Are Lutein, Lycopene, and β-carotene Lost through the Digestive Process?

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Keywords: carotenoids, iron, metmyoglobin, in vitro digestion, metabolism, carotenoid degradation
Abstract

The bioavailability of many carotenoids has been assessed, but little attention has been given to the metabolism of these antioxidant compounds during digestion. The isomerization and loss of lutein, lycopene, and β-carotene incorporated into a lipid-rich liquid meal was determined in vitro through the gastric, duodenal, and jejunal phases in the presence and absence of digestive enzymes, and in the presence and absence of known oxidizing agents often found in mixed meals (metmyoglobin in red meat and ferrous sulfate in supplemental iron). Carotenoids were quantitated using HPLC-PDA. In the absence of enzymes, lutein and lycopene were lost during earlier phases of the digestive process. In the presence of enzymes, lutein and lycopene were robust through the gastric and duodenal phases, with statistically significant losses of 40% and 20%, respectively, observed only during the jejunal phase. Regardless of the presence or absence of enzymes, an initial 25% of β-carotene was lost during the gastric phase, but no further loss was observed. Ferrous sulfate had no significant impact on any carotenoid level. Metmyoglobin had no impact on lutein, but significantly reduced lycopene and β-carotene levels by 30% and 80%, respectively, by the end of the jejunal phase. No significant isomerization was observed between the initial and jejunal phases for any of the carotenoids.
1. Introduction

The consumption of carotenoid-containing foods has been associated with a reduced risk of developing a number of different types of diseases, such as various types of cancer and cardiovascular disease. The contribution of oxidative stress to these and other chronic conditions has been well established. Thus, for many years, the prevailing hypothesis was that carotenoids absorbed from carotenoid-containing foods exerted antioxidant and singlet oxygen quenching effects *in vivo*, ultimately leading to a reduction in chronic diseases.

However, low carotenoid concentrations in areas where oxidative damage is a concern, as well as inconsistent results from high-dose clinical studies, have called into question the plausibility of this hypothesis.

In contrast, higher carotenoid concentrations found in the lumen of the gastrointestinal tract (i.e. after the consumption of a carotenoid-containing meal) more convincingly support the theory that these compounds may act as antioxidants in the gut. In fact, work from our group and others has demonstrated the capacity of carotenoids to confer protection to unsaturated fatty acids under gastric and micellar (i.e. duodenal-like) conditions. Carotenoids also have a notoriously low bioaccessibility and bioavailability relative to other fat-soluble antioxidants like tocopherols and omega-3-fatty acids. It is possible that at least a portion of this low bioavailability may be due to loss of the carotenoid via oxidation during the digestive process. Indeed, carotenoids are known to be sensitive to increased temperature, pro-oxidative species, and an acidic pH, three factors found during digestion. Furthermore, foods are consumed and co-consumed in a multitude of combinations. The essential metal iron is present in various oxidation states and complexes in the diet, and has also been shown to be a strong carotenoid oxidizing...
agent. The oxidation effect of iron may be perpetuated in the presence of unsaturated fatty acids, where lipid peroxidation products can ultimately co-oxidize carotenoids in solution.

There is still no prevailing consensus on the mechanism(s) of action of non-provitamin A carotenoids once absorbed in the human body. More recent theories suggest that carotenoids may serve as “pro-bioactives”, i.e. the parent carotenoid consumed from a fruit or vegetable may be chemically or enzymatically converted into a biologically active metabolite(s) that ultimately exerts a disease-protective effect. This hypothesis is further supported by the fact that non-provitamin A metabolites of the most commonly consumed carotenoids, i.e. \( \beta \)-carotene, lycopene, and lutein, have been identified in blood plasma and various fruits, vegetables, and food products.\(^{27-29}\) It is not clear if these products were 1) absorbed directly from the foods themselves, 2) formed during digestion, 3) formed after parent carotenoid absorption, or a combination thereof.

To better understand the fate of carotenoids during digestion, \textit{in vitro} methods were employed to study carotenoid stability after each phase (initial, post-gastric, post-duodenal, and post-jejunal). Three of the most widely consumed carotenoids were chosen for study: lutein, \( \beta \)-carotene, and lycopene. Experiments were carried out both with and without digestive enzymes to better understand their influence. Finally, the impact of different dietary iron sources (i.e. ferrous sulfate commonly prescribed as an iron supplement, and metmyoglobin as the primary source of oxidized iron in red meat) were also investigated.

2. \textbf{Materials & Methods}

2.1 \textit{Materials} – For digestion experiments, food-grade lutein powder (product number 1EAA6165; 69\% lutein by weight, as determined by molar extinction coefficient of all-\textit{trans} lutein and HPLC-PDA analysis; geometrical isomer breakdown: 95\% all-\textit{trans} and 5\% \textit{cis}-lutein) and food-grade lycopene-containing oleoresin (product number 1EAA9272; 4.5\% lycopene by weight as
determined by molar extinction coefficient of all-trans-lycopene and LC-PDA analysis; geometrical isomer breakdown: 78% as all-trans-, 11% 5-cis- and 11% other-cis-lycopene) were kindly donated by Simona Birtic of Naturex (Montfavet, France). Fluka brand β-carotene (≥ 97% pure; geometrical isomer breakdown: 96% all-trans- and 4% cis-β-carotene) from Sigma-Aldrich (Saint-Quentin-Fallavier, France) was used both for digestion experiments and for analytical quantitation. White granular sugar and 100% sunflower oil were purchased from a local supermarket (Auchan, Avignon, France). Food-grade phospholipid (Ovolife IF 50, containing 50% w/w phospholipid derived from egg yolk with a maltodextrin excipient) was kindly donated by Lecico, Inc. (Hamburg, Germany). Deionized water was obtained through filtration via a Millipore Q-Plus. HPLC grade methyl tert-butyl ether (MTBE) and LC-MS grade methanol were purchased from Fisher Scientific (Illkirch, France). Ammonium acetate (≥ 98% pure, reagent grade), pepsin (pepsin A, from porcine gastric mucosa), bile extract (porcine origin), pancreatin (from porcine pancreas), mucin (type III, from porcine stomach), α-amylase (from Bacillus subtilis), myoglobin (≥ 90% from equine heart), sodium chloride (NaCl), sodium citrate tribasic dehydrate, sodium bicarbonate, calcium chloride dihydrate, potassium phosphate, disodium ethylenediaminetetraacetic acid (EDTA); Fluka brand potassium chloride, Extrasynthese brand lycopene (analytical grade), and Sigma brand pyrogallol (>99%) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Hydrochloric acid (HCl) was purchased from VWR. Tardyferon® was used as the source of ferrous sulfate (Boulogne, France). O-TRENSOX® (used to chelate metmyoglobin30) was a kind gift from Gisele Gellon.

2.2 Carotenoid in oil preparation for the test meals – β-Carotene (2 mg) or powdered lutein (3.1 mg) were added to 0.6 g sunflower oil, while the lycopene oleoresin (63 mg) was combined with 0.84 g sunflower oil. The oil mixtures were stirred overnight in the absence of light and under
argon. Note that the quantity of each carotenoid-containing product mixed with oil was dosed to deliver approximately 1.0 mg of carotenoid in 0.3 g oil.

2.3 Test meal preparation - Quantities of the test meal ingredients are shown in Table 1. Water and sugar were first mixed together in a beaker until the sugar was dissolved. The phospholipid containing powder (Ovolife IF 50) was added to the mixture and homogenized using a Heidolph Silent Crusher M (Schwabach, Germany) operated at 24,000 rpm for 2 min. Sunflower oil was then added and the mixture was again homogenized using the same conditions, followed by probe sonication using a Q700 QSonica (Newton, USA) with 40% amplitude and 30 sec sonication followed by 30 sec rest, repeated 8 x in sequence. After the removal of 18 mL of solution (which served to provide 3 “control” meals), the carotenoid in oil was added to the test meal remaining in the beaker. The meal was again homogenized and probe sonicated as described before.

2.4 Ferrous sulfate ($Fe^{II}$) solution – A Tardyferon® tablet containing 80 mg of iron was powdered, and 20% of the final powder weight was added to 45 mL of a 0.9% aq. NaCl solution. The solution was homogenized for 30 sec and then bath sonicated for 10 min to enhance dissolution (final concentration = 5.7 mM $Fe^{II}$/6 mL).

2.5 Metmyoglobin ($MbFe^{III}$) solution – The Sigma product myoglobin ($MbFe^{II}$) was dissolved in water and tested via UV-Visible spectrometry to indeed confirm by $\lambda_{max}$ that the product had been completely oxidized to metmyoglobin ($MbFe^{III}$)$^{31}$. $MbFe^{III}$ (21 mg) was dissolved in 10 mL of a 0.9% aq. NaCl solution (final concentration = 40 $\mu$M $MbFe^{III}$/2 mL).

2.6 Inhibitor solutions – Inhibitor solutions were prepared to halt any further chemical degradation of the digesta samples during extraction. The pyrogallol solution (used to quench samples of the digesta of carotenoid alone) was prepared by dissolving 125 mg pyrogallol in 25 mL water. The $Na_2$EDTA solution (to quench the carotenoid + $Fe^{II}$ digesta) was made by dissolving 140 mg
Na₂EDTA and 100 mg pyrogallol in 20.2 mL of water. The O-TRENSOX® solution (to quench carotenoid + MbFe³⁺ digesta) was made by dissolving 8.6 mg O-TRENSOX® and 100 mg pyrogallol in 20.2 mL of water.

2.7 Digestion with enzymes – Following the initial in vitro digestion method of Garret et al., modifications were made as previously described, as well as the addition of a jejunal phase. Each digestive condition with carotenoid was tested in triplicate, and the test beakers were placed on a magnetic stir plate in an oven (in the absence of light) at 37 °C during each phase. The digestions were prepared by adding 6 mL of test meal (delivering 80 μg carotenoid) to 32 mL of a 0.9% aq. NaCl solution (carotenoid alone), 30 mL of a 0.9% aq. NaCl solution containing 2 mL of the MbFe³⁺ solution (carotenoid + MbFe³⁺), or 26 mL of a 0.9% aq. NaCl solution containing 6 mL of the Fe²⁺ solution (carotenoid + Fe²⁺). The buccal phase (10 min), gastric and duodenal phases (each 30 min) were mimicked as previously described. The jejunal phase was mimicked by adjusting the pH to 7 by adding ~1.2 mL 0.9M aq. NaHCO₃ and digesting for 30 min. In addition, during the duodenal and jejunal phases, the beaker headspace was backfilled with argon to displace any air and thus any dioxygen molecules. Samples (0.5 mL) were taken immediately after the test meal was added to the digestion beaker (t = 0), and after the gastric, duodenal, and jejunal phases, and quenched with an equivalent volume of their respective inhibitor solutions.

2.8 Digestion without enzymes – The procedures were followed as described above, with the exception that the respective digestion buffers alone were added in place of the buffers with enzymes.

2.9 Sample extraction – To each sample already combined with inhibitor solution, methanol (0.5 mL) was added and the vial was shaken for 1 minute. Next, hexane was added (2 mL), the sample vortexed for 1 min. The upper hexane phase was transferred to a clean glass vial, and
dichloromethane (2 mL) was then added to the remaining sample. The sample was again vortexed (1 min), and then centrifuged for 2 min at 3,000 rpm (Jouan MR1822, ThermoFisher Scientific, Walham, MA, U.S.A.) to induce a clean phase separation. The lower dichloromethane phase was removed and pooled with the hexane extract and dried under a stream of argon at 30°C. Dried extracts were stored at -20°C for no more than 2 days before analysis.

2.8 HPLC Analysis – Extracts were redissolved in 50 μL MTBE, to which 50 μL of MeOH was added and samples were briefly sonicated (< 5 sec) in a sonication bath for dissolution. Samples were analyzed using an HP1100 (Agilent Technologies, Santa Clara, CA, U.S.A.) HPLC system equipped with a photodiode array detector. Lutein and β-carotene samples were separated using a YMC C30 column (150 mm x 2.0 mm, 3 μm particle size) using a previously published liquid chromatography method\textsuperscript{34} employing solvent A (80:18:2 MeOH/water/2% aq. ammonium acetate) and solvent B (20:78:2 MeOH/MTBE/2% aq. ammonium acetate), but halting the gradient at 26 min and injecting 10 μL. Note that this method separated all-trans-β-carotene and all-trans-lutein from cis geometrical isomers (9-cis-β-carotene was putatively determined by coincident PDA spectra and elution order just after all-trans-β-carotene\textsuperscript{35}, while all other cis-isomers of β-carotene and all cis isomers of lutein were determined by PDA spectra and elution prior to the all-trans configuration). Lycopene samples were separated using a method designed to partially separate 5-cis-lycopene (which elutes just after the all-trans configuration on a C30 column\textsuperscript{36}) from all-trans-lycopene, as well as other cis-isomers (which elute prior to the all-trans configuration). A YMC C30 column (150 mm x 4.6 mm, 3 μm particle size) column was employed using the same solvent system as above, with the following gradient: beginning at 40% B, increasing linearly to 80% B over 23 min, holding at 80% B for 1 min, returning to 40% B over 3 min. The column was held at a cooler temperature of 20°C (which afforded better separation of the lycopene isomers\textsuperscript{37}), the
flow rate was 1.3 mL/min, and 10 μL samples were injected. Carotenoids were quantitated using external calibration curves generated from authentic standards by integrating the peak areas at 445 nm (lutein), 450 nm (β-carotene), and 471 nm (lycopene). Total carotenoid calculated at each step incorporated any previous digesta volume change (with the addition of enzyme/buffer solution and previous sample removal) over the course of the experiment.

2.10 Statistical Analysis - Data was analyzed using R statistical software\textsuperscript{38}. A mixed factorial design with repeated measures ANOVA was employed to test the fixed factors of meal (between subject factor with 3 levels consisting of control, metmyoglobin, and ferrous sulfate), digestion phase (within subject factor with 4 levels i.e. initial, gastric, duodenal, and jejunal), and meal*digestion phase, on carotenoid level (dependent variable). The error factor controlled for within flask variability. A \( P \) value < 0.05 was considered statistically significant. Post-hoc analysis was performed using the Bonferroni correction for multiple comparisons to determine statistically significant interactions within the same meal over multiple digestion phases, and between meals at the same digestion phase.

3. Results

Figure 1 reveals the loss of lutein in the 3 different meals during the course of \textit{in vitro} digestion. In the absence of enzymes (Figure 1A), approximately 25% of the initial lutein was lost during the gastric phase regardless of the meal (i.e. control, metmyoglobin, or ferrous sulfate). During the duodenal and jejunal phases, no additional loss of lutein was observed with lutein alone or lutein + ferrous sulfate. Only in the presence of metmyoglobin was an additional 20% of lutein lost during the duodenal phase. Figure 1B reveals that lutein was more robust through the gastric and duodenal phases of digestion when enzymes were present, with no difference observed between conditions
or phases. However, by the jejunal phase, the results mimicked that of the experiment without enzymes, and a total of 60% of the lutein remained regardless of meal.

Figure 2 demonstrates the loss of lycopene during digestion. In the absence of enzymes (Figure 2A), gradual loss of lycopene was observed at each stage, resulting in a 35% overall loss post-jejunal phase. The presence of metmyoglobin or ferrous sulfate did not significantly alter lycopene degradation at any digestion stage. In comparison, the experiment with enzymes (Figure 2B) revealed no change in lycopene levels with lycopene alone or lycopene + ferrous sulfate until the jejunal phase, where a ~20% loss was observed. In contrast, a more pronounced lycopene loss was observed after the duodenal and jejunal phases (12% and 19%, respectively) in the presence of metmyoglobin.

The loss of β-carotene during digestion is shown in Figure 3. In the absence of enzymes (Figure 3A), an immediate 20% post-gastric loss of β-carotene was observed under all meal conditions, but there was no further significant β-carotene loss when alone or together with ferrous sulfate during the rest of the experiment. In contrast, an additional 20% of β-carotene was lost in the presence of metmyoglobin during the duodenal phase. In the presence of enzymes (Figure 3B), a 25% loss of β-carotene when alone was observed post-gastric phase, but no additional significant loss was observed through the duodenal and jejunal phases. When ferrous sulfate was added together with β-carotene, this resulted in a ~40% β-carotene loss during the gastric phase, but no further loss through the duodenal and jejunal phases was observed. By comparison, when metmyoglobin was added to β-carotene, a 50% β-carotene loss was observed during the gastric phase and a further 30% loss from initial β-carotene level during the duodenal phase of digestion.
No significant change in % cis-isomers was observed between the starting material and the jejunal phase for any of the 3 carotenoids tested. Thus, the bars in Figures 1, 2, and 3 represent total carotenoid (i.e. the summation of all-trans + cis isomers) at each stage.

4. Discussion

The relative susceptibility of these three carotenoids to loss under various conditions of digestion presents some intriguing results. We begin by comparing the behavior of carotenoids alone digested without enzymes, as compared to carotenoids digested with enzymes (solid bars, A vs. B in Figures 1, 2, and 3). Our experiments clearly reveal that a greater percentage of lutein and lycopene survived the gastric and duodenal phases of digestion in the presence of enzymes, as compared to the experiments without enzymes. In the experiments with enzymes, lipases and bile salts work in concert to reduce the size of emulsified lipid droplets and create mixed micelles during the duodenal phase. In contrast, the digestion experiments without enzymes were performed to better understand the impact of chemical conditions alone on carotenoid degradation. Thus, these experiments contained the emulsified test meal throughout each phase. Studies on the stability of β-carotene and lutein emulsions have demonstrated that carotenoid stability is directly proportional to lipid droplet size. Consequently, we expected the larger emulsified lipid droplets produced in the experiments without enzyme to be more protective than small particle micelles in the experiments with enzymes. Therefore, it was initially quite surprising to observe no difference in β-carotene degradation, and a more rapid lutein and lycopene degradation in the experiments without enzymes. However, the enzyme experiments also contained various proteins, such as amylase during the initial sample treatment, pepsin added during the gastric phase, and the porcine pancreatin preparation added during the duodenal phase. Previous in vitro studies have demonstrated that certain whey proteins further protect β-carotene and lutein from degradation.
in emulsification model systems, as these proteins are believed to rest at the interface between the lipid droplet and the aqueous solution.\textsuperscript{41} Similarly, a whey-protein encapsulated lycopene has been shown to successfully deliver lycopene in humans, presumably via at least partial protection of lycopene through gastro-intestinal conditions.\textsuperscript{42} In short, our results suggest that protein solutions confer additional protection to lutein and lycopene against chemical oxidation during the gastric and duodenal phases of digestion, but any advantage gleaned from protein is lost during the jejunal phase (likely due to carotenoid transfer to and retention in less-protective micelles).

\textit{β}-carotene was equally susceptible to degradation, regardless of the absence or presence of enzymes (solid bars, Figure 3A vs. 3B). It should be noted that these experiments with \textit{β}-carotene were repeated (with the repeat experiments also performed in triplicate), with the same average \textit{β}-carotene level and variability observed each time. Previous research has demonstrated that the % carotenoid transferred from emulsion lipid droplet to micelle is inversely proportional to carotenoid hydrophobicity, with lutein > \textit{β}-carotene > lycopene.\textsuperscript{39} Thus, we would expect that any “emulsion effect” would more dramatically impact lutein as compared to \textit{β}-carotene. Similarly, we would anticipate that protein confers the same type of protection to \textit{β}-carotene as compared to lutein or lycopene.\textsuperscript{41} Thus, it is not clear why no difference in \textit{β}-carotene was observed regardless of the absence or presence of enzymes.

Of those tested, lycopene alone was found to be the most stable carotenoid through the entire digestive process with enzymes, with a 20% loss overall. In contrast, 40% of lutein alone and \textit{β}-carotene alone were lost during their respective digestions with enzymes. The lycopene and \textit{β}-carotene results are in good agreement with Blanquet-Diot et al., who reported a 20% and \textasciitilde30% loss, respectively using a dynamic \textit{in vitro} digestion model.\textsuperscript{43} In contrast, Courraud et al., reported a 50% loss of \textit{β}-carotene after the gastric phase, and a further 20% loss during the intestinal
phase. These differences in β-carotene stability are likely due to Courraud et al.’s direct application of β-carotene dissolved in acetone to the mimicked digesta, and a much lower pH of 2 used during a prolonged gastric phase. Lutein loss was similar to that reported by Courraud et al. in raw spinach. Other studies by Blanquet-Diot et al., and Chitchumroonchokchai et al., observed no change in lutein levels over the course of in vitro digestion. It is likely that the use of a tomato food matrix and the absence of a jejunal digestion phase are the source of this divergence.

Regardless of the presence or absence of enzymes, ferrous sulfate had no additional impact on carotenoid loss during the experiments (white bars, Figures 1, 2, and 3). Previous work by our group has demonstrated that in a gastric-like micellar model, increasing levels of ferrous iron relative to β-carotene (0-10 equivalents) result in increasing levels of β-carotene oxidation, as compared to no iron. However, excess levels of ferrous iron relative to β-carotene (50 equivalents) resulted in an initial oxidative loss of β-carotene over 5-10 min, but surprisingly less β-carotene oxidation through 120 min. It was postulated that when ferrous iron is present in excess, various perferryl and ferryl intermediates are formed, which further react with ferrous iron to produce high levels of a diferric ether product, which is inert. The dose of ferrous sulfate and carotenoid used in this study were calculated to reproduce a plausible scenario of a single iron tablet prescribed as a supplement (delivering 80 mg of divalent iron) consumed with a meal containing 20 mg carotenoid. Under these conditions, the iron to carotenoid equivalent is 37 for meals containing lycopene or β-carotene + ferrous sulfate, and 39 for meals containing lutein + ferrous sulfate. Based upon our results, we speculate that the ferrous sulfate levels were sufficiently high to result in the formation of inert diferric ether, resulting in no additional carotenoid oxidation in these groups. It
is also possible that the gelatin coating and maltodextrin excipient of the iron tablet exerted a protective carotenoid effect during the initial phase of digestion.\textsuperscript{46}

The concentration of metmyoglobin used in our studies (40 μM) is reflective of the quantity expected from the consumption of a 255 g (9 oz.) beef steak (estimated to deliver 30-50 μM metmyoglobin).\textsuperscript{47–49} In the \textit{in vitro} experiments with enzymes, metmyoglobin had no impact on lutein stability (striped bars, Figure 1B), but a more pronounced impact on carotene stability (striped bars, Figures 2B and 3B), especially that of β-carotene. These results are in good agreement with similar work observing the capacity of various carotenoids to inhibit metmyoglobin-induced peroxidation of linoleic acid in an emulsion model.\textsuperscript{16} Binary concentrations at 50% of maximal linoleic acid peroxidation (IC\textsubscript{50}) values were measured, and were shown to follow the order of β-carotene > lycopene > xanthophylls derived from bacteria.\textsuperscript{16} In other words, a larger concentration of β-carotene was needed to protect linoleic acid relative to the other carotenoids. Further measures of the peroxide kinetics suggested that this difference may lie in the fact that carotenoids sit in the center of the lipid droplet, and thus their only mechanism of defense is to halt further fatty acid peroxide propagation. In contrast, bacterial xanthophylls sit at the lipid-water interface and likely inhibit peroxidation initiation altogether.\textsuperscript{16} It is not clear why β-carotene was more sensitive than lycopene to metmyoglobin-induced oxidation. It is possible that at the concentrations of lycopene used, lycopene microcrystals may have formed in the digesta mixture\textsuperscript{50}, which may have conferred physico-chemical protection, while β-carotene likely remained dissolved.

We intentionally used un-stripped sunflower oil in all of our experiments to create a realistic liquid beverage that would be safe for use in a human clinical study. However, the endogenous tocopherols and tocotrienols present likely protected carotenoids and reduced losses
due to oxidation, as studies have previously demonstrated that alpha-tocopherol protects lycopene\textsuperscript{51,52}, \(\beta\)-carotene\textsuperscript{53}, and lutein\textsuperscript{40} from various forms of oxidation in emulsion systems.

It should be noted that under typical digestive conditions (i.e. with enzymes), levels of lutein alone and lycopene alone were not significantly different from the initial meal through the duodenal phase. Immunohistochemical staining demonstrates the presence of apical membrane transporters SR-B1 and CD-36 in the duodenum and jejunum of humans\textsuperscript{54}, with Western blotting showing significantly higher SR-B1 protein expression in the duodenum\textsuperscript{55} and higher expression of CD-36 in the jejunum\textsuperscript{56} of rodents. Thus, we anticipate a fair portion of lutein and lycopene to be absorbed in the duodenum (before degradation), and any resulting loss in the jejunum would probably have a limited impact on overall bioavailability. However, our results suggest that partial degradation of \(\beta\)-carotene alone under these types of meal conditions may ultimately reduce the quantity of \(\beta\)-carotene that is bioavailable.

We noted no significant change from the initial \(\%\) \textit{cis} isomers to the \(\%\) \textit{cis} isomers remaining in jejunal phase. These results are in good agreement with previous studies using static \textit{in vitro} digestion, which have demonstrated that the \(\%\) \textit{cis} isomer contribution to total carotenoid content is well maintained for lycopene\textsuperscript{57,58}, lutein\textsuperscript{59}, and \(\beta\)-carotene\textsuperscript{60}. These results are further corroborated with human studies on gastric digesta for lycopene and \(\beta\)-carotene\textsuperscript{61}. The same human study also investigated duodenal digesta, and found no change in lycopene but a slight increase in \(\%\) \textit{cis} \(\beta\)-carotene (relative to starting \(\%\) \textit{cis} \(\beta\)-carotene).\textsuperscript{61} We would attribute this disparity to the dynamic nature of absorption in humans, and transporter selectivity for all-\textit{trans-}\(\beta\)-carotene\textsuperscript{62}.

While our primary objective was to understand carotenoid loss during digestion, we also anticipated observing carotenoid degradation products in our samples. Indeed, the extraction and
HPLC-PDA methods employed for the analysis of β-carotene- and lycopene-meal digesta would have revealed the presence of β-apo-carotenals and apo-lycopenals, respectively, if they had been present in sufficiently high quantities for PDA detection. Furthermore, previous work by our group has demonstrated that under certain mimicked conditions of gastric digestion and in the presence of metmyoglobin, β-apo-carotenals can be observed by PDA. However, no oxidation products were observed in any of the samples in this study. This discrepancy with previous work may be due to the previous use of non-biological surfactants (Tween 20 and Brij 35) to create micelle-like conditions. Similarly, aldehydes have the capacity to react irreversibly with proteins to form Schiff bases and thus it is possible that aldehyde products were formed in our study, but were not able to be extracted due to protein sequestration. While apo-carotenaldehydes were not observed, the loss of lutein and lycopene in the jejunal phase, and the immediate loss of β-carotene in the gastric phase, indicate that at least some derivatives were created. Further work is needed to identify these products, and to determine if they are absorbed, and/or if they might have biological effects in the jejunum or colon.

In conclusion, some of the chemical factors that reduce carotenoid stability in an in vitro emulsion model are counteracted in the presence of digestive enzymes. In the presence of enzymes, lutein and lycopene were robust to the effects of the digestive process throughout the gastric and duodenal phases, with the most pronounced losses occurring during the jejunal phase. β-carotene was most susceptible to degradation during the gastric phase, but no further loss was observed. Our data suggests that a commonly prescribed dose of supplemental iron consumed with a carotenoid-containing meal does not have an impact on carotenoid stability. However, co-consumption of red meat with lycopene- or β-carotene-containing foods is likely to reduce the quantity of carotenoid available for absorption, and may generate carotenoid oxidation products.
Further work is needed to determine which products are produced, and whether they have biologically relevant actions.
Conflict of Interest

There are no conflicts of interest to declare.

Acknowledgements

We would like to thank Simona Birtic of Naturex, Inc. who kindly provided the lutein powder and lycopene oleoresin used in this study. We would also like to thank Gisele Gellon of the University of Grenoble Alpes (DCM UMR CNRS 5250, CS 40700, F-38058 Grenoble, France) for providing the O-TRENSOX®. This work was supported by a grant from the PACA (Provence-Alpes-Côte d’Azur) region. Rachel Kopec received support from the European Union, in the framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreenSkills fellowship (under grant agreement n° 267196).

Abbreviations:

DCM dichloromethane
HPLC high-performance liquid chromatography
LC-MS liquid chromatography-mass spectrometry
MeOH methanol
MTBE methyl tert-butyl ether
PDA photodiode array detection
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Table 1. Composition of the test meals

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Water</td>
<td>90 g</td>
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<tr>
<td>Sucrose</td>
<td>9 g</td>
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<tr>
<td>Phospholipid containing powder\textsuperscript{a,b}</td>
<td>150 mg</td>
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<tr>
<td>Sunflower oil</td>
<td>3 g</td>
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<tr>
<td>Carotenoid-oil preparation\textsuperscript{b}</td>
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</tbody>
</table>

\textsuperscript{a} Delivering 75 mg of phospholipid derived from egg, to match the 1:40 ratio of phospholipid to lipid representative of a western diet\textsuperscript{64}.

\textsuperscript{b} See Materials and Methods for additional details.
**Figure 1.** Loss of initial lutein through the gastric, duodenal, and jejunal phases of digestion in the absence (A) and presence (B) of digestive enzymes. Bars represent average % lutein ± standard deviation (n=3 for each meal). Different letters indicate statistically significant differences within the same meal at different digestion phases ($P < 0.05$). There was no significant difference between meal types at any of the digestion phases ($P < 0.05$).
Figure 2. Loss of initial lycopene through the gastric, duodenal, and jejunal phases of digestion in the absence (A) and presence (B) of digestive enzymes. Bars represent average % lycopene ± standard deviation (n=3 for each meal). Different letters indicate statistically significant differences within the same meal at different digestion phases (P < 0.05). Statistically significant differences between meal types in the same digestion phase are noted with an asterisk, described below.

*Significantly different from Lycopene + FeII after the same digestion phase (P < 0.001)

**Significantly different from Lycopene Alone and Lycopene + FeII after the same digestion phase (P < 0.0001)
Figure 3. Loss of initial β-carotene through the gastric, duodenal, and jejunal phases of digestion in the absence (A) and presence (B) of digestive enzymes. Bars represent average % β-carotene ± standard deviation (n=3 for each meal). Different letters indicate statistically significant differences within the same meal at different digestion phases ($P < 0.05$). Statistically significant differences between meal types in the same digestion phase are noted with an asterisk, described below.

*Significantly different from β-Carotene + FeII after the same digestion phase ($P < 0.005$)

**Significantly different from β-Carotene Alone after the same digestion phase ($P = 0.006$)

***Significantly different from β-Carotene Alone and β-Carotene + FeII after the same digestion phase ($P < 0.0001$)