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

2

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31 **Abstract**

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

47

48 **1. Introduction**

49 The consumption of carotenoid-containing foods has been associated with a reduced risk of
50 developing a number of different types of diseases, such as various types of cancer¹ and
51 cardiovascular disease.² The contribution of oxidative stress to these and other chronic conditions
52 has been well established.³⁻⁵ Thus, for many years, the prevailing hypothesis was that carotenoids
53 absorbed from carotenoid-containing foods exerted antioxidant and singlet oxygen quenching
54 effects *in vivo*, ultimately leading to a reduction in chronic diseases.⁶⁻⁸
55 However, low carotenoid concentrations in areas where oxidative damage is a concern⁹, as well as
56 inconsistent results from high-dose clinical studies, have called into question the plausibility of
57 this hypothesis.¹⁰⁻¹²

58 In contrast, higher carotenoid concentrations found in the lumen of the gastrointestinal tract
59 (i.e. after the consumption of a carotenoid-containing meal) more convincingly support the theory
60 that these compounds may act as antioxidants in the gut.^{13,14} In fact, work from our group¹⁵⁻¹⁸ and
61 others^{19,20} has demonstrated the capacity of carotenoids to confer protection to unsaturated fatty
62 acids under gastric and micellar (i.e. duodenal-like) conditions. Carotenoids also have a
63 notoriously low bioaccessibility²¹ and bioavailability²² relative to other fat-soluble antioxidants
64 like tocopherols²³ and omega-3-fatty acids.²⁴ It is possible that at least a portion of this low
65 bioavailability may be due to loss of the carotenoid via oxidation during the digestive process.
66 Indeed, carotenoids are known to be sensitive to increased temperature, pro-oxidative species, and
67 an acidic pH, three factors found during digestion.^{13,14} Furthermore, foods are consumed and co-
68 consumed in a multitude of combinations. The essential metal iron is present in various oxidation
69 states and complexes in the diet, and has also been shown to be a strong carotenoid oxidizing

70 agent.¹⁵ The oxidation effect of iron may be perpetuated in the presence of unsaturated fatty acids,
71 where lipid peroxidation products can ultimately co-oxidize carotenoids in solution.¹⁶

72 There is still no prevailing consensus on the mechanism(s) of action of non-provitamin A
73 carotenoids once absorbed in the human body. More recent theories suggest that carotenoids may
74 serve as “pro-bioactives”, i.e. the parent carotenoid consumed from a fruit or vegetable may be
75 chemically or enzymatically converted into a biologically active metabolite(s) that ultimately
76 exerts a disease-protective effect.^{25,26} This hypothesis is further supported by the fact that non-
77 provitamin A metabolites of the most commonly consumed carotenoids, i.e. β -carotene, lycopene,
78 and lutein, have been identified in blood plasma and various fruits, vegetables, and food
79 products.^{27–29} It is not clear if these products were 1) absorbed directly from the foods themselves,
80 2) formed during digestion, 3) formed after parent carotenoid absorption, or a combination thereof.

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

88 **2. Materials & Methods**

89 *2.1 Materials* – For digestion experiments, food-grade lutein powder (product number 1EAA6165;
90 69% lutein by weight, as determined by molar extinction coefficient of all-*trans* lutein and HPLC-
91 PDA analysis; geometrical isomer breakdown: 95% all-*trans*- and 5% *cis*-lutein) and food-grade
92 lycopene-containing oleoresin (product number 1EAA9272; 4.5% lycopene by weight as

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

113 *2.2 Carotenoid in oil preparation for the test meals* – β -Carotene (2 mg) or powdered lutein (3.1
114 mg) were added to 0.6 g sunflower oil, while the lycopene oleoresin (63 mg) was combined with
115 0.84 g sunflower oil. The oil mixtures were stirred overnight in the absence of light and under

116 argon. Note that the quantity of each carotenoid-containing product mixed with oil was dosed to
117 deliver approximately 1.0 mg of carotenoid in 0.3 g oil.

118 *2.3 Test meal preparation* - Quantities of the test meal ingredients are shown in Table 1. Water
119 and sugar were first mixed together in a beaker until the sugar was dissolved. The phospholipid
120 containing powder (Ovolife IF 50) was added to the mixture and homogenized using a Heidolph
121 Silent Crusher M (Schwabach, Germany) operated at 24,000 rpm for 2 min. Sunflower oil was
122 then added and the mixture was again homogenized using the same conditions, followed by probe
123 sonication using a Q700 QSonica (Newton, USA) with 40% amplitude and 30 sec sonication
124 followed by 30 sec rest, repeated 8 x in sequence. After the removal of 18 mL of solution (which
125 served to provide 3 “control” meals), the carotenoid in oil was added to the test meal remaining in
126 the beaker. The meal was again homogenized and probe sonicated as described before.

127 *2.4 Ferrous sulfate (Fe^{II}) solution* – A Tardyferon[®] tablet containing 80 mg of iron was powdered,
128 and 20% of the final powder weight was added to 45 mL of a 0.9% aq. NaCl solution. The solution
129 was homogenized for 30 sec and then bath sonicated for 10 min to enhance dissolution (final
130 concentration = 5.7 mM Fe^{II} /6 mL).

131 *2.5 Metmyoglobin ($MbFe^{III}$) solution* – The Sigma product myoglobin ($MbFe^{II}$) was dissolved in
132 water and tested via UV-Visible spectrometry to indeed confirm by λ_{max} that the product had been
133 completely oxidized to metmyoglobin ($MbFe^{III}$)³¹. $MbFe^{III}$ (21 mg) was dissolved in 10 mL of a
134 0.9% aq. NaCl solution (final concentration = 40 μ M $MbFe^{III}$ /2 mL).

135 *2.6 Inhibitor solutions* – Inhibitor solutions were prepared to halt any further chemical degradation
136 of the digesta samples during extraction. The pyrogallol solution (used to quench samples of the
137 digesta of carotenoid alone) was prepared by dissolving 125 mg pyrogallol in 25 mL water. The
138 Na_2EDTA solution (to quench the carotenoid + Fe^{II} digesta) was made by dissolving 140 mg

139 Na₂EDTA and 100 mg pyrogallol in 20.2 mL of water. The O-TRENTOX® solution (to quench
140 carotenoid + MbFe^{III} digesta) was made by dissolving 8.6 mg O-TRENTOX® and 100 mg
141 pyrogallol in 20.2 mL of water.

142 *2.7 Digestion with enzymes* – Following the initial *in vitro* digestion method of Garret et al.³²,
143 modifications were made as previously described³³, as well as the addition of a jejunal phase. Each
144 digestive condition with carotenoid was tested in triplicate, and the test beakers were placed on a
145 magnetic stir plate in an oven (in the absence of light) at 37 °C during each phase. The digestions
146 were prepared by adding 6 mL of test meal (delivering 80 µg carotenoid) to 32 mL of a 0.9% aq.
147 NaCl solution (carotenoid alone), 30 mL of a 0.9% aq. NaCl solution containing 2 mL of the
148 MbFe^{III} solution (carotenoid + MbFe^{III}), or 26 mL of a 0.9% aq. NaCl solution containing 6 mL of
149 the Fe^{II} solution (carotenoid + Fe^{II}). The buccal phase (10 min), gastric and duodenal phases (each
150 30 min) were mimicked as previously described.³³ The jejunal phase was mimicked by adjusting
151 the pH to 7 by adding ~1.2 mL 0.9M aq. NaHCO₃ and digesting for 30 min. In addition, during the
152 duodenal and jejunal phases, the beaker headspace was backfilled with argon to displace any air
153 and thus any dioxygen molecules. Samples (0.5 mL) were taken immediately after the test meal
154 was added to the digestion beaker (t = 0), and after the gastric, duodenal, and jejunal phases, and
155 quenched with an equivalent volume of their respective inhibitor solutions.

156 *2.8 Digestion without enzymes*– The procedures were followed as described above, with the
157 exception that the respective digestion buffers alone were added in place of the buffers with
158 enzymes.

159 *2.9 Sample extraction* – To each sample already combined with inhibitor solution, methanol (0.5
160 mL) was added and the vial was shaken for 1 minute. Next, hexane was added (2 mL), the sample
161 vortexed for 1 min. The upper hexane phase was transferred to a clean glass vial, and

162 dichloromethane (2 mL) was then added to the remaining sample. The sample was again vortexed
163 (1 min), and then centrifuged for 2 min at 3,000 rpm (Jouan MR1822, ThermoFisher Scientific,
164 Walham, MA, U.S.A.) to induce a clean phase separation. The lower dichloromethane phase was
165 removed and pooled with the hexane extract and dried under a stream of argon at 30°C. Dried
166 extracts were stored at -20°C for no more than 2 days before analysis.

167 *2.8 HPLC Analysis* – Extracts were redissolved in 50 µL MTBE, to which 50 µL of MeOH was
168 added and samples were briefly sonicated (< 5 sec) in a sonication bath for dissolution. Samples
169 were analyzed using an HP1100 (Agilent Technologies, Santa Clara, CA, U.S.A.) HPLC system
170 equipped with a photodiode array detector. Lutein and β-carotene samples were separated using a
171 YMC C30 column (150 mm x 2.0 mm, 3 µm particle size) using a previously published liquid
172 chromatography method³⁴ employing solvent A (80:18:2 MeOH/water/2% aq. ammonium acetate)
173 and solvent B (20:78:2 MeOH/MTBE/2% aq. ammonium acetate), but halting the gradient at 26
174 min and injecting 10 µL. Note that this method separated all-*trans*-β-carotene and all-*trans*-lutein
175 from *cis* geometrical isomers (9-*cis*-β-carotene was putatively determined by coincident PDA
176 spectra and elution order just after all-*trans*-β-carotene³⁵, while all other *cis*-isomers of β-carotene
177 and all *cis* isomers of lutein were determined by PDA spectra and elution prior to the all-*trans*
178 configuration). Lycopene samples were separated using a method designed to partially separate 5-
179 *cis*-lycopene (which elutes just after the all-*trans* configuration on a C30 column³⁶) from all-*trans*-
180 lycopene, as well as other *cis*-isomers (which elute prior to the all-*trans* configuration). A YMC
181 C30 column (150 mm x 4.6 mm, 3 µm particle size) column was employed using the same solvent
182 system as above, with the following gradient: beginning at 40% B, increasing linearly to 80% B
183 over 23 min, holding at 80% B for 1 min, returning to 40% B over 3 min. The column was held at
184 a cooler temperature of 20°C (which afforded better separation of the lycopene isomers³⁷), the

185 flow rate was 1.3 mL/min, and 10 μ L samples were injected. Carotenoids were quantitated using
186 external calibration curves generated from authentic standards by integrating the peak areas at 445
187 nm (lutein), 450 nm (β -carotene), and 471 nm (lycopene). Total carotenoid calculated at each step
188 incorporated any previous digesta volume change (with the addition of enzyme/buffer solution and
189 previous sample removal) over the course of the experiment.

190 *2.10 Statistical Analysis* - Data was analyzed using R statistical software³⁸. A mixed factorial
191 design with repeated measures ANOVA was employed to test the fixed factors of meal (between
192 subject factor with 3 levels consisting of control, metmyoglobin, and ferrous sulfate), digestion
193 phase (within subject factor with 4 levels i.e. initial, gastric, duodenal, and jejunal), and
194 meal*digestion phase, on carotenoid level (dependent variable). The error factor controlled for
195 within flask variability. A *P* value < 0.05 was considered statistically significant. Post-hoc analysis
196 was performed using the Bonferroni correction for multiple comparisons to determine statistically
197 significant interactions within the same meal over multiple digestion phases, and between meals
198 at the same digestion phase.

199 **3. Results**

200 Figure 1 reveals the loss of lutein in the 3 different meals during the course of *in vitro* digestion.
201 In the absence of enzymes (Figure 1A), approximately 25% of the initial lutein was lost during the
202 gastric phase regardless of the meal (i.e. control, metmyoglobin, or ferrous sulfate). During the
203 duodenal and jejunal phases, no additional loss of lutein was observed with lutein alone or lutein
204 + ferrous sulfate. Only in the presence of metmyoglobin was an additional 20% of lutein lost during
205 the duodenal phase. Figure 1B reveals that lutein was more robust through the gastric and duodenal
206 phases of digestion when enzymes were present, with no difference observed between conditions

207 or phases. However, by the jejunal phase, the results mimicked that of the experiment without
208 enzymes, and a total of 60% of the lutein remained regardless of meal.

209 Figure 2 demonstrates the loss of lycopene during digestion. In the absence of enzymes (Figure
210 2A), gradual loss of lycopene was observed at each stage, resulting in a 35% overall loss post-
211 jejunal phase. The presence of metmyoglobin or ferrous sulfate did not significantly alter lycopene
212 degradation at any digestion stage. In comparison, the experiment with enzymes (Figure 2B)
213 revealed no change in lycopene levels with lycopene alone or lycopene + ferrous sulfate until the
214 jejunal phase, where a ~20% loss was observed. In contrast, a more pronounced lycopene loss was
215 observed after the duodenal and jejunal phases (12% and 19%, respectively) in the presence of
216 metmyoglobin.

217 The loss of β -carotene during digestion is shown in Figure 3. In the absence of enzymes
218 (Figure 3A), an immediate 20% post-gastric loss of β -carotene was observed under all meal
219 conditions, but there was no further significant β -carotene loss when alone or together with ferrous
220 sulfate during the rest of the experiment. In contrast, an additional 20% of β -carotene was lost in
221 the presence of metmyoglobin during the duodenal phase. In the presence of enzymes (Figure 3B),
222 a 25% loss of β -carotene when alone was observed post-gastric phase, but no additional significant
223 loss was observed through the duodenal and jejunal phases. When ferrous sulfate was added
224 together with β -carotene, this resulted in a ~40% β -carotene loss during the gastric phase, but no
225 further loss through the duodenal and jejunal phases was observed. By comparison, when
226 metmyoglobin was added to β -carotene, a 50% β -carotene loss was observed during the gastric
227 phase and a further 30% loss from initial β -carotene level during the duodenal phase of digestion.

228 No significant change in % *cis*-isomers was observed between the starting material and the
229 jejunal phase for any of the 3 carotenoids tested. Thus, the bars in Figures 1, 2, and 3 represent
230 total carotenoid (i.e. the summation of all-*trans* + *cis* isomers) at each stage.

231 **4. Discussion**

232 The relative susceptibility of these three carotenoids to loss under various conditions of
233 digestion presents some intriguing results. We begin by comparing the behavior of carotenoids
234 alone digested without enzymes, as compared to carotenoids digested with enzymes (solid bars, A
235 vs. B in Figures 1, 2, and 3). Our experiments clearly reveal that a greater percentage of lutein and
236 lycopene survived the gastric and duodenal phases of digestion in the presence of enzymes, as
237 compared to the experiments without enzymes. In the experiments with enzymes, lipases and bile
238 salts work in concert to reduce the size of emulsified lipid droplets and create mixed micelles
239 during the duodenal phase.³⁹ In contrast, the digestion experiments without enzymes were
240 performed to better understand the impact of chemical conditions alone on carotenoid degradation.
241 Thus, these experiments contained the emulsified test meal throughout each phase. Studies on the
242 stability of β -carotene^{40,41} and lutein⁴⁰ emulsions have demonstrated that carotenoid stability is
243 directly proportional to lipid droplet size. Consequently, we expected the larger emulsified lipid
244 droplets produced in the experiments without enzyme to be more protective than small particle
245 micelles in the experiments with enzymes. Therefore, it was initially quite surprising to observe
246 no difference in β -carotene degradation, and a more rapid lutein and lycopene degradation in the
247 experiments without enzymes. However, the enzyme experiments also contained various proteins,
248 such as amylase during the initial sample treatment, pepsin added during the gastric phase, and the
249 porcine pancreatin preparation added during the duodenal phase. Previous *in vitro* studies have
250 demonstrated that certain whey proteins further protect β -carotene⁴¹ and lutein⁴⁰ from degradation

251 in emulsification model systems, as these proteins are believed to rest at the interface between the
252 lipid droplet and the aqueous solution.⁴¹ Similarly, a whey-protein encapsulated lycopene has been
253 shown to successfully deliver lycopene in humans, presumably via at least partial protection of
254 lycopene through gastro-intestinal conditions.⁴² In short, our results suggest that protein solutions
255 confer additional protection to lutein and lycopene against chemical oxidation during the gastric
256 and duodenal phases of digestion, but any advantage gleaned from protein is lost during the jejunal
257 phase (likely due to carotenoid transfer to and retention in less-protective micelles).

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

268 Of those tested, lycopene alone was found to be the most stable carotenoid through the
269 entire digestive process with enzymes, with a 20% loss overall. In contrast, 40% of lutein alone
270 and β -carotene alone were lost during their respective digestions with enzymes. The lycopene and
271 β -carotene results are in good agreement with Blanquet-Diot et al., who reported a 20% and ~30%
272 loss, respectively using a dynamic *in vitro* digestion model.⁴³ In contrast, Courraud et al., reported
273 a 50% loss of β -carotene after the gastric phase, and a further 20% loss during the intestinal

274 phase⁴⁴. These differences in β -carotene stability are likely due to Courraud et al.'s direct
275 application of β -carotene dissolved in acetone to the mimicked digesta, and a much lower pH of 2
276 used during a prolonged gastric phase.⁴⁴ Lutein loss was similar to that reported by Courraud et al.
277 in raw spinach.⁴⁴ Other studies by Blanquet-Diot et al., and Chitchumroonchokchai et al., observed
278 no change in lutein levels over the course of *in vitro* digestion^{43,45}. It is likely that the use of a
279 tomato food matrix⁴³ and the absence of a jejunal digestion phase⁴⁵ are the source of this
280 divergence.

281 Regardless of the presence or absence of enzymes, ferrous sulfate had no additional impact
282 on carotenoid loss during the experiments (white bars, Figures 1, 2, and 3). Previous work by our
283 group has demonstrated that in a gastric-like micellar model, increasing levels of ferrous iron
284 relative to β -carotene (0-10 equivalents) result in increasing levels of β -carotene oxidation, as
285 compared to no iron. However, *excess* levels of ferrous iron relative to β -carotene (50 equivalents)
286 resulted in an initial oxidative loss of β -carotene over 5-10 min, but surprisingly less β -carotene
287 oxidation through 120 min. It was postulated that when ferrous iron is present in excess, various
288 perferryl and ferryl intermediates are formed, which further react with ferrous iron to produce high
289 levels of a diferric ether product, which is inert.¹⁵ The dose of ferrous sulfate and carotenoid used
290 in this study were calculated to reproduce a plausible scenario of a single iron tablet prescribed as
291 a supplement (delivering 80 mg of divalent iron) consumed with a meal containing 20 mg
292 carotenoid. Under these conditions, the iron to carotenoid equivalent is 37 for meals containing
293 lycopene or β -carotene + ferrous sulfate, and 39 for meals containing lutein + ferrous sulfate. Based
294 upon our results, we speculate that the ferrous sulfate levels were sufficiently high to result in the
295 formation of inert diferric ether, resulting in no additional carotenoid oxidation in these groups. It

296 is also possible that the gelatin coating and maltodextrin excipient of the iron tablet exerted a
297 protective carotenoid effect during the initial phase of digestion.⁴⁶

298 The concentration of metmyoglobin used in our studies (40 μ M) is reflective of the quantity
299 expected from the consumption of a 255 g (9 oz.) beef steak (estimated to deliver 30-50 μ M
300 metmyoglobin).⁴⁷⁻⁴⁹ In the *in vitro* experiments with enzymes, metmyoglobin had no impact on
301 lutein stability (striped bars, Figure 1B), but a more pronounced impact on carotene stability
302 (striped bars, Figures 2B and 3B), especially that of β -carotene. These results are in good
303 agreement with similar work observing the capacity of various carotenoids to inhibit
304 metmyoglobin-induced peroxidation of linoleic acid in an emulsion model.¹⁶ Inhibitory concentrations
305 at 50% of maximal linoleic acid peroxidation (IC₅₀) values were measured, and were shown to
306 follow the order of β -carotene > lycopene > xanthophylls derived from bacteria.¹⁶ In other words,
307 a larger concentration of β -carotene was needed to protect linoleic acid relative to the other
308 carotenoids. Further measures of the peroxide kinetics suggested that this difference may lie in the
309 fact that carotenes sit in the center of the lipid droplet, and thus their only mechanism of defense
310 is to halt further fatty acid peroxide propagation. In contrast, bacterial xanthophylls sit at the lipid-
311 water interface and likely inhibit peroxidation initiation altogether.¹⁶ It is not clear why β -carotene
312 was more sensitive than lycopene to metmyoglobin-induced oxidation. It is possible that at the
313 concentrations of lycopene used, lycopene microcrystals may have formed in the digesta mixture⁵⁰,
314 which may have conferred physico-chemical protection, while β -carotene likely remained
315 dissolved.

316 We intentionally used un-stripped sunflower oil in all of our experiments to create a
317 realistic liquid beverage that would be safe for use in a human clinical study. However, the
318 endogenous tocopherols and tocotrienols present likely protected carotenoids and reduced losses

319 due to oxidation, as studies have previously demonstrated that alpha-tocopherol protects
320 lycopene^{51,52}, β -carotene⁵³, and lutein⁴⁰ from various forms of oxidation in emulsion systems.

321 It should be noted that under typical digestive conditions (i.e. with enzymes), levels of lutein
322 alone and lycopene alone were not significantly different from the initial meal through the
323 duodenal phase. Immunohistochemical staining demonstrates the presence of apical membrane
324 transporters SR-B1 and CD-36 in the duodenum and jejunum of humans⁵⁴, with Western blotting
325 showing significantly higher SR-B1 protein expression in the duodenum⁵⁵ and higher expression
326 of CD-36 in the jejunum⁵⁶ of rodents. Thus, we anticipate a fair portion of lutein and lycopene to
327 be absorbed in the duodenum (before degradation), and any resulting loss in the jejunum would
328 probably have a limited impact on overall bioavailability. However, our results suggest that partial
329 degradation of β -carotene alone under these types of meal conditions may ultimately reduce the
330 quantity of β -carotene that is bioavailable.

331 We noted no significant change from the initial % *cis* isomers to the % *cis* isomers remaining
332 in jejunal phase. These results are in good agreement with previous studies using static *in vitro*
333 digestion, which have demonstrated that the % *cis* isomer contribution to total carotenoid content
334 is well maintained for lycopene^{57,58}, lutein⁵⁹, and β -carotene.⁶⁰ These results are further
335 corroborated with human studies on gastric digesta for lycopene and β -carotene.⁶¹ The same
336 human study also investigated duodenal digesta, and found no change in lycopene but a slight
337 increase in % *cis* β -carotene (relative to starting % *cis* β -carotene).⁶¹ We would attribute this
338 disparity to the dynamic nature of absorption in humans, and transporter selectivity for all-*trans*-
339 β -carotene.⁶²

340 While our primary objective was to understand carotenoid loss during digestion, we also
341 anticipated observing carotenoid degradation products in our samples. Indeed, the extraction and

342 HPLC-PDA methods employed for the analysis of β -carotene- and lycopene-meal digesta would
343 have revealed the presence of β -apo-carotenals and apo-lycopenals, respectively, if they had been
344 present in sufficiently high quantities for PDA detection. Furthermore, previous work by our group
345 has demonstrated that under certain mimicked conditions of gastric digestion and in the presence
346 of metmyoglobin, β -apo-carotenals can be observed by PDA.¹⁵ However, no oxidation products
347 were observed in any of the samples in this study. This discrepancy with previous work may be
348 due to the previous use of non-biological surfactants (Tween 20 and Brij 35) to create micelle-like
349 conditions.¹⁵ Similarly, aldehydes have the capacity to react irreversibly with proteins to form
350 Schiff bases⁶³ and thus it is possible that aldehyde products were formed in our study, but were
351 not able to be extracted due to protein sequestration. While apo-carotenaldehydes were not
352 observed, the loss of lutein and lycopene in the jejunal phase, and the immediate loss of β -carotene
353 in the gastric phase, indicate that at least some derivatives were created. Further work is needed to
354 identify these products, and to determine if they are absorbed, and/or if they might have biological
355 effects in the jejunum or colon.

356 In conclusion, some of the chemical factors that reduce carotenoid stability in an *in vitro*
357 emulsion model are counteracted in the presence of digestive enzymes. In the presence of enzymes,
358 lutein and lycopene were robust to the effects of the digestive process throughout the gastric and
359 duodenal phases, with the most pronounced losses occurring during the jejunal phase. β -carotene
360 was most susceptible to degradation during the gastric phase, but no further loss was observed.
361 Our data suggests that a commonly prescribed dose of supplemental iron consumed with a
362 carotenoid-containing meal does not have an impact on carotenoid stability. However, co-
363 consumption of red meat with lycopene- or β -carotene-containing foods is likely to reduce the
364 quantity of carotenoid available for absorption, and may generate carotenoid oxidation products.

365 Further work is needed to determine which products are produced, and whether they have
366 biologically relevant actions.

367

368 **Conflict of Interest**

369 There are no conflicts of interest to declare.

370

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379

380 **Abbreviations:**

381 DCM dichloromethane

382 HPLC high-performance liquid chromatography

383 LC-MS liquid chromatography-mass spectrometry

384 MeOH methanol

385 MTBE methyl *tert*-butyl ether

386 PDA photodiode array detection

387

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558

559 **Table 1.** Composition of the test meals

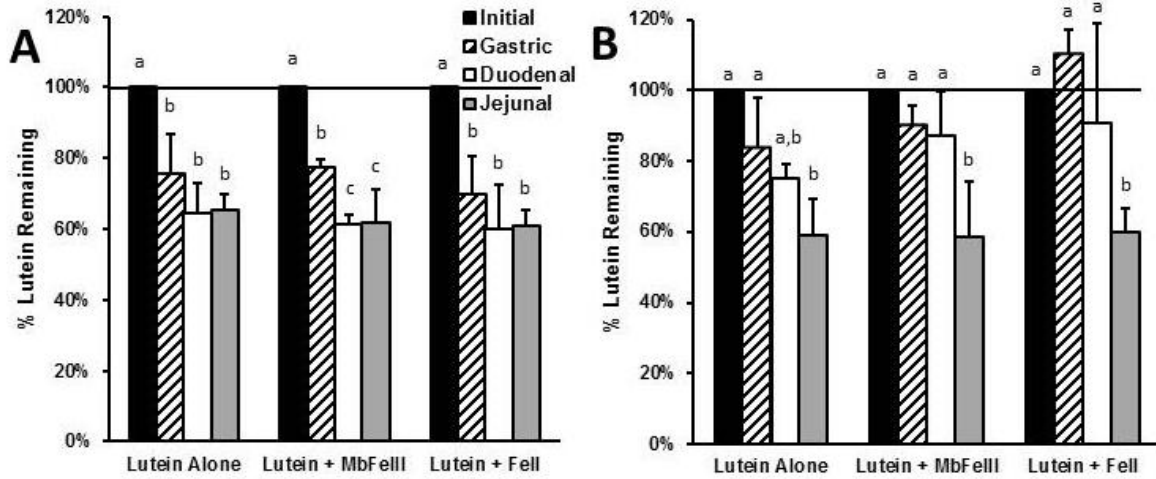
Water	90 g
Sucrose	9 g
Phospholipid containing powder ^{a, b}	150 mg
Sunflower oil	3 g
Carotenoid-oil preparation ^b	0.3 g

560 a) Delivering 75 mg of phospholipid derived from egg, to match the 1:40 ratio of
561 phospholipid to lipid representative of a western diet⁶⁴.

562 b) See Materials and Methods for additional details.

563

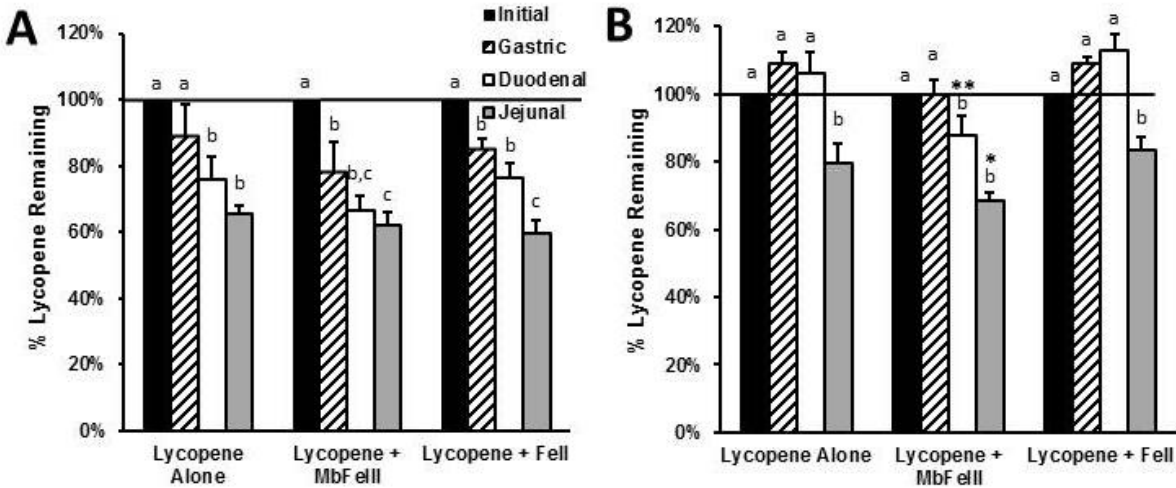
564



565

566 **Figure 1.** Loss of initial lutein through the gastric, duodenal, and jejunal phases of digestion in
 567 the absence (A) and presence (B) of digestive enzymes. Bars represent average % lutein \pm
 568 standard deviation (n=3 for each meal). Different letters indicate statistically significant
 569 differences within the same meal at different digestion phases ($P < 0.05$). There was no
 570 significant difference between meal types at any of the digestion phases ($P < 0.05$).

571



572

573 **Figure 2.** Loss of initial lycopene through the gastric, duodenal, and jejunal phases of digestion

574 in the absence (A) and presence (B) of digestive enzymes. Bars represent average % lycopene ±

575 standard deviation (n=3 for each meal). Different letters indicate statistically significant

576 differences within the same meal at different digestion phases ($P < 0.05$). Statistically significant

577 differences between meal types in the same digestion phase are noted with an asterisk, described

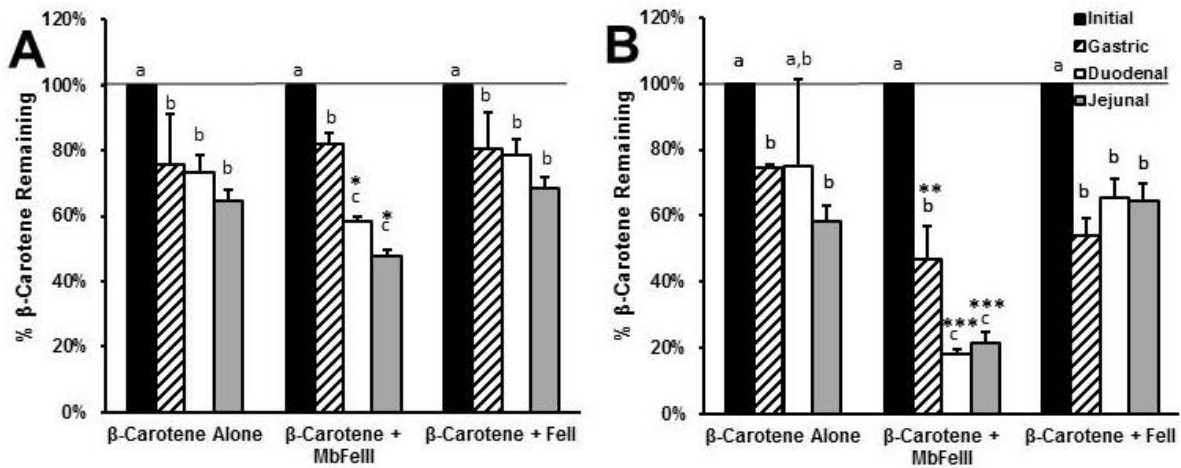
578 below.

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

580 **Significantly different from Lycopene Alone and Lycopene + FeII after the same digestion

581 phase ($P < 0.0001$)

582



583

584 **Figure 3.** Loss of initial β -carotene through the gastric, duodenal, and jejunal phases of digestion
 585 in the absence (A) and presence (B) of digestive enzymes. Bars represent average % β -carotene \pm
 586 standard deviation (n=3 for each meal). Different letters indicate statistically significant
 587 differences within the same meal at different digestion phases ($P < 0.05$). Statistically significant
 588 differences between meal types in the same digestion phase are noted with an asterisk, described
 589 below.

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

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

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

594