenal (>95%, used as internal standard to calculate recovery yield during carotenoid extraction) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Fetal bovine serum (FBS) was purchased from Biomedia (Issy-les-Moulineaux, France). Dulbecco's modified eagle medium (DMEM) containing 4.5 g/L glucose and trypsin/EDTA (500 and 200 mg/L, respectively), nonessential amino acids, penicillin/streptomycin, PBS, and PBS containing 0.1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ (PBSCM) were from Invitrogen (Cergy-Pontoise, France). Hexane, methanol, dichloromethane, and methyl-*t*-butylether were of HPLC grade and were purchased from Carlo Erba Reactifs SDS (Val-de-Reuil, France). The small kit of the Uptima bicinchoninic acid (BCA) assay for protein quantification was from Interchim (Montluçon, France).

### 2.2 Test meals used in the in vitro digestion experiments for assessing carotenoid bioaccessibility

Experiments were carried out twice for each carotenoid: (i) with pure substances to measure the intrinsic bioaccessibility of carotenoids (i.e., without the effect of the food matrix), and (ii) with foods rich in each carotenoid studied. For experiments with pure carotenoids, stock carotenoid solutions in hexane were dispersed in commercial groundnut oil, which contained no quantifiable amounts of carotenoids as checked by HPLC, and the solvent was evaporated to obtain concentrations of ca. 750 μmol/L in oil. The first digestions were conducted on the oil alone, while for experiments with foods, the food sources of carotenoids were codigested with a standard meal (Table 1). This was done to approach nutritional conditions where carotenoid sources are usually ingested together with other foods. Foods were chosen such that (i) they contained no detectable amounts of carotenoids (checked by HPLC), and (ii) their mix gave a meal macronutrient composition (lipids, carbohydrates, and proteins) close to US recommended dietary intake of macronutrients (see legend to Table 1). Potatoes were boiled in tap water, peeled, and handpureed. Meat was fried in a frying pan without added fat. Potato purée and fried meat were divided into aliquots and frozen at −20°C. These food sources of carotenoids were chosen for their high amounts of the main carotenoid of interest: carrot purée for $\beta$-carotene, tomato pulp for lycopene, chopped spinach for lutein, and salmon for astaxanthin

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**Table 1.** Composition of the test meals used in the in vitro digestion experiments performed to measure carotenoid bioaccessibility

| Experimental conditions | Components | Amounts[a)] (g) |
|---|---|---|
| Pure carotenoids | Groundnut oil (containing ca. 40 μg pure carotenoids) | 0.1 ± 0.02 |
| | NaCl 0.9% | 32.0 ± 0.01 |
| Carotenoid-rich foods | Potato pulp | 6.7 ± 0.02 |
| | Beef meat | 1.2 ± 0.02 |
| | Olive oil | 0.1 ± 0.02 |
| | Carotenoid-rich food | 4.0 ± 0.02[b)] |
| | NaCl 0.9% | 32.0 ± 0.01 |

The test meal without the carotenoid-rich food contained 64.5% energy as carbohydrates, 14.5% as fat, and 21.0% as proteins. When, e.g., 4 g tomato pulp was added as the lycopene source, the meal contained 66.8% energy as carbohydrates, 13.2% as fat, and 20% as proteins (the macronutrient composition and percent energy were estimated using dietary software, Nutrilog 1.3, Nutrilog SAS, Marans, France). These proportions are close to US recommended dietary intake of macronutrients, namely 45–65% carbohydrates, 20–35% fat, and 10–35% proteins.
a) Means ± SD of the amounts added in three experiments.
b) The detailed carotenoid content of each test meal is reported in Table 2.

(Table 2). Foods were used immediately after unpacking and cut into pieces of similar size to minimize variability due to food preparation.

### 2.3 In vitro digestion experiments to assess carotenoid bioaccessibility

We used the in vitro digestion model developed by Garrett et al. [15]. The experimental conditions were slightly modified [16] to take better account of the physicochemical conditions prevailing in the human gastrointestinal lumen [17] and to reduce as much as possible the amount of triglycerides left at the end of the digestion experiments. A preliminary experiment had shown that if the digestion period is too short, residual triglycerides trap a fraction of carotenoids, leading to underestimation of carotenoid bioaccessibility. Since almost all triglycerides are hydrolyzed in vivo [18], it was important to avoid this artifact by ensuring almost complete hydrolysis by the end of the in vitro digestion. Meal components, or only groundnut oil when the bioaccessibility of carotenoids was studied without the effect of the food matrix (Table 1), were mixed with 32 mL of 0.9% NaCl in twice-distilled water and homogenized in a shaking water bath at 37°C for 10 min. Then 2.5 mL of artificial saliva [19] ($NaHCO_3$ 62 mmol/L, NaCl 15.1 mmol/L, KCl 6.4 mmol/L, $CaCl_2.2H_2O$ 3 mmol/L, $K_2HPO_4$ 6 mmol/L, mucin 2.16 g/L, and α-amylase 13 g/L) was added and the medium was blended (Ultra-Turrax IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 5000 rpm for 30 s to mimic chewing and incubated at 37°C for a further 10 min. The next steps in the in vitro digestion procedure were similar to those described previously [16]. Each experiment with the different carotenoid sources was run in triplicate.

Natural mixed micelles produced during the in vitro digestion were separated from oil droplets and food particles by centrifuging (2500 × *g*, for 1 h at 10°C in a Jouan GR412-W rotor, (Saint-Herblain, France), followed by filtration (0.8 and then 0.22 μm filter with Millex®-GS membrane, Millipore MF, Cork, Ireland). A control experiment had shown that this separation method isolated the same particle size range as ultracentrifuging (20 000 rpm, 18 h) followed by filtration at 0.22 μm [16]. Aliquots were stored at −80°C under a blanket of nitrogen until either the tests of intestinal cell uptake in Caco-2 cells or the HPLC analysis of carotenoids.

**Table 2.** Carotenoid content of the test meals used in the in vitro digestion experiments performed to measure carotenoid bioaccessibility

| Carotenoid tested | | Carotenoid source | Amount of carotenoids added in the in vitro digestion experiments[a)] (μg) |
|---|---|---|---|
| Pure carotenoids | β-Carotene | (All-*E*)-β-carotene > 95%, synthetic | 40 |
| | Lycopene | (All-*E*)-lycopene > 90%, natural | 40 |
| | Lutein | (All-*E*)-lutein > 98%, synthetic | 40 |
| | Astaxanthin | (All-*E*)-astaxanthin > 98%, synthetic | 40 |
| Carotenoids in commercial foods | β-Carotene | Carrot puree | 44.2 ± 5.8[b)] |
| | Lycopene | Tomato pulp | 29.9 ± 5.5[b)] |
| | Lutein | Chopped spinach | 215.1 ± 1.0[b)] |
| | Astaxanthin | Smoked salmon | 35.9 ± 5.5[b)] |

a) Means ± SD of three measurements.
b) Amounts of only the main carotenoids found in these foods, but note carrots also contain α-carotene, tomato also contains neurosporene and β-carotene, spinach also contains zeaxanthin and β-carotene, and salmon also contains canthaxanthin.

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### 2.4 Determination of the intrinsic ability of carotenoids to be incorporated into synthetic mixed micelles

Synthetic mixed micelles were prepared as previously described [20] to mimic the lipid composition of the mixed micelles recovered in the human duodenum during digestion [18, 21]. Appropriate volumes of stock trichloromethane solutions of the following compounds were transferred to glass flasks to obtain the following final concentrations: 0.04 mmol/L phosphatidylcholine, 0.16 mmol/L lysophosphatidylcholine, 0.3 mmol/L monoolein, 0.1 mmol/L free cholesterol, 0.5 mmol/L oleic acid. Mixed micelle size was measured by photon correlation spectroscopy (Zetasizer Nano Zs, Malvern Instruments, Malvern, UK). The results showed that only one particle size range was present. Its mean hydrodynamic radius was around 3 nm, close to the size observed for micelles in vivo, i.e., between 3 and 6 nm [22].

To measure the maximum amount of carotenoid that could be incorporated into a fixed amount of synthetic mixed micelles, an appropriate volume of stock solution providing an excess of pure carotenoids (50 nmol) was mixed with appropriate volumes of stock solutions of mixed micelle lipid components. The solvent was then evaporated under nitrogen and the residue homogenized in 10 mL of a solution of 0.9% NaCl and 5 mmol/L taurocholate in twice-distilled water. Synthetic mixed micelles were then formed by sonication as previously described [20]. The 0.22 μm filtered solutions were stored at −80°C until the percentage of carotenoid incorporated into synthetic mixed micelles was measured as described below.

To measure the transfer of carotenoids from their solid form into synthetic mixed micelles solubilized in water, an aqueous solution (2 mL) of carotenoid-free synthetic mixed micelles in 0.9% NaCl and 5 mmol/L taurocholate was poured onto 10 nmol of dry carotenoid powder in a glass tube. After a 10-s sonication to disperse the carotenoid powder into the aqueous solution of synthetic micelles, the transfer of the carotenoids into the synthetic mixed micelles was followed for 6 h. This was done under moderate stirring (250 rpm) at 37°C and protection from light. Every 30 min, a sample of the aqueous medium was collected and filtered through a 0.22 μm filter (same reference as above). The amount of carotenoids incorporated into the synthetic mixed micelles was measured as described below.

### 2.5 Cell culture

Caco-2 clone TC-7 cells were a gift from Dr. M. Rousset (UMR S872, Paris, France). Cells, passages 25-80, were grown as previously described [23]. Cells were incubated at 37°C in a humidified atmosphere of air:$CO_2$ (90:10, v/v) and the medium was changed every 48 h. Monolayers were subcultured with a 4-day passage frequency, when they reached a confluence of approximately 80%, by treatment with 25% trypsin/EDTA.

### 2.6 In vitro carotenoid uptake by Caco-2 cell monolayers

At the beginning of each experiment, cell monolayers, seeded and grown on Transwells as previously described [20], were washed twice with 1 mL PBS. Before each experiment, the integrity of the cell monolayers was checked by measuring transepithelial electrical resistance with a voltohmmeter equipped with a chopstick electrode (Millicell ERS; Millipore, Saint-Quentin-en-Yvelines, France). The apical side of the cell monolayers received 1 mL of carotenoid-rich natural mixed micelles, i.e., mixed micelles from the in vitro digestion experiments, diluted one-fifth in DMEM. Micelles were diluted because previous experiments had shown that concentrations of bile salts in nondiluted micelles lysed the cells. The basolateral side received 2 mL of normal medium. Cell monolayers were incubated at 37°C for 3 h. The incubation time was chosen from preliminary experiments so as to obtain sufficient amounts of absorbed carotenoids for accurate measurements. After the incubation period, media from each side of the membrane were harvested. Cell monolayers were washed twice with 1 mL of PBS to eliminate adsorbed carotenoids, scraped, and collected in 500 μL of PBS. All the samples were stored at −80°C under nitrogen until carotenoid extraction and HPLC analysis.

### 2.7 Measurement of carotenoid bioavailability in an animal model

The animal model used to study carotenoid bioavailability was a multiple-dose gavage experiment lasting 3 days in young (6-week-old) male albino Wistar rats of normal weight (about 200 g). The experiments complied with animal ethics rules and were approved by the local ethical committee of Aix-Marseille University. Five groups of eight rats were included in the study: a control group was force-fed with 1 mL of groundnut oil without carotenoids and four groups were force-fed with the same amount of oil containing pure carotenoids ($\beta$-carotene, lycopene, lutein, or astaxanthin).

The protocol was as follows: after 1-week acclimatizing, eight rats randomly chosen among the 40 were kept without food the night before blood collection. A blood sample (600 μL) collected by intracardiac venipuncture was used as control (baseline of plasma carotenoid concentrations). Gavage was performed each day over 3 days with pure carotenoids incorporated in 1 mL of groundnut oil. The rats had free access to standard chow diet and water until the evening before the last day of the gavage experiments. Pure carotenoids were incorporated in oil as follows: carotenoids and oil were dissolved in hexane solutions, mixed, and the solvent was evaporated under nitrogen. The doses of carotenoids

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incorporated in oil and given to the rats were chosen so as to provide 55 nmol carotenoids/rat/day, corresponding to 0.15 mg carotenoid/kg/day. The third gavage experiment was carried out on fasting rats (no access to food the night before). Just before this last gavage, an intracardiac venipuncture was performed on anaesthetized rats to evaluate the effect of the 2-day gavage with carotenoids on fasting plasma carotenoid concentrations. The third gavage was then performed and the rats were killed exactly 4 h later, i.e., at the time of peak blood concentration of carotenoids [10]. Blood and tissue samples (liver and adipose tissue) were collected to evaluate (i) the effect of a meal rich in carotenoids on the increase in postprandial plasma concentration of carotenoids, which is an acceptable estimate of carotenoid absorption efficiency, and (ii) the effect of a 3-day gavage with carotenoids on carotenoid concentration in their main storage tissues (adipose tissue and liver). For the adipose tissue, samples were parts of the epididymal adipose tissue, which is easily collected and a good model of the global adipose tissue in rats. All the tissue samples were immediately immersed in liquid nitrogen and kept at −80°C until carotenoid analysis.

### 2.8 Extraction of carotenoids from the various in vitro and in vivo samples

The procedure was adapted to the diverse types of samples (aqueous fractions from the in vitro digestion experiments and from Caco-2 experiments, or animal tissues).

#### 2.8.1 Extractions from digesta, micellar fractions, and from Caco-2 experiment fractions

In a glass tube, 500 μL of internal standard (apo-8′-carotenal in ethanol, used as internal standard to calculate recovery yield during carotenoid extraction) and 2 mL of hexane were added to 500 μL of sample. The mixture was homogenized for 5 min in a vortex blender and then centrifuged (1200 × *g*, for 5 min at 4°C in a Jouan GR412-W rotor). The upper phase was collected and 2 mL dichloromethane added to the lower phase. After a further 5 min homogenization and 5 min centrifugation, the lower phase was collected, pooled with the previously collected volume and evaporated under nitrogen.

#### 2.8.2 Extractions from plasma samples

The diverse blood samples from rats, collected in tubes with heparin, were immediately centrifuged to separate the plasma, which was then placed in liquid nitrogen and kept at −80°C. Extractions were then conducted as described above for the in vitro samples.

#### 2.8.3 Extractions from liver and adipose tissue samples

About 150 mg was mixed with 300 μL of PBS buffer and crushed with two 3-mm diameter stainless steel balls in 2 mL Eppendorf tubes using an MM301 ball mill (Retsch, Eragny-sur-Oise, France). A 50-μL aliquot was collected for protein assay. Another 250-μL aliquot was transferred into a glass tube, mixed with 150 μL of PBS, 500 μL of trichloromethane, and 1 mL of methanol. After 5 min homogenization in a vortex blender, 500 μL of trichloromethane and 500 μL of distilled water were added. The mixture was homogenized for 1 min in a vortex blender and centrifuged (1200 × *g*, 10 min, 10°C). The lower phase was collected and evaporated to dryness under nitrogen. The subsequent procedure depended on the tissue.

For liver samples, the dry residue was taken up in 500 μL of distilled water, 500 μL of internal standard (apo-8′-carotenal in ethanol), and 2 mL of hexane. The mixture was then homogenized for 1 min on a vortex blender and centrifuged (1200 × *g*, 5 min, 4°C). The upper phase was collected and 2 mL of dichloromethane was added to the lower phase. After a further 1 min homogenization and 10 min centrifugation, the lower phase was collected, pooled with the previously collected volume and evaporated under nitrogen.

For adipose tissue samples, the dry residue was taken up in 1 mL of ethanol-KOH (5.5%, m/v) and 100 μL of freshly prepared ethanol-pyrogallol (1.2%, m/v). The mixture was incubated at 37°C with stirring for saponification of the lipid esters from the adipose tissue. After 90-min incubation, 1 mL of distilled water, 250 μL of internal standard (apo-8′-carotenal in ethanol), and 3 mL of hexane were added. The mixture was homogenized for 1 min on a vortex blender and centrifuged (1200 × *g*, 10 min, 4°C). The upper phase was collected and rinsed with 1 mL of distilled water and 1 mL of ethanol. After a further 1 min homogenization and 5-min centrifugation, the upper phase was collected and evaporated under nitrogen.

### 2.9 HPLC analysis of the carotenoids

After evaporation to dryness, all the dried extracts were dissolved in 200 μL of methanol:dichloromethane (65:35, v/v). Carotenoids were quantified, as recently described [24], by reverse-phase HPLC on a Dionex system (equipped with in-line degasser, a P680 pump, a cooled automatic sample injector ASI-100, and a UV/visible diode-array detector UVD340U, Dionex France, Voisins-le-Bretonneux, France). Carotenoids and apo-8′-carotenal (used as internal standard to calculate recovery yield during carotenoid extraction) were detected at their maximum absorption wavelength (445 nm for lutein, 455 nm for $\beta$-carotene, 470 nm for lycopene and astaxanthin, 454 nm for apo-8′-carotenal). They were identified by their retention times and UV-visible spectra (from 300 to 550 nm) against pure standards. Quantification was performed using

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Dionex Chromeleon software (Dionex Chromatography Management system, version 6.80).

### 2.10 Protein assay on the tissue samples from the in vivo study

Tissue proteins were titrated by the BCA assay on 96-well microplates. The 50-μL aliquots of liver samples and adipose tissue samples were diluted, respectively, to 1/200 and 1/15 in PBS buffer before titration.

### 2.11 RNA isolation from rat adipose tissue and qPCR

Total cellular RNA was extracted from control rat liver and epididymal fat pads using TRIzol reagent according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA using random primers and Moloney murine leukemia virus reverse transcriptase [5]. Real-time quantitative RT-PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA) as previously described [25]. Expression of Scavenger Receptor class B type I (SR-BI) and Cluster Determinant 36 (CD36) membrane transporters was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold method. Data were expressed as relative expression ratio.

### 2.12 Calculations and statistics

All the in vitro experiments were run in triplicate. Bioaccessibility was defined as the percentage of carotenoids recovered in the micellar fraction after in vitro digestion, relative to the amount of carotenoids measured in the digestive medium just before addition of artificial saliva. The solubility of the carotenoids in synthetic mixed micelles was defined as the percentage of carotenoids recovered in the micellar fraction, relative to the total carotenoids present in the medium. Uptake efficiency of the carotenoids was defined as the percentage of carotenoids recovered in scraped Caco-2 cells, relative to the amount of carotenoids initially added on the apical sides of the cell monolayers. Results were expressed as means and standard deviations (SDs). Differences between means were assessed using ANOVA followed by the post hoc Tukey–Kramer test for parametric data (in vitro results). In the case of nonparametric data (in vivo results), they were assessed using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test when the Kruskal–Wallis test showed significant differences between groups. The relationships between two variables were assessed by both linear regression analyses (Pearson's parametric test) and Kendall's tau (τ) coefficient (nonparametric hypothesis test). All $p$ values under 0.05 (two-sided) were considered significant. Statistical comparisons were performed using StatView software, version 5.0 (SAS Institute Inc., Cary, NC, USA).

## 3 Results

### 3.1 Bioaccessibility of carotenoids as measured by the in vitro digestion model

The prolonged two-step digestion procedure (gastric digestion for 1 h and duodenal digestion for 2 h, i.e., the longest durations reported in previous similar procedures [26]), along with the chosen concentration of oil (100 mg in 50 mL digestion medium) gave about 90% lipolysis at the end of the process (lipolysis was estimated by the mass of remaining lipids extracted by the method of Folch). We note that not all the carotenoids were recovered at the end of the digestion experiments. This is probably because a fraction was degraded to metabolites. We observed new peaks, suggesting the appearance of these metabolites in the HPLC chromatograms. However, we had no standard substances to identify them. It is also possible that a fraction of carotenoids was adsorbed on the labware. Lutein and astaxanthin were the most extensively recovered, with 87 ± 2% and 93 ± 3% of the initial content detected at the end of digestion, respectively. The residual amount of $\beta$-carotene was 69 ± 1%. Lycopene was the least abundantly recovered, with only 57 ± 4% detected at the end of the digestion experiment.

The two pure xanthophylls, lutein and astaxanthin, had similar bioaccessibilities, with about 50% of the initial carotenoid content recovered in the natural mixed micelles fraction (Fig. 1). The two pure carotenes, $\beta$-carotene and lycopene, were, respectively, about 4 and 16 times less bioaccessible than the two xanthophylls. Chopped spinach lutein was the most bioaccessible food carotenoid, followed by carrot purée $\beta$-carotene, smoked salmon astaxanthin, and tomato pulp lycopene (Fig. 1). Compared with pure carotenoids, $\beta$-carotene and lutein from the whole vegetables were transferred into natural mixed micelles in similar proportions. Lycopene was again the least micellarized carotenoid. Also, its bioaccessibility from tomato pulp was about half, although nonsignificantly different, that observed in the absence of the food matrix. The result for astaxanthin was the most surprising: smoked salmon astaxanthin was more than seven times less bioaccessible than food-matrix-free astaxanthin.

### 3.2 Incorporation of pure carotenoids into synthetic mixed micelles

When homogenized with phospholipids, cholesterol and glycerol esters (mixed micelle lipids) before the formation of synthetic mixed micelles, $\beta$-carotene, lutein, and astaxanthin were efficiently incorporated into synthetic mixed micelles (Table 3). Lutein showed a particularly high incorporation efficiency (about 89%), followed by $\beta$-carotene and

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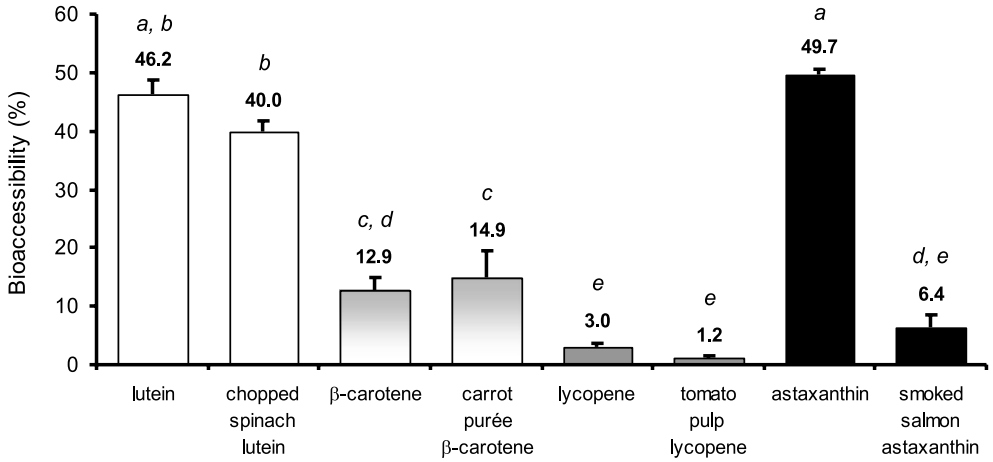


**Figure 1.** Bioaccessibility of pure and food carotenoids using the in vitro digestion model. Means ± SD of three independent experiments. Different letters indicate significant ($p < 0.05$) differences between means (ANOVA and Tukey–Kramer test).

astaxanthin. Lycopene was by far the least efficiently incorporated (2–3%).

The concentration versus time course of the transfer of pure carotenoids from carotenoid powders to preformed synthetic mixed micelles in water is shown in Fig. 2. In all cases, the maximum solubilization rates were nearly reached in the first 30–60 min. The maximum transfer of carotenoids measured at the plateau revealed that $\beta$-carotene and lutein were significantly more abundantly transferred from powder into synthetic mixed micelles (ca. 8%) than astaxanthin and lycopene (<1%). Also, maximum percentage values of carotenoids transferred into synthetic mixed micelles were much lower than for the incorporation of carotenoids during the preparation of synthetic mixed micelles (Table 3).

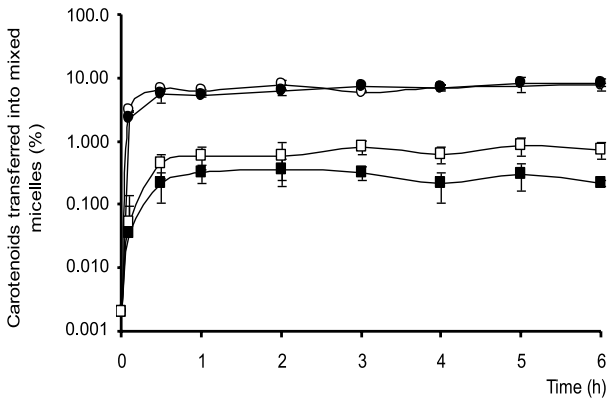


**Figure 2.** Amount of pure carotenoids transferred from carotenoid powders to synthetic mixed micelles solubilized in water as a function of time. Pure (○) lutein, (●) $\beta$-carotene, (□) astaxanthin, and (■) lycopene. Experiments performed protected from light at 37°C. Means ± SD of three independent experiments. Different letters indicate significant ($p < 0.05$) differences between means (ANOVA and Tukey–Kramer test).

### 3.3 Uptake of carotenoids by Caco-2 cells

The intestinal epithelium model was incubated with carotenoid-rich natural mixed micelles coming from the in vitro digestions of the test meals and the uptake efficiencies of the four carotenoids were compared. We note that no carotenoid was detected in the basolateral media. This was not surprising as it is well known that Caco-2 hardly secretes any lipoproteins, which carry carotenoids in the basolateral medium. We also note that the $\beta$-carotene metabolites, retinol and retinyl palmitate, were not detected in the cells, suggesting that the incubation conditions did not allow

**Table 3.** Maximum proportions of carotenoids incorporated into synthetic mixed micelles or transferred from their solid form to preformed synthetic mixed micelles solubilized in water

| | Lutein | $\beta$-Carotene | Lycopene | Astaxanthin |
|---|---|---|---|---|
| Incorporation efficiency (%)[a)] | 89.3 ± 3.2[a] | 48.6 ± 3.2[b] | 2.5 ± 0.7[c] | 37.2 ± 2.5[b] |
| Transfer efficiency (%)[b)] | 8.1 ± 1.3[a] | 8.3 ± 2.2[a] | 0.4 ± 0.2[b] | 0.9 ± 0.3[b] |

a) Maximum proportion of carotenoids incorporated into synthetic mixed micelles during their preparation (mixture of lipids and carotenoids followed by sonication).
b) Maximum proportion of pure carotenoids transferred from their solid form into synthetic mixed micelles solubilized in water during concentration versus time experiments (see Fig. 2). Values are means ± SD of three experiments. For each row, different letters indicate significant differences ($p < 0.05$) between means as assessed by Kruskal–Wallis and Mann–Whitney *U*-test.

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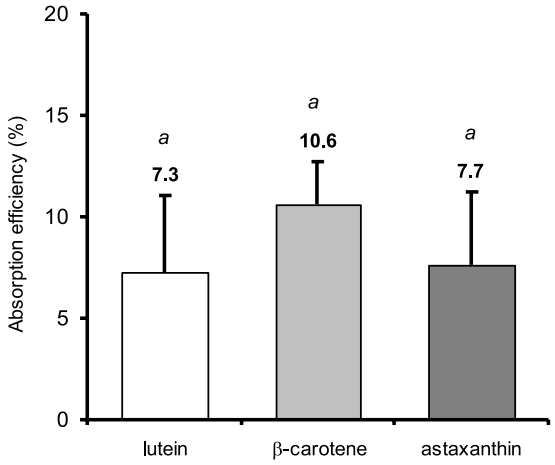


**Figure 3.** Uptake, by Caco-2 cell monolayers, of carotenoids incorporated in mixed micelles that came from in vitro digestion experiments of test meals containing food sources of carotenoids, e.g., chopped spinach for lutein, carrot purée for $\beta$-carotene, and smoked salmon for astaxanthin. Uptake efficiency was expressed as percent of the carotenoid amount added onto cell monolayers. Means $\pm$ SD of three independent experiments. Similar letters indicate nonsignificant ($p < 0.05$) differences between means (ANOVA).

$\beta,\beta$-carotene-15,15′-monooxygenase [27–29] to significantly cleave $\beta$-carotene.

All four carotenoids were taken up (Fig. 3). However, lycopene was hardly detected in Caco-2 cells (0.5 ng/well). This was probably because of its poor bioaccessibility, as observed in Fig. 1, which resulted in very low concentrations in natural mixed micelles. Thus, its uptake efficiency showed a high SD and was not shown in the figure. The three other carotenoids had close, nonsignificantly different uptake efficiencies (7–11%).

### 3.4 Blood and tissue carotenoid responses (changes from initial concentrations) to carotenoid gavage in rats

No carotenoids were detected either in rat plasma before the gavage administration, or in plasma and tissues of rats that were force-fed with carotenoid-free oil (data not shown). Concentrations of carotenoids in plasma and tissues of the other groups after a 2- or 3-day gavage are shown in Table 4. We note that retinyl palmitate, the main metabolite produced by $\beta,\beta$-carotene-15,15′-monooxygenase from provitamin A carotenoids, was detected in plasma and liver, and that no significant difference was observed among the diverse groups of rats, before or after gavage with the carotenoid-enriched oils. After the 2-day gavage, the amounts of carotenoids in the plasma of rats (deprived of food the night before blood withdrawal) were around 1–2 nmol/L, except for lutein (16 nmol/L). Plasma carotenoid levels were higher after the 3-day gavage in the postprandial period (4 h after the gavage administration) than at fast after the 2-day gavage period, except in the case of lutein, which exhibited similar levels. The concentration of lycopene in the liver was clearly higher than that of the three other carotenoids. Carotenoid concentration in adipose tissue was maximum with lutein and minimal with astaxanthin. Figure 4 shows the total amounts of carotenoids found in each compartment studied. The most noteworthy findings were the very large amounts of lycopene in the liver and the large amounts of lutein in the adipose tissue. Figure 4 also gives the relative proportions of each carotenoid in plasma and both tissues 4 h after the last gavage. In all cases except astaxanthin, a minor fraction of the carotenoids was recovered in plasma. About half of the lutein was recovered in the liver and about one-third in the adipose tissue, whereas the two carotenes ($\beta$-carotene and lycopene) were found mostly (ca. 90%) in the liver.

### 3.5 Relative expression of SR-BI and CD36 rat adipose tissue and liver

The expression of SR-BI and CD36 was measured in rats not force-fed with groundnut oil. The results showed that CD36 was much more strongly expressed in adipose tissue than in the liver (about 45 times more). By contrast, SR-BI was more strongly expressed in the liver than in adipose tissue (about five times more) (data not shown).

## 4 Discussion

It is assumed that the second key factor that governs carotenoid absorption, after the first key factor, taken to be their release from the food matrix, is their incorporation into mixed micelles during digestion, i.e., their bioaccessibility [30]. To assess whether known physicochemical properties of all-*E* carotenoids, reported in Table 5, affect their incorporation, or transfer from other structures into mixed micelles, we performed three different experiments.

First, we measured the maximum amount of carotenoids that could be incorporated/solubilized into synthetic mixed micelles during their preparation. The results obtained (Table 3), which were in very close agreement with data published on the incorporation of lutein and $\beta$-carotene into micelles [31], revealed a significant positive relationship ($r = 0.99$, $p = 0.015$; $\tau = 1$, $p = 0.041$) between the efficiency of incorporation of carotenoids into synthetic mixed micelles and carotenoid melting point (Table 5). This suggests that the higher the crystallinity of the carotenoids, the higher their incorporation into micelles. We then measured the transfer of carotenoids from their solid state (carotenoid dry powders) into preformed synthetic micelles in water. This can mimic what can happen when carotenoid crystals are ingested, e.g., in some supplements or in some vegetables. The first observation was that the transfer of carotenoids from this solid state into

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**Table 4.** Concentrations of carotenoids in plasma, liver, and adipose tissues after the gavage experiments[a)]

| | After 2-day gavage[b)] | After 3-day gavage[c)] | | |
|---|---|---|---|---|
| | Plasma (nmol/L) | Plasma (nmol/L) | Liver (nmol/g proteins) | Adipose tissue (nmol/g proteins) |
| Lutein | $15.8 \pm 7.0^{a}$ | $13.6 \pm 7.0^{a}$ | $0.3 \pm 0.2^{c}$ | $0.9 \pm 0.9^{a}$ |
| $\beta$-Carotene | $0.7 \pm 0.01^{b}$ | $6.7 \pm 0.9^{a,b}$ | $0.6 \pm 0.1^{a}$ | $0.2 \pm 0.3^{a,b}$ |
| Lycopene | $2.0 \pm 1.3^{b}$ | $2.9 \pm 1.3^{b}$ | $2.5 \pm 0.7^{b}$ | $0.5 \pm 0.3^{a}$ |
| Astaxanthin | $0.7 \pm 0.6^{b}$ | $14.5 \pm 7.6^{a}$ | $0.1 \pm 0.2^{c}$ | $0.1 \pm 0.2^{b}$ |

a) Eight rats per group were force-fed each day with 1 mL of groundnut oil containing 55 nmol of pure carotenoids (for more details, see Material and Methods). Values are means ± SD of six to eight rats (some rats died after the first two gavages). In each column, different letters indicate significant differences ($p < 0.05$) between means as assessed by Kruskal–Wallis and Mann–Whitney *U*-test.
b) In rats deprived of food the last night before the third day. Blood withdrawal just before the last gavage.
c) During the postprandial period (4 h after the third gavage administration).

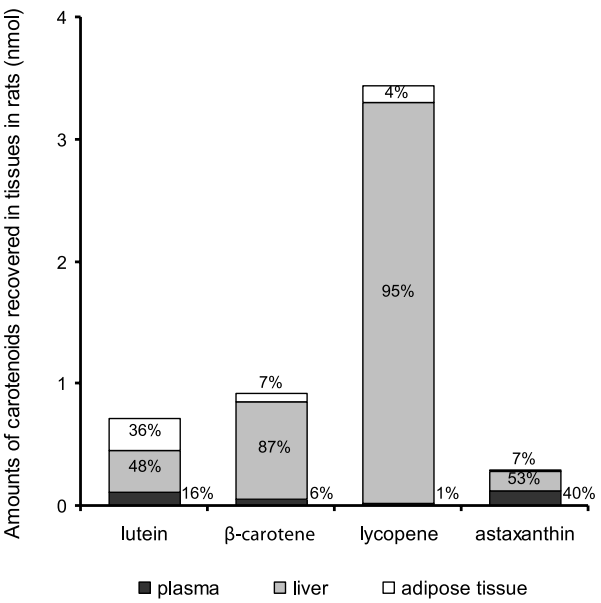


**Figure 4.** Amounts of carotenoids recovered in rat tissues after Wistar rats were force-fed for 3 days with a mixture of groundnut oil and pure carotenoids (55 nmol carotenoids/rat/day). Numbers show the relative distributions (%) of these carotenoids between plasma (black section), liver (gray section), and adipose tissue (white section).

synthetic micelles was significantly less efficient than the incorporation observed in the experiment on the incorporation of carotenoids during synthetic mixed micelle preparation (Table 3). This is probably because carotenoids are insoluble in water, so that the transfer requires micelles to come into contact with carotenoid powders/crystals to be transferred. We also hypothesized that part of the carotenoid powder could be in an organized state (possibly crystalline) that is much more resistant to solubilization. Unlike in the experiment on the incorporation of carotenoids during synthetic mixed micelle preparation, we found that the carotenoid transfer efficiency was not related to any of the physicochemical properties of carotenoids reported in Table 5. However, we again observed that carotenoids with terminal six-membered rings were more easily transferred than lycopene. Overall, when initially dispersed with phospholipids, cholesterol, and lipid digestion products, carotenoids with cyclic end moieties were ultimately much better incorporated into synthetic mixed micelles than lycopene. This trend was only partially reflected in the maximum transfer efficiencies of carotenoids from the solid state to preformed synthetic mixed micelles. Astaxanthin, despite its structural resemblance to lutein, was much less efficiently transferred than the latter. This observation can be at least partially explained by stronger interactions between astaxanthin molecules in the solid state (due to the additional polar keto groups), as suggested by its higher melting point (a difference of ca. 20°C). The good incorporation of $\beta$-carotene into synthetic mixed micelles shows that polar ends are not required. By contrast, the apparent poor affinity of lycopene for the synthetic mixed micelles may be due to its elongated linear shape that could cause the pigment molecule to protrude from the micelles into the surrounding aqueous environment.

A third experiment was set up to measured the transfer efficiency of pure carotenoids incorporated in triglycerides to natural mixed micelles produced during in vitro digestion (Fig. 1). This transfer is assumed to be the most common one in the gastrointestinal tract, as it is assumed that due to their hydrophobicity, most carotenoids are transferred into dietary triglycerides before transferral into micelles. In contrast to the two previous experiments, we found a strongly significant inverse relationship ($r = -1$, $p = 0.005$, $\tau = -1$, $p = 0.041$) between carotenoid hydrophobicity (log *P*) and carotenoid transfer into natural mixed micelles. A similar relationship was observed with log *D* at pH 5.5 and 7.4. Finally, we note that a similar experiment was performed with newly identified bacterial carotenoids [32, 33], carotenoids with still unknown physicochemical properties, but which are probably more polar than astaxanthin and lutein because they are substituted with hexose sugars. The results of this experiment

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**Table 5.** Main physicochemical properties of the tested carotenoids[a)]

| | Lutein | β-Carotene | Lycopene | Astaxanthin |
|---|---|---|---|---|
| log *P*[b)] | 8.55 | 11.12 | 11.93 | 8.05 |
| log *D* (pH 5.5 and 7.4)[c)] | 11.52 | 14.76 | 15.19 | 8.16 |
| Freely rotatable bonds (*n*) | 12 | 10 | 16 | 12 |
| Polar surface area ($A^{\circ 2}$) | 603.29 | 584.45 | 591.58 | 606.29 |
| van der Waals volume ($A^{\circ 3}$) | 40.46 | 0 | 0 | 74.60 |
| Molar volume ($cm^3$) | 566.11 | 570.13 | 604.21 | 557.10 |
| Surface tension (dyne/cm) | 40.90 | 36.36 | 31.09 | 44.67 |
| Melting point (°C)[d)] | 196.0 | 183.0 | 175.0 | 182.5 |
| Flash point (°C) | 269.07 | 345.95 | 350.74 | 435.84 |
| Boiling point (mm Hg) | 702.27 | 654.68 | 660.91 | 774.04 |
| Density ($g/cm^3$) | 1.01 | 0.94 | 0.89 | 1.07 |
| Enthalpy of vaporization (kJ/mol) | 117.48 | 92.93 | 93.73 | 128.45 |

a) ChemSpider free chemical database (chemSpider.com). Predicted data generated using ACD/Lab's ACD/physchem suite software (ACD/Labs.com) and ChemAxon (chemicalize.org) for log *P*.
b) log *P*: this is the partition coefficient of the carotenoids in a water: *n*-octanol system. It has been adopted as the standard measure of lipophilicity.
c) log *D* is the partition coefficient at a given pH. Similar values at pH 5.5 and 7.4 for the studied carotenoids.
d) Experimental (Human Metabolome Database version 2.5).

(data not shown) showed that the bioaccessibility of these bacterial carotenoids was higher than that of all the tested carotenoids in this study, consistent with the relationship observed. Thus, carotenoid hydrophobicity affects incorporation/transfer into micelles only when carotenoids are solubilized in triglycerides. We therefore conclude that it is mainly the interaction between carotenoids and triglycerides, which is modulated by carotenoid hydrophobicity, that governs the transfer efficiency of carotenoids from triglycerides into micelles.

To our knowledge, all the studies that have reported carotenoid bioaccessibility values were performed with carotenoids in foods or supplements. We therefore hypothesize that the values measured in these studies were due to both (i) the effect of carotenoid hydrophobicity on bioaccessibility demonstrated here, and (ii) the additive effect of the food matrices. To differentiate between these two effects, we compared the bioaccessibility values obtained with the pure carotenoids (Fig. 1) with the values obtained with the same carotenoids in their usual food matrices (Fig. 1). This comparison suggests that carrot puree and chopped spinach have no marked effect on β-carotene and lutein bioaccessibility, respectively. Conversely, tomato pulp has an inhibitory effect (about 50%) although nonsignificant due to the variability in the ANOVA test on lycopene bioaccessibility. This effect is well known [34, 35] and is explained by encapsulation within cellular compartments that are disrupted by thermal [36] and mechanical processing. Finally, some factor associated with the salmon flesh matrix apparently had a significant inhibitory effect on astaxanthin bioaccessibility. We suggest that there may be a competition of some as yet unspecified salmon-derived lipophilic components for the micellar space. Additional experiments will be required to explore this possibility. Finally, the comparison between bioaccessibility values obtained with the pure carotenoids and with the food matrix also showed that there was no significant effect of the type of oil incorporated in the digestion medium, olive or groundnut oil, on carotenoid bioaccessibility.

To accurately assess the effect of the food matrix, or of effectors of absorption [13] in future studies, we advocate subtracting the bioaccessibility values obtained with pure carotenoids from the crude measured bioaccessibility values. We can thereby conclude that the effect of common food matrices on lutein bioaccessibility is low because published values on lutein bioaccessibility, using the same in vitro model and the same method to isolate mixed micelles, lay between 38% and 57% for different vegetables [16], values very close to the value obtained for pure lutein (around 46%). For β-carotene, the value obtained here with pure β-carotene (around 13%) and the values published with the same methodologies [16], suggest that β-carotene bioaccessibility is not markedly affected by carrot juice or carrot puree matrices, or by boiled spinach matrix. By contrast, β-carotene bioaccessibility is significantly affected by raw tomato matrix. Finally, concerning lycopene, the value obtained with the pure substance (around 3%) suggests that its bioaccessibility is intrinsically low due to its high hydrophobicity and that some food matrices, e.g., raw tomato [16] and tomato purée here can even have an additive inhibitory effect. Finally, we note that because different bioaccessibility values were obtained by different teams using the in vitro digestion model, probably due to the method used to isolate mixed micelles, we consider that each team should measure its own values with pure substances to accurately correct the effect of carotenoid hydrophobicity on bioaccessibility.

Uptake by intestinal cells is the second key step governing carotenoid absorption. We therefore compared, in the Caco-2 model, the uptake efficiency of pure carotenoids in natural mixed micelles, i.e., micelles produced in the in vitro

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digestion experiments. The values obtained were close to those reported in previous studies [37–39]. They also showed that the uptake efficiencies of lutein, $\beta$-carotene, and astaxanthin were not significantly different from one another (Fig. 3). There was no significant relationship between the uptake efficiency of carotenoids by Caco-2 and any of the tested physicochemical properties of carotenoids (Table 5), whether or not we used the lycopene data. Thus, the physicochemical properties of carotenoids, once these are incorporated into mixed micelles, are apparently not a key factor in their uptake by intestinal cells. This conclusion is further supported by the results of a Caco-2 experiment with bacterial carotenoids (see above), which showed similar absorption efficiency between these probably more polar carotenoids and the carotenoids studied here (data not shown).

After absorption, carotenoids are incorporated into chylomicrons and transported by the blood to the liver. The liver stores a fraction of the carotenoids and secretes another fraction in VLDLs, which transport carotenoids to other tissues. Liver and adipose tissue [14] are the main storage tissues for carotenoids. Our gavage study in rats was dedicated to assessing the role of carotenoid physicochemical properties in blood and liver and adipose tissue responses to carotenoid intake. Blood concentrations of carotenoids were measured either at fast, after 2-day gavage, or in the postprandial period, 4 h after the third day gavage. Fasting and postprandial plasma concentrations of carotenoids were different because in the postprandial period, plasma contains both carotenoids previously absorbed and transported in VLDL, LDL, and HDL [40], and carotenoids newly absorbed and transported into chylomicrons. Interestingly, the ranking of plasma carotenoid concentrations observed in the postprandial period correlated well with the ranking observed in a supplementation study in rainbow trout [41]. There was a significant inverse relationship ($r = -0.99$, $p = 0.006$; $\tau = -1$, $p = 0.041$) between carotenoid hydrophobicity (log $P$) and postprandial plasma carotenoid concentration, which is assumed to reflect carotenoid absorption efficiency. A similar relationship was observed with log $D$ ($\tau = -1$, $p = 0.042$). Finally, we note that the fact that postprandial plasma carotenoid concentration was correlated with bioaccessibility ($r = 0.99$, $p = 0.005$; $\tau = 1$, $p = 0.042$) suggests that bioaccessibility is the main step governing carotenoid absorption efficiency.

Concerning tissue responses to carotenoid intake, the results obtained first showed that the amount and distribution of carotenoids between plasma, liver, and adipose tissue were very different according to the carotenoid (Table 4, Fig. 4). In agreement with previous studies, the liver was the major depot organ for lycopene [9–11]. The proportion of lycopene in adipose tissue (4%, Fig. 4) was also in good agreement with the results reported in a previous publication (around 8%) [11]. The distribution of carotenoids in tissues is due to several factors, e.g., amount transported to tissues by lipoproteins, rate of metabolization in each tissue, and amount resecreted by each tissue. The borderline positive relationship, nonsignificant in the parametric test ($r = 0.82$, $p = 0.17$) but significant in the nonparametric test ($\tau = 1$, $p = 0.041$), between carotenoid hydrophobicity (log $P$ as well as log $D$) and carotenoid concentration in the liver, suggests that the most hydrophobic carotenoids accumulate more abundantly in the liver than the less hydrophobic ones. However, many factors could explain this finding, such as the time it takes to equilibrate for the adipose tissue versus liver and the physiological function of the liver, which includes metabolic clearance of compounds that have no physiological function. Since no essential function for lycopene has yet been demonstrated in humans, there may be a higher accumulation in the liver for higher elimination. Concerning adipose tissue, we did not find any relationship between any carotenoid physicochemical properties reported in Table 5 and carotenoid concentration. This shows that other factors are predominant for carotenoid accumulation in this tissue. We hypothesize that membrane transporters involved in cellular uptake of carotenoids [7] may play an important role. As CD36 was more strongly expressed in adipose tissue than in liver of rats, whereas the opposite was the case for SR-BI, and because these scavenger receptors have different affinities for the different vehicles that carry carotenoids in the blood, e.g., mostly lipoproteins but also possibly albumin, we hypothesize that CD36 has a higher affinity for the vehicles that carry lutein than for those that carry other carotenoids, leading to a higher accumulation of this xanthophyll in adipose tissue. By contrast, $\beta$-carotene and lycopene may be incorporated into blood vehicles that have an affinity for SR-BI, e.g., HDL, but also LDL [42], VLDL [43], and chylomicrons [44], and thus preferentially accumulate in the liver, while xanthophylls are preferentially incorporated into vehicles that have a lower affinity for SR-BI. These hypotheses require further experiments for validation.

In summary, our experiments show that the hydrophobicity of carotenoids is a key factor affecting their bioaccessibility in naturally produced mixed micelles as well as their postprandial blood response. By contrast, the physicochemical properties of carotenoids reported in Table 5 do not significantly affect the efficiency of carotenoid uptake into intestinal cells or their accumulation in adipose tissue.

*We thank the company DSM (Switzerland) for its gift of carotenoids, and Maria-Erica Lopez (head of the laboratory animal house), and Jean-Marc Feuerstein and Mélanie Piednoel for their technical assistance in animal experiments. The present work received research funding from the European Community's Sixth Framework Programme. The funding was attributed to the project entitled New Sources of Natural, Gastric Stable, Food Additives, Colorants and Novel Functional Foods (COLORSPORE project number 207948) which was a small collaborative project within the work programme topic (Bio-)Technologies for the production of food additives, colorants, and flavors (KBBE-2007-2-3-03). This publication reflects only the authors' view. The Colorspore community is not liable for any use that may be made of the results.*

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