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# The effect of an iron supplement on lycopene metabolism and absorption during digestion in healthy humans

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**Keywords:** carotenoid, apo-lycopenals/apo-lycopenoids, gastrointestinal lumen, ferrous sulfate, bioavailability

**Abbreviations:** AUC = Area under the time-versus-concentration curve, BCO2 =  $\beta$ -carotene oxygenase 2, DCM = dichloromethane, EDTA = ethylenediaminetetraacetic acid, LC-MS/MS = high pressure liquid chromatography-tandem mass spectrometry, methanol = MeOH, methyl *tert*-butyl ether = MTBE, TRL = triglyceride-rich lipoprotein fraction

36

37

38 **ABSTRACT**

39 *Scope:* To investigate the formation and absorption of lycopene (LYC) metabolites in the human  
40 upper gastrointestinal lumen, in the absence and presence of iron.

41 *Methods:* Healthy males (n=7) consumed test meals delivering ~22 mg LYC + ~0.3 mg apo-  
42 lycopenals from oleoresin without (-FeSO<sub>4</sub>) and with ferrous sulfate (160 mg, +FeSO<sub>4</sub>).

43 Subjects were intubated with a naso-gastric/naso-duodenal tube. Digesta, blood plasma, and the  
44 triglyceride-rich lipoprotein (TRL) fractions of plasma were analyzed using LC-MS/MS, to  
45 measure lycopene and apo-lycopenoids.

46 *Results:* Digesta LYC concentrations increased with time ( $P = 1.2 \times 10^{-7}$ ), decreased with  
47 time\*iron ( $P = 1.1 \times 10^{-5}$ ), and remained ~200x higher than apo-lycopenals/lycopenone. Digesta  
48 apo-8', -10', -12', -14', -15-lycopenal and apo-13-lycopenone concentrations increased with  
49 time ( $P < 0.01$ ), apo-12', -14', -15-lycopenal, apo-13-lycopenone increased with iron ( $P < 0.05$ ),  
50 and time\*iron decreased apo-8', -10', -12', -14', -15-lycopenal, apo-13-lycopenone  
51 concentrations ( $P < 0.01$ ). A 1.9-fold decrease in LYC TRL area-under-the-time-concentration-  
52 curve was observed after the test meal +FeSO<sub>4</sub> vs. the test meal -FeSO<sub>4</sub> ( $P = 0.02$ ). Apo-  
53 lycopenals were detected in later TRL fractions. No apo-lycopenols or apo-lycopenoic acids  
54 were observed in any samples.

55 *Conclusions:* FeSO<sub>4</sub> reduces lycopene absorption. Apo-lycopenals appear to be absorbed from  
56 foods, and not made in significant quantities during digestion.

57

## 58 INTRODUCTION

59 Numerous studies, previously reviewed<sup>[1]</sup>, have associated increased lycopene (LYC)  
60 consumption with reduced chronic disease risk. However, only a small portion of LYC, the  
61 pigment conferring red hues to tomatoes, watermelon, and pink grapefruit, is absorbed by  
62 humans. Under even the most ideal meal conditions, not more than 23%-33.9% is taken up.<sup>[2, 3]</sup>  
63 Low solubility resulting from its physical-chemical properties reduces absorption,<sup>[4]</sup> and a  
64 portion may also be lost to enzymatic or chemical degradation during digestion. Trace metals in  
65 water or meal co-factors like iron, which remain active in the acidic environment of the stomach,  
66 have been shown to induce lipid and LYC oxidation and co-oxidation in some,<sup>[5-9]</sup> but not all,<sup>[10]</sup>  
67 *in vitro* models of digestive conditions. Likewise, body temperature, peristaltic gastric  
68 contractions, the presence of oxygen and lipid or peroxy radicals, and the shift from immiscible  
69 liquid phases to an emulsion system together favor LYC oxidation.<sup>[11, 12]</sup> In studies showing  
70 oxidation under digestive conditions, the LYC is ultimately consumed by oxidative reactions,<sup>[8,</sup>  
71 <sup>13, 14]</sup> and metabolites are presumably formed in the process.

72 Limited LYC absorption also suggests that the portion remaining in the digestive tract  
73 could protect the gastrointestinal system from oxidative damage. Higher intakes of lycopene  
74 have been associated with a reduced fecal blood, mucus and pus in subjects with ulcerative  
75 colitis in remission.<sup>[15]</sup> Likewise, animal studies of colitis have demonstrated a reduction in  
76 myeloperoxidase, a marker of colon inflammation, after lycopene administration both with<sup>[16]</sup> or  
77 without<sup>[17]</sup> iron. One plausible mechanism of action is that LYC acts directly to protect the  
78 gastrointestinal lumen. However, it is also possible that LYC is converted to bioactive  
79 metabolites<sup>[18, 19]</sup> in the intestinal tract which confer protection.<sup>[20, 21]</sup> Recombinant studies have  
80 demonstrated that recombinant  $\beta$ -carotene oxygenase 1 (BCO1) obtained from humans cleaves

81 LYC *in vitro*,<sup>[22]</sup> and the enzyme  $\beta$ -carotene oxygenase 2 (BCO2) obtained from mouse and ferret  
82 has the ability to cleave LYC,<sup>[23]</sup> with a preference for *cis*-LYC isomers.<sup>[24]</sup> Its expression by  
83 human intestinal cells further supports the hypothesis that it may act during digestion.<sup>[25]</sup> Indeed,  
84 LYC metabolites have been found circulating in human plasma,<sup>[26-28]</sup> although the origin of these  
85 metabolites has not been established.

86 *In vitro* studies reveal ~20% loss of LYC over the digestive period,<sup>[10, 29]</sup> regardless of  
87 presence or absence of iron. However, *in vivo* studies of the loss of LYC during digestion in the  
88 presence of dietary iron have not been performed. Likewise, the formation and potential  
89 absorption of LYC metabolites made during digestion in healthy humans has not been observed.  
90 Thus, the primary objective of this study was to follow LYC metabolism in the upper digestive  
91 tract of healthy humans, in the absence and presence of dietary iron. Postprandial concentrations  
92 of LYC and apo-lycopenoid metabolites (i.e. apo-lycopenals, apo-lycopenols, or apo-lycopenoic  
93 acids) in the gastrointestinal lumen and in the chylomicron-rich triglyceride-lipoprotein (TRL)  
94 fraction of blood (representing newly absorbed lipophilic analytes<sup>[30]</sup>) were measured.

95

## 96 MATERIALS & METHODS

### 97 *Chemicals*

98 Food-grade oleoresin (4.5% lycopene by weight, product number 1EAA9272) was kindly  
99 provided by Dr. Simona Birtic of Naturex (Montfavet, France). The oleoresin was extracted  
100 from tomato and consisted of tomato phospholipids, in addition to minor amounts of tocopherols,  
101 phytoene and phytofluene. The isomer composition was 78% all-*trans*, 11% 5-*cis*, and 11% as  
102 other *cis*-isomers. MS grade methanol and LC grade methyl tert-butyl ether (MTBE) were  
103 purchased from Fisher Scientific (Illkirch, France). Dichloromethane (DCM) was purchased  
104 from Fisher Scientific (Pittsburgh, PA, USA). Double deionized water was produced by a  
105 Millipore Q-Plus. MS grade formic acid (>98% pure) was purchased from Sigma-Aldrich  
106 (Saint-Quentin-Fallavier, France). Apo-6'-, -8'-, and -12'-lycopenal standards were purchased  
107 from CaroteNature (Münsingen, Switzerland). Apo-10'-lycopenal, apo-10'-lycopenol, apo-10'-  
108 lycopenoic acid, apo-14'-lycopenal, apo-14'-lycopenol, apo-14'-lycopenoic acid, apo-11-  
109 lycopenal standards were synthesized previously.<sup>[31]</sup> The glycerol phosphate oxidase assay was  
110 from Abliance (Compiègne, France).

111 Sunflower oil, white granular sugar, and demineralized water were purchased at local  
112 supermarkets (Avignon and Marseille, France). Lecico, Inc. kindly donated the Ovolife IF 50  
113 phospholipid (Hamburg, Germany), consisting of 50% *w/w* phospholipid derived from egg yolk.  
114 The ferrous sulfate (FeSO<sub>4</sub>) tablet (Tardyferon®, 80 mg of iron per capsule) was purchased from  
115 a local pharmacy and powdered before blending with the test meal, described below.

### 116 *Preparation of LYC test meals*

117 The LYC-containing oleoresin was mixed with sunflower oil, aliquoted into amber glass  
118 ampoules (targeting 10 mg LYC + 0.25 mg apo-lycopenals/apo-lycopenone in 2.5 g total oil),

119 closed under argon, and stored under refrigeration (4° C) before test meal preparation. For iron-  
120 containing meals, Tardyferon® tablets were finely powdered and the equivalent of 2 tablets  
121 sealed in separate amber ampoules under argon.

122 Test and control meal preparations and macronutrient composition were previously  
123 published,<sup>[32]</sup> with LYC replacing <sup>13</sup>C β-carotene for this study. For test meals containing iron  
124 (i.e. +FeSO<sub>4</sub>), just before serving, an ampoule of powdered Tardyferon® (delivering 160 mg  
125 Fe<sup>+2</sup>) was added, and the meal blended for an additional 30 s.

126 Because LYC crystals in the oleoresin complicated the aliquoting of LYC-rich oil into  
127 each ampoule, samples of each individual test meal (0.5 mL) were taken to measure the final  
128 concentration of LYC and apo-lycopenoids dosed (see **Table 1**).

### 129 *Clinical Subjects and Experimental Design*

130 The study was performed at the Clinical Investigation Center of the Hôpital de la  
131 Conception in Marseille, France. The regional Institutional Review Board Comité de Protection  
132 des Personnes Sud Méditerranée I, France (Protocol #2013-A01398-37) approved the study,  
133 which was in accordance with the Declaration of Helsinki of 1975 as revised in 1983. Further  
134 details are provided at [clinicaltrials.gov](https://clinicaltrials.gov), study #NCT03492593. Baseline characteristics of the  
135 subjects are provided in **Table 2**. Criteria of inclusion and exclusion match those published  
136 previously,<sup>[32]</sup> with an additional exclusion criteria of LYC supplement consumption in the last 3  
137 mo. Written, informed consent was obtained from all subjects before participation. Twelve  
138 healthy male subjects were screened for the study, with 9 subjects meeting the  
139 inclusion/exclusion criteria and randomly assigned to consume the test or control meals by the  
140 study coordinator (n = 7 for LYC-containing test meals, n=2 for control meals, i.e. the test meal  
141 with sunflower oil in place of the LYC-rich oleoresin). Sample size was estimated based on

142 previously published work of the study of carotenoids and vitamins A and E in the human  
143 digestive tract.<sup>[33, 34]</sup>

144 Overnight fasted subjects arrived at the clinic, and a nasoduodenal tube was inserted and  
145 positioned in the antrum of the stomach or the inferior descending duodenum, as confirmed by  
146 X-ray. Afterward, a blood sample (0 h) was taken. Test meal subjects then consumed either the  
147 liquid test meal alone (i.e. -FeSO<sub>4</sub>) or liquid test meal +FeSO<sub>4</sub>. Likewise, control meal subjects  
148 consumed a liquid control meal -FeSO<sub>4</sub>, or a liquid control meal +FeSO<sub>4</sub>. Meals were drunk in  
149 5 min. and chased with demineralized water (50 mL) used to rinse the meal preparation  
150 glassware. Samples of digesta (either gastric or duodenal) were taken as previously described<sup>[32]</sup>  
151 at 30 min, 1, 2, 3, and 4 hours postprandially. Exact digesta sample volume was measured, to  
152 which an aqueous “inhibitor” solution (containing 0.5% pyrogallol and 0.7% EDTA) was added  
153 at half the volume. Samples were immediately vortexed, aliquoted, and stored at -80 °C. Plasma  
154 samples were taken at 0, 1, 2, 4, 5, 6, and 7 h, and TRL fractions were prepared as previously  
155 described.<sup>[32]</sup> Samples were transported on dry ice between facilities, and held at -80 °C until  
156 extraction. Subjects consumed the same meal -FeSO<sub>4</sub> twice, and the same meal +FeSO<sub>4</sub> twice  
157 (once to obtain gastric digesta and a second time to obtain duodenal digesta, randomized for  
158 order). Because subjects were required to wait  $\geq 2$  weeks between the consumption of each  
159 meal, the total study period for each subject was  $\geq 6$  weeks.

#### 160 *Digesta, Plasma, and TRL extraction*

161 Samples were thawed for 10 min in cold tap water, and extracted as previously  
162 described.<sup>[32]</sup> Extracts were immediately dried under argon and stored for  $\leq 2$  days at -80 °C  
163 before LC-MS/MS analysis.

#### 164 *LC-MS/MS analyses*



165 Extracts of digesta, plasma, TRL and test meal were redissolved in 1:1 MTBE/MeOH,  
166 and analyzed using the same LC-MS parameters described previously,[32] except for those noted  
167 below. The gradient method began with 0% B, holding 0% B for 1 min, increasing to 30% B  
168 over 4 min, increasing to 55% B over 3 min., increasing to 90% B over 7 min, increasing to  
169 100% B over 2 min., holding at 100% B for 4 min, and returning and holding 0% B for 3 min.  
170 Quadrupole 1 resolution = 0.7 units, quadrupole 3 resolution = 2.0 units, for all  
171 precursor>product combinations monitored (provided in **Supplemental Table 1**).

172 Identities of apo-6'-, -8'-, -10'-, -12'-, -14'- and -11-lycopenal, apo-10'-, -14'- and apo-10'-,  
173 -14'-lycopenoic acid were confirmed with retention time and precursor > product ions coincident  
174 with authentic standards, and quantitated with external standard curves. The retention times,  
175 precursor ions, and product ions of apo-15-lycopenal and apo-13-lycopenone were determined  
176 using a LYC extract rich in apo-lycopenals, and quantitated using apo-14'-lycopenal equivalents.  
177 Putative retention times, precursor and product ions of the remaining apo-lycopenols and apo-  
178 lycopenoic acids *not* available as authentic standards were determined by studying ionization,  
179 fragmentation, and retention time behavior of the available acids and alcohols (see **Online**  
180 **Supplemental Material** and **Supplemental Table 1** for additional details of quantitation).

#### 181 *Caco-2 cell experiments*

182 LYC was oxidized as previously described.<sup>[35]</sup> Approximately 0.4 mg of the resulting  
183 apo-lycopenals/lycopenone mixture was solubilized in chloroform (0.5 mL) before incorporation  
184 into artificial mixed micelles composed of oleic acids, bile salts,  $\alpha$ -tocopherol, and  
185 phosphatidylcholines, as previously described.<sup>[36]</sup> The mixed micelles were diluted 1:4 with  
186 DMEM containing 500  $\mu$ mol/L phenol red, and the solution immediately incubated with Caco-2  
187 cells (2 mL/well), as previously described.<sup>[36]</sup>

188 Caco-2 human intestinal cells (HTB 37, passages 27-31) were grown in 6-well plates on  
189 membrane inserts for 21 days after reaching confluence. The freshly prepared DMEM containing  
190 the apo-lycopenals/lycopenone micelle solution was applied to the apical surface of the cells and  
191 incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air for 7 h. Monolayer integrity was  
192 monitored by phenol red transfer from the apical to basolateral compartments, as detailed  
193 previously.<sup>[37]</sup> After incubation, the apical and basolateral media, as well as the washed cell  
194 monolayer, were collected and frozen at -80 °C.

195 The apical and basolateral fractions were extracted as described in *Digesta, Plasma, and*  
196 *TRL extraction*. Cells were extracted by combining with an equal volume of MeOH and probe  
197 sonication (Microson XL-2000, Misonix, Farmingdale, NY) for 30 sec, placed on ice for 1 min,  
198 repeated twice more. Then DCM (4 parts) was added, followed by 30 sec of probe sonication and  
199 centrifugation (Allegra 6R, Beckman Coulter, Indianapolis IL) at 4 °C for 5 min at 162 x g. The  
200 lower DCM layer was transferred to a clean glass vial and the extraction repeated, with DCM  
201 layers combined. All extracts were dried under argon gas and analyzed immediately using the  
202 same LC-MS/MS method described on a Vanquish UHPLC interfaced with a Quantiva triple  
203 quadrupole (Thermo Fisher Scientific, Waltham, MA). Species were identified by comparison  
204 with an oxidized apo-lycopenal/lycopenone mixture.<sup>[26]</sup>

#### 205 *Statistics*

206 Analyses were performed using R, version 3.1.0.<sup>[38]</sup> Visual inspection of residual plots  
207 confirmed assumptions of homoscedasticity and normality. LYC bioavailability in the TRL  
208 fraction was baseline-corrected before calculation of the area under the time-concentration curve  
209 (AUC) using trapezoidal approximation. To determine the effect of iron on LYC bioavailability  
210 within the same subject, AUC values of the test meal -FeSO<sub>4</sub> vs. the test meal +FeSO<sub>4</sub> were

211 compared using a paired t-test, with  $P < 0.05$  considered statistically significant. Repeated  
212 measures ANOVA assessed the effect of time, iron, and time\* iron on the percentage of all-  
213 *trans*-, *5-cis*-, and other-*cis*-LYC isomers in the TRL fraction.<sup>[10]</sup> Because of the absence of  
214 duodenal digesta samples in different subjects (due to inability to aspirate sample at that time  
215 point), a mixed model was chosen to determine the influence of digesta type, time, iron, and  
216 time\*iron on metabolite formation. The lme4 package with mixed effects regression<sup>[39]</sup> was used  
217 to determine the relationship between digesta type, time, iron, and time\*iron on the percentage of  
218 all-*trans*-, *5-cis*, and other-*cis*-LYC to total LYC in digesta. The relationship between digesta  
219 type, time, iron, and time\*iron on the absolute values of total LYC, LYC isomers, and apo-  
220 lycopinals/apo- lycopene in digesta was also determined using this approach. Fixed effects  
221 included sampling time (0, 0.5, 1, 2, 3, 4 h), digesta type (gastric or duodenal), and iron (+FeSO<sub>4</sub>  
222 or -FeSO<sub>4</sub>) were considered, and the mixed effect time\*iron was modeled as a new variable.  
223 Random effects included subject, for which individual intercepts were determined. The  
224 likelihood ratio test (LRT) of the full model was compared to the model minus the effect in  
225 question to understand the influence of the fixed effects on the goodness of fit of the model, with  
226  $P < 0.05$  statistically significant. To calculate percentage loss between the two meals, LYC  
227 digesta levels of the test meal -FeSO<sub>4</sub> vs. the test meal +FeSO<sub>4</sub> within the gastric and duodenal  
228 compartments were made using AUC comparisons ( $P < 0.05$ ).  
229

## 230 RESULTS

### 231 *Subject Recruitment and Sample Collection*

232 **Supplemental Figure 1** provides an overview of subject randomization, sampling, and  
233 attrition.

### 234 *LC-MS/MS method development*

235 Details are provided in **Online Supplemental Material** and **Supplemental Figures 2, 3,**  
236 **and 4**. The method limit of detection (LOD), defined as signal/noise = 3, was ~0.4 fmol on  
237 column for apo-6'-, -10'-lycopenal, and ~2 fmol on column for apo-8'-, -12'-, -14'-lycopenal.

### 238 *Digesta concentrations of LYC and apo-lycopenals/apo-lycopenone*

239 Digesta concentrations of total LYC increased 4.5-fold in the test meal -FeSO<sub>4</sub> and  
240 remained the same in the test meal + FeSO<sub>4</sub>, between initial concentration (t = 0) and after 4 h of  
241 digestion (**Figure 1A**). Over the course of 4 hours of digestion, the test meal +FeSO<sub>4</sub> resulted in  
242 44% less LYC in the gastric compartment and 66% less LYC in the duodenal compartment, as  
243 compared to the test meal -FeSO<sub>4</sub> (as determined by comparing digesta AUC).

244 With regards to isomer composition, the test meal -FS delivered 84.4% of the LYC dose  
245 as all-*trans*-, 8.7% as 5-*cis*-, and 6.9% as other-*cis*-LYC. The test meal +FeSO<sub>4</sub> delivered 83.8%  
246 of the LYC dose as all-*trans*-, 7.9% as 5-*cis*-, and 8.3% as other-*cis*-LYC. There was no  
247 statistical significance between the LYC isomer composition between the two test meals,  
248 although the absolute level of LYC delivered was higher in the test meal -FeSO<sub>4</sub> as compared to  
249 the test meal +FeSO<sub>4</sub> (**Table 1**). There was no significant change in LYC isomer profile within a  
250 subject over time, regardless of the presence or absence of iron, or the digesta type sampled.

251 The test meal also delivered apo-lycopenals/apo-lycopenone, noted in **Table 1**. The test  
252 meal +FS delivered significantly more apo-10'-, -12'-, -14'-, -15-lycopenal and apo-13-

253 lycopene as compared to the test meal  $-FeSO_4$  ( $P < 0.05$ ). **Figure 1A** shows the  
254 concentrations of total LYC and **Figure 1B-H** each of the apo-lycopenals/apo-lycopenone in the  
255 gastric and duodenal digesta samples after the test meal fed  $-FS$  and  $+FS$ . A breakdown of  
256 digesta LYC by isomer type is shown in **Supplemental Figure 5**. The mixed modeling results  
257 are provided in **Table 3**. Across all subjects, time was positively associated with the digesta  
258 concentrations of total LYC, as well as the concentrations of each isomer individually. There was  
259 a non-significant trend of total LYC concentration being higher in gastric digesta relative to  
260 duodenal digesta. In contrast, the interaction factor of time\*iron was significantly negatively  
261 associated with digesta total LYC concentrations and the concentrations of each individual  
262 isomer.

263         Digesta concentrations of apo-lycopenals/apo-lycopenone increased up to 9-fold more  
264 than initial test meal concentrations at their peak (**Figure 1B-H**), however they remained ~200  
265 fold lower than LYC concentration in the same samples. A non-significant trend was seen for  
266 gastric digesta type positively associated with the concentrations of apo-8', -12'-lycopenal and  
267 apo-13-lycopenone (**Table 3**). The concentrations of digesta apo-8', -10', -12', -14', -15-  
268 lycopene and apo-13-lycopenone were significantly increased with time. Iron was significantly  
269 associated with increased digesta concentrations of the shorter apo products, i.e. apo-12', -14', -  
270 15-lycopenal and apo-13-lycopenone. In contrast, the time\*iron effect was significantly  
271 associated with decreased concentrations of apo-8', -10', -12', -14', -15-lycopenal and apo-13-  
272 lycopene. Interestingly, no factors were significantly associated with digesta concentrations of  
273 apo-6'-lycopenal.

274 *Plasma and TRL concentrations of LYC and apo-lycopenals/lycopenone*

275 LYC was observed circulating in the fasted plasma of all subjects ( $0.43 \pm 0.31 \mu\text{mol/L}$ ).  
276 Similarly, apo-lycopenals/apo-lycopenone, but no apo-lycopenols nor apo-lycopenoic acids,  
277 were detected in the plasma of control meal and test meal subjects, and concentrations remained  
278 stable over the 7 h after test-meal consumption. Mean concentrations at 0 h, i.e. after an  
279 overnight fast, for apo-6'-lycopenal =  $0.53 \pm 0.57 \text{ nmol/L}$ , apo-8'-lycopenal =  $0.17 \pm 0.10$   
280 nmol/L, apo-10'-lycopenal =  $0.34 \pm 0.38 \text{ nmol/L}$ , apo-12'-lycopenal =  $0.40 \pm 0.54 \text{ nmol/L}$ , apo-  
281 14'-lycopenal =  $0.74 \pm 0.76 \text{ nmol/L}$ , and apo-13-lycopenone =  $0.47 \pm 0.59 \text{ nmol/L}$ . Apo-15-  
282 lycopenal was only consistently detectible in the plasma of 2 subjects. These levels remained  
283 constant throughout the study.

284 The AUC of total LYC observed over 7 hours in the TRL fraction after test meal -FeSO<sub>4</sub>  
285 consumption was on average  $118 \pm 58.7 \text{ nmol}\cdot\text{h/L}$  plasma, and after test meal +FeSO<sub>4</sub>  
286 consumption was  $62.9 \pm 20.2 \text{ nmol}\cdot\text{h/L}$  plasma (**Figure 2**). This ~1.9 fold lower AUC after test  
287 meal +FeSO<sub>4</sub> consumption was determined to be statistically significant using a paired t-test ( $P =$   
288  $0.0218$ ). It should also be noted that LYC was more bioavailable from the meal -FeSO<sub>4</sub> vs. the  
289 meal + FeSO<sub>4</sub> in 6 of 7 subjects tested. The LYC isomer profile remained fairly stable over 0-7  
290 hours, with a non-significant trend towards an increase in all-*trans*-LYC and a decrease in other-  
291 *cis*-LYC over time ( $P = 0.06$ ) in the TRL fraction of plasma (**Supplemental Figure 6**).

292 Apo-lycopenals/lycopenone were also detected in some of the TRL fractions of plasma in  
293 test meal subjects (but none observed in any TRL fractions of control meal subjects). **Figure 3**  
294 depicts an LC-MS/MS chromatogram of a separated TRL fraction extract at 6 h from one subject  
295 after consuming the test meal -FeSO<sub>4</sub>. Apo-8'-, -10'-, -12'-, -and -14'-lycopenal were detectible  
296 in the TRL of all subjects at hours 5, 6, and 7, however only ~35% of the samples were above  
297 the quantitative signal to noise cutoff (i.e. signal to noise  $\geq 10$ , or  $\geq \sim 67 \text{ pmol/L}$ ). Apo-6'-

298 lycopenal and apo-13-lycopenone were only detected in the TRL fraction a few subjects, and  
299 apo-15-lycopenal was only detected at a few time points in two subjects.

300 Apo-lycopenols and apo-lycopenoic acids were not observed in any of the digesta,  
301 plasma, or TRL extracts. The possibility that apo-lycopenals may be reduced and esterified to  
302 produce apo-lycopenyl esters circulating in plasma was also investigated using a mild  
303 saponification procedure to test plasma from 2 subjects. However, no apo-lycopenols were  
304 observed in these saponified plasma extracts.

#### 305 *Caco-2 cell experiments*

306 The oxidized LYC mixture contained apo-8', -10', -12', -14', -15-lycopenal and apo-  
307 13-lycopenone, but no LYC or apo-6'-lycopenal remained after the reaction. The same  
308 metabolites were visible in the media applied to the apical membrane at 0 h and after 7 h of  
309 incubation (**Supplemental Figure 7**). In the cell and basolateral extracts, the same metabolites  
310 minus apo-10'-lycopenal were observed.

311

## 312 **DISCUSSION**

313           The metabolism of LYC in the upper digestive tract of healthy humans was observed,  
314 exploring the effects of digestion alone and for the first time digestion in the presence of the  
315 common dietary oxidant iron. Ferrous sulfate was chosen because it is prescribed as a  
316 supplemental source for those who are iron deficient,<sup>[40]</sup> and a dose of 160 mg per day is in line  
317 with the 150-200 mg/day dose prescribed by medical professionals for these individuals.<sup>[41]</sup>  
318 Likewise, consumption of inorganic iron from sources also rich in ascorbic acid (like fruits and  
319 vegetables) results in ferric iron being reduced to ferrous iron during digestion.<sup>[42]</sup>

320           An initial decrease in LYC digesta concentrations from 0 h (i.e. test meal) to the first  
321 aspirated sample at 0.5 h was observed, as previously noted in a similar study with  $\beta$ -carotene,<sup>[32]</sup>  
322 and is hypothesized to be a “dilution effect” as endogenous fluid is secreted into the digestive  
323 lumen. After this initial drop, increasing concentrations of LYC in the gastric compartment over  
324 time may be due to LYC floating with lipid droplets in the early stages of digestion,<sup>[43]</sup> and better  
325 access to this lipid phase observed as the compartment is emptied. This result contrasts with a  
326 previous study where tomato puree LYC (10 mg) slowly decreased over 3 h of gastric  
327 digestion.<sup>[33]</sup> Differences could be attributed to differences of LYC localization in the food  
328 matrix, with tomato puree LYC remaining in the food matrix and thus emptied regularly from the  
329 stomach into the duodenum<sup>[33]</sup> as compared to LYC already dissolved in oil. Duodenal digesta  
330 LYC concentration also increased with time, similar to a previous study with  $^{13}\text{C}$   $\beta$ -carotene,<sup>[32]</sup>  
331 and may have occurred as bigger lipid droplets are transferred to the duodenum at the end of the  
332 gastric emptying phase.

333           Over time, the test meal +FeSO<sub>4</sub> led to a significant decrease in digesta total LYC (and  
334 each isomer individually) relative to the test meal –FeSO<sub>4</sub>. This result is in contrast to *in vitro*



335 studies where no change in the loss of LYC (-20%) was observed over 2-3 h, regardless of  
336 presence of  $\text{Fe}^{2+}$ .<sup>[10, 29]</sup> At 2 h, the study herein observed a 15% loss in stomach and a 40% loss  
337 in the duodenum of LYC, after the  $+\text{FeSO}_4$  as compared to the  $-\text{FeSO}_4$  meal (**Figure 1A**),  
338 suggesting oxidation of LYC directly via  $\text{Fe}^{2+}$ , or co-oxidation with meal lipid, did occur in the  
339 human system. Besides oxidation, the  $\text{Fe}^{2+}$  physical-chemical properties as a divalent metal may  
340 also reduce LYC micellarization and uptake, as supported by an *in vitro* study where a dose-wise  
341 reduction in micellarization of LYC was observed with increasing concentrations up to 12.5  
342 mmol  $\text{Fe}^{2+}$ /L digesta.<sup>[44]</sup> Significant reductions were also observed in a human study when LYC  
343 was fed with  $\text{Ca}^{2+}$ , another divalent metal, plausibly due to change in zeta-potential absolute  
344 charge.<sup>[45]</sup> Similarly, complexation of iron with free fatty acids and/or bile salts could also reduce  
345 micelle formation, and ultimately LYC uptake.<sup>[44, 46]</sup> LYC bioavailability (as measured by TRL  
346 AUC) was significantly lower with the test meal  $+\text{FeSO}_4$  as compared to the test meal  $-\text{FeSO}_4$ ,  
347 further supporting the loss of LYC during digestion in the presence of iron. The isomer profile  
348 in the TRL had a non-significant trend towards an increase in all-*trans* LYC and decrease in  
349 other-*cis* LYC over time, confirming previous work demonstrating that the isomer profile is  
350 dependent on the profile of the test meal fed.<sup>[2, 3, 47, 48]</sup>

351 With regard to digesta concentrations of apo-lycopenals/lycopenone, a presumed  
352 “dilution effect” was also observed between the test meal concentrations of apo-lycopenals and  
353 the 0.5 h digesta sampling. Time was positively associated with increasing apo-  
354 lycopenal/lycopenone concentrations for all except apo-6'-lycopenal in digesta, which matches  
355 the trend observed for LYC. Iron was expected to result in greater concentrations of all apo-  
356 lycopenal/lycopenone in the digesta due to increased oxidation of lycopene.<sup>[11, 12]</sup> While iron  
357 increased concentrations in the initial test meal preparation (**Table 1**), iron was positively

358 correlated with only shorter chain apo-products in the digesta (i.e. apo-12'-, -14'-, -15-lycopenals  
359 and apo-13-lycopenone, which could be derived either from LYC or from apo-6'-, -8'-, or -10'-  
360 lycopenal). Conversely, time\*iron was associated with decreased concentrations of all apo-  
361 lycopenal/lycopenone except apo-6'-lycopenal, as demonstrated both in **Figure 1** and **Table 1**.  
362 This loss in the digesta was most markedly observed in the concentration drop from 2 h to 4 h in  
363 the presence of iron, likely due to continued oxidation. Indeed, pre-formed apo-lycopenals  
364 inhibit lipid peroxidation induced by another form of dietary iron, metmyoglobin, and are likely  
365 destroyed in the process.<sup>[6]</sup> Thus, apo-lycopenals may be transient metabolites of LYC when iron  
366 is present, as noted by their increase in the test meal prepared +FeSO<sub>4</sub> vs. -FeSO<sub>4</sub> (**Table 1**), but  
367 further as yet un-identified metabolites produced from their oxidation during digestion.

368         The concentrations of apo-lycopenals/lycopenone noted in blood plasma (both fasting  
369 and post-prandial) are on par with those reported previously.<sup>[26]</sup> It was unanticipated that the  
370 source of LYC used for the test meal would be rich in apo-lycopenals, providing 10-100x more  
371 apo-lycopenals than that delivered from a raw tomato or tomato-product,<sup>[26]</sup> and providing an  
372 unexpected opportunity to study whether apo-lycopenals are absorbed directly. Apo-  
373 lycopenal/lycopenone were observed in later TRL fractions, regardless of test meal  
374 consumption -FeSO<sub>4</sub> or +FeSO<sub>4</sub>. These results were surprising considering the lack of <sup>13</sup>C apo-  
375 lycopenal reported in blood plasma after the feeding of a single 12 mg dose of <sup>13</sup>C LYC in oil  
376 (no <sup>13</sup>C apo-lycopenals were reported in the dose).<sup>[2]</sup> A study by Cooperstone et al. detected apo-  
377 6'-apo-lycopenal in 29 subjects, of which it was quantifiable in 14 (averaging 0.82 nmol/L) after  
378 4 weeks of daily tomato juice consumption, but no other apo-lycopenals/lycopenone were  
379 observed.<sup>[49]</sup> The study reported herein fed 3.5 times more apo-6'-lycopenal, an equivalent dose  
380 of apo-8'-lycopenal, and 3-40 times more of the remaining apo-lycopenals/lycopenone, all

381 delivered with a large quantity of lipid. Furthermore, the LOD is estimated at ~2-20 pmol apo-  
382 lycopenals/lycopenone per L of plasma, as compared to an LOD of 100 pmol/L plasma estimated  
383 by Cooperstone et al. Thus, the difference in dose and instrument sensitivity are the most  
384 plausible reasons for the detection of apo-lycopenals in the TRL of these study subjects.

385 The parent Caco-2 cell line does not express BCO1,<sup>[50]</sup> and has not been reported to  
386 express BCO2. Likewise, mammals do not synthesize lycopene (it can only be obtained from the  
387 diet).<sup>[51]</sup> Thus, the uptake and basolateral secretion of apo-lycopenals observed in the Caco-2  
388 experiments is due to absorption, and not from further BCO1 or BCO2 cleavage during  
389 incubation. These results further support the hypothesis that these products are being absorbed  
390 from the test meal. Taken together with the human results, evidence collectively suggests that the  
391 bulk of apo-lycopenals observed in circulation originate from foods.

392 One limitation of this study was the lack of labeled apo-lycopenals, which would more  
393 conclusively prove their absorption from the digestive tract. Future studies feeding a labeled  
394 dose  $\geq 1$  mg of each of the apo-lycopenal/lycopenone species would better capture the kinetics of  
395 uptake and transport, and could provide TRL concentrations above the limit of quantitation.  
396 Also, this study was conducted in healthy male subjects, and differences could be observed in  
397 other demographic groups. Finally, only apo-lycopenoids (i.e. < 40 carbon metabolites) were  
398 studied in this work, to the exclusion of other reported LYC epoxides and diols, which have been  
399 previously examined.<sup>[27, 28]</sup>

400 In summary, LYC from a lipid-rich test meal appeared to be robust to loss and to  
401 isomerization during digestion, and apo-lycopenal/lycopenone concentrations were largely  
402 constant relative to LYC concentrations over 4 h. The feeding of inorganic iron resulted in a loss  
403 of LYC both in the digesta and in the newly-absorbed TRL fraction, and reduced digesta apo-

404 lycopenal/lycopenone concentrations over time. The presence of apo-lycopenals/lycopenone in  
405 the later chylomicron-rich TRL fractions supports absorption from the test meal, and a lack of  
406 apo-lycopenols and apo-lycopenoic acids in all biological samples tested suggests a limit of  
407 detection that is too high, or that apo-lycopenals may be metabolized quite differently from  
408 retinal. In short, if maximal absorption of LYC and/or apo-lycopenals/apo-lycopenone is  
409 desired, then LYC-rich meals should be consumed in the absence of inorganic iron.

410

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421

422 **CONFLICT OF INTEREST**

423 The authors declare no conflict of interest.

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**Table 1.** Lycopene and apo-lycopenals/apo-lycopenone measured in the test meals<sup>1</sup>

Carotenoid	-FeSO <sub>4</sub> (mg/meal)	+FeSO <sub>4</sub> (mg/meal)
Total Lycopene	23.1 ± 1.4	21.3 ± 1.5
all- <i>trans</i> -lycopene	19.5 ± 1.3	17.9 ± 1.4
5- <i>cis</i> -lycopene	2.0 ± 0.30	1.7 ± 0.25
other- <i>cis</i> -lycopene	1.6 ± 0.16	1.8 ± 0.22
Apo-6'-lycopenal	0.16 ± 0.029	0.15 ± 0.030
Apo-8'-lycopenal	0.043 ± 0.009	0.048 ± 0.007
Apo-10'-lycopenal <sup>2</sup>	0.012 ± 0.002	0.023 ± 0.002
Apo-12'-lycopenal <sup>2</sup>	0.017 ± 0.002	0.027 ± 0.002
Apo-14'-lycopenal <sup>2</sup>	0.029 ± 0.006	0.078 ± 0.007
Apo-15'-lycopenal <sup>3</sup>	0.009 ± 0.004	0.019 ± 0.005
Apo-13'-lycopenone <sup>2,3</sup>	0.032 ± 0.010	0.080 ± 0.013

<sup>1</sup>Values are means ± SEM,  $n = 11$  for both test meals, with sampling taken just after meal preparation (see *Methods* for details). Note that neither apo-lycopenols nor apo-lycopenoic acids were observed in the test meals.

<sup>2</sup>The amount of this compound between meal types was statistically different (two-tailed unpaired Student's t-test ( $P < 0.05$ )).

<sup>3</sup>Provided as apo-14'-lycopenal equivalents.



**Table 2.** Characteristics of the subjects<sup>1</sup>

	Age (years)	Weight (kg)	BMI (kg/m <sup>2</sup> )	Plasma total cholesterol concentration (mg/dL)	Plasma triacylglycerol concentration (mg/dL)	Plasma glucose (mg/dL)	Hemoglobin (g/dL)
Test Meals	43.0 ± 6.8	80 ± 7.9	24.1 ± 2.3	162 ± 38.9	55.7 ± 23.0 <sup>2</sup>	90 ± 6.1	15.4 ± 0.87
Control Meals	27.5 ± 4.9	77 ± 3.7	23.9 ± 2.3	170 ± 14.1	80.0 ± 0 <sup>2</sup>	83 ± 4.2	15.8 ± 0.21

<sup>1</sup>Values are means ± standard deviation at the screening visit of subjects who completed the study,  $n = 7$  for the two test meals, i.e. one with lycopene oleoresin -FeSO<sub>4</sub>, the other with lycopene oleoresin +FeSO<sub>4</sub>,  $n = 2$  for the control meals, i.e. one -FeSO<sub>4</sub> and one +FeSO<sub>4</sub>.

<sup>2</sup>Characteristics between meal types are statistically different from one another using a two-tailed Welch's (i.e. unpaired) t-test ( $P < 0.05$ )

BMI = Body mass index.

**Table 3.** Mixed model results for digesta concentrations of lycopene and apo-lycopenals/apo-lycopenone<sup>1</sup>

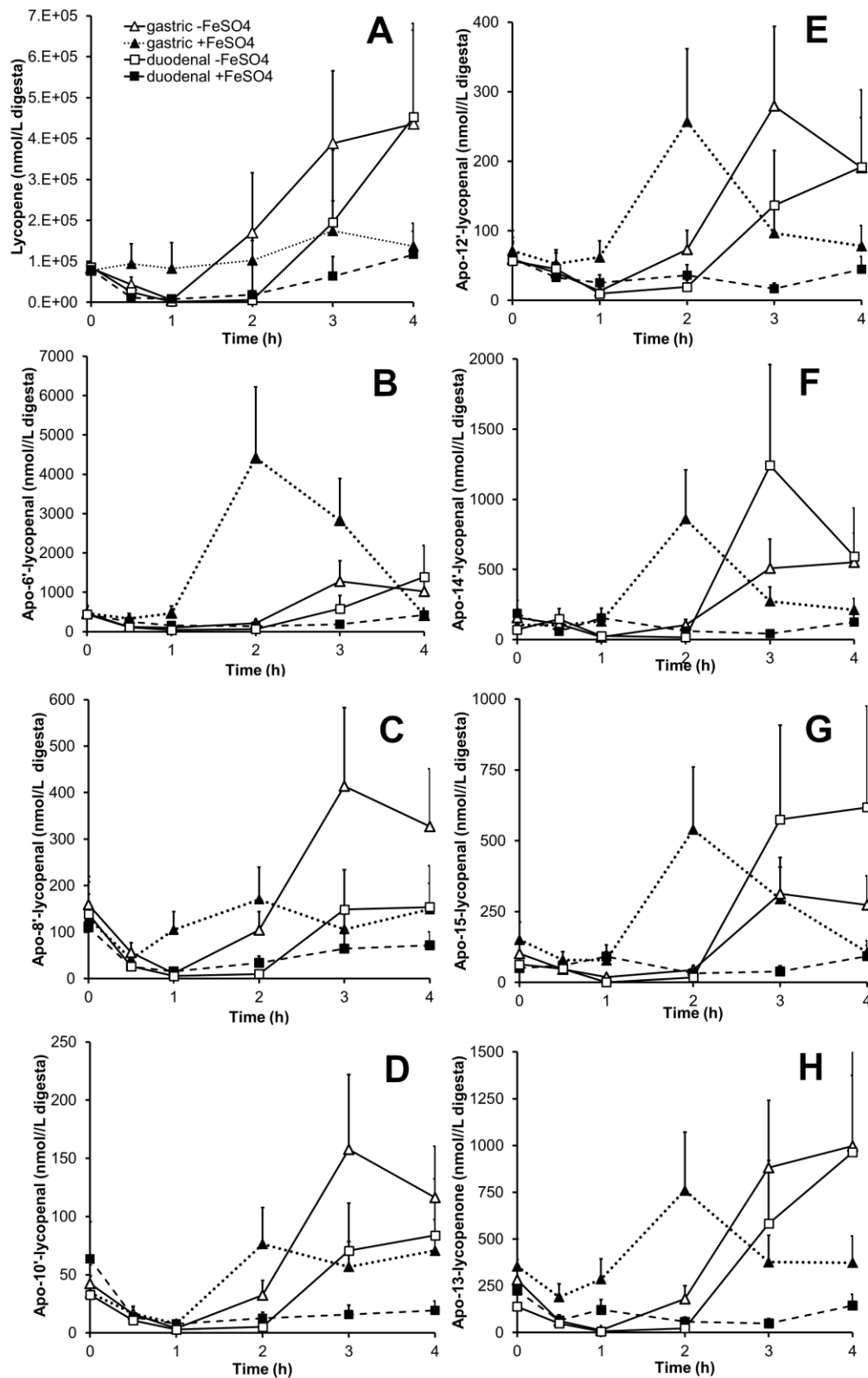
Analyte	Coefficient (± SE)	<i>Time</i>		Coefficient (± SE)	<i>Digesta</i>		Coefficient (± SEM)	<i>Iron</i>		Coefficient (± SEM)	<i>Time*Iron</i>	
		$\chi^2$	<i>P</i> - value <sup>2</sup>		$\chi^2$	<i>P</i> - value <sup>2</sup>		$\chi^2$	<i>P</i> - value <sup>2</sup>		$\chi^2$	<i>P</i> - value <sup>2</sup>
total lycopene <sup>2</sup>	1.0E+5(±1.8E+4)	28.1	1.2E-7 <sup>3</sup>	6.4E+4(±3.7E+4)	3.08	0.08	6.6E+4(±5.5E+4)	1.47	0.23	-8.1E+4(±2.5E+4)	10.7	1.1E-5 <sup>3</sup>
all- <i>trans</i> - lycopene <sup>2</sup>	1.0E+5(±1.8E+4)	21.1	4.3E-6 <sup>3</sup>	6.3E+4(±3.6E+4)	2.78	0.10	6.6E+4(±5.4E+4)	1.19	0.27	-8.1E+4(±2.4E+4)	7.22	7.2E-3 <sup>3</sup>
5- <i>cis</i> - lycopene <sup>2</sup>	2.3E+4(±4.6E+3)	22.9	1.7E-6 <sup>3</sup>	1.4E+4(±9.1E+3)	2.36	0.12	1.5E+4(±1.4E+4)	1.15	0.28	-1.9E+4(±6.3E+3)	9.24	2.4E-3 <sup>3</sup>
other- <i>cis</i> - lycopene <sup>2</sup>	2.9E+4(±5.0E+3)	31.2	2.3E-8 <sup>3</sup>	1.0E+4(±1.4E+4)	1.1	0.30	1.9E+4(±1.5E+4)	1.47	0.23	-2.3E+4(±6.9E+3)	11.53	6.8E-4 <sup>3</sup>
apo-6'- lycopenal	1.2E+5(±1.1E+5)	1.11	0.29	2.8E+5(±2.2E+5)	1.50	0.22	3.0E+5(±3.4E+5)	0.74	0.39	-4.4E+4(±1.5E+5)	0.08	0.77
apo-8'- lycopenal	4.4E+4(±1.5E+4)	9.21	2.4E-3 <sup>3</sup>	4.8E+4(±2.9E+4)	2.86	0.09	4.4E+4(±4.4E+4)	1.01	0.32	-4.2E+4(±2.0E+4)	4.48	3.4E-2 <sup>3</sup>
apo-10'- lycopenal	1.8E+4(±5.4E+3)	11.6	6.6E-4 <sup>3</sup>	1.7E+4(±1.1E+4)	2.43	0.12	2.8E+4(±1.6E+4)	2.87	0.09	-1.9E+4(±7.3E+3)	6.65	9.9E-3 <sup>3</sup>
apo-12'- lycopenal	3.9E+4(±9.8E+3)	15.3	9.0E-5 <sup>3</sup>	3.5E+4(±2.0E+4)	3.13	0.08	6.2E+4(±3.0E+4)	6.25	0.012 <sup>3</sup>	-4.0E+4(±1.3E+4)	9.10	2.5E-3 <sup>3</sup>
apo-14'- lycopenal	1.5E+5(±3.2E+4)	20.0	7.8E-6 <sup>3</sup>	1.6E+4(±6.4E+4)	0.06	0.81	2.5E+5(±9.7E+4)	6.38	0.012 <sup>3</sup>	-1.7E+5(±4.3E+4)	14.3	1.5E-4 <sup>3</sup>
apo-15- lycopenal <sup>4</sup>	8.0E+4(±1.8E+4)	18.5	1.7E-5 <sup>3</sup>	1.3E+4(±3.7E+4)	0.12	0.73	1.1E+5(±5.6E+4)	4.08	4.3E-2 <sup>3</sup>	-7.3E+4(±2.5E+4)	8.73	3.1E-3 <sup>3</sup>
apo-13- lycopenone <sup>4</sup>	1.5E+5(±3.7E+4)	16.7	4.3E-5 <sup>3</sup>	1.3E+5(±7.5E+4)	3.08	0.08	3.3E+5(±1.1E+5)	8.35	3.8E-3 <sup>3</sup>	-1.9E+5(±5.0E+4)	14.1	1.7E-4 <sup>3</sup>

<sup>1</sup>All test subject-samples at were included in determining the mixed model coefficients provided above ( $n=7$  gastric -FeSO<sub>4</sub>,  $n=7$  gastric +FeSO<sub>4</sub>,  $n=4$  duodenal -FeSO<sub>4</sub>, and  $n=6$  duodenal +FeSO<sub>4</sub> subject samples at  $t = 0, 0.5, 1, 2, 3, 4$ )

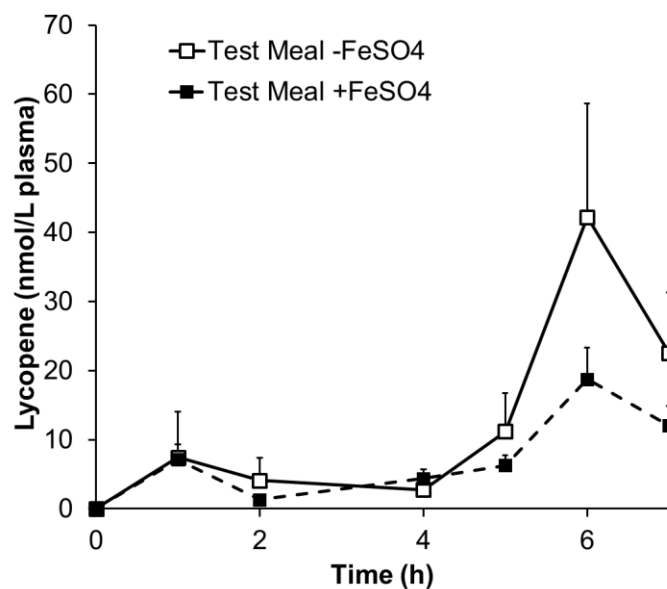
<sup>2</sup>As determined by the likelihood test ratio, degrees of freedom = 1 for all factor evaluations

<sup>3</sup> $P < 0.05$  was considered statistically significant.

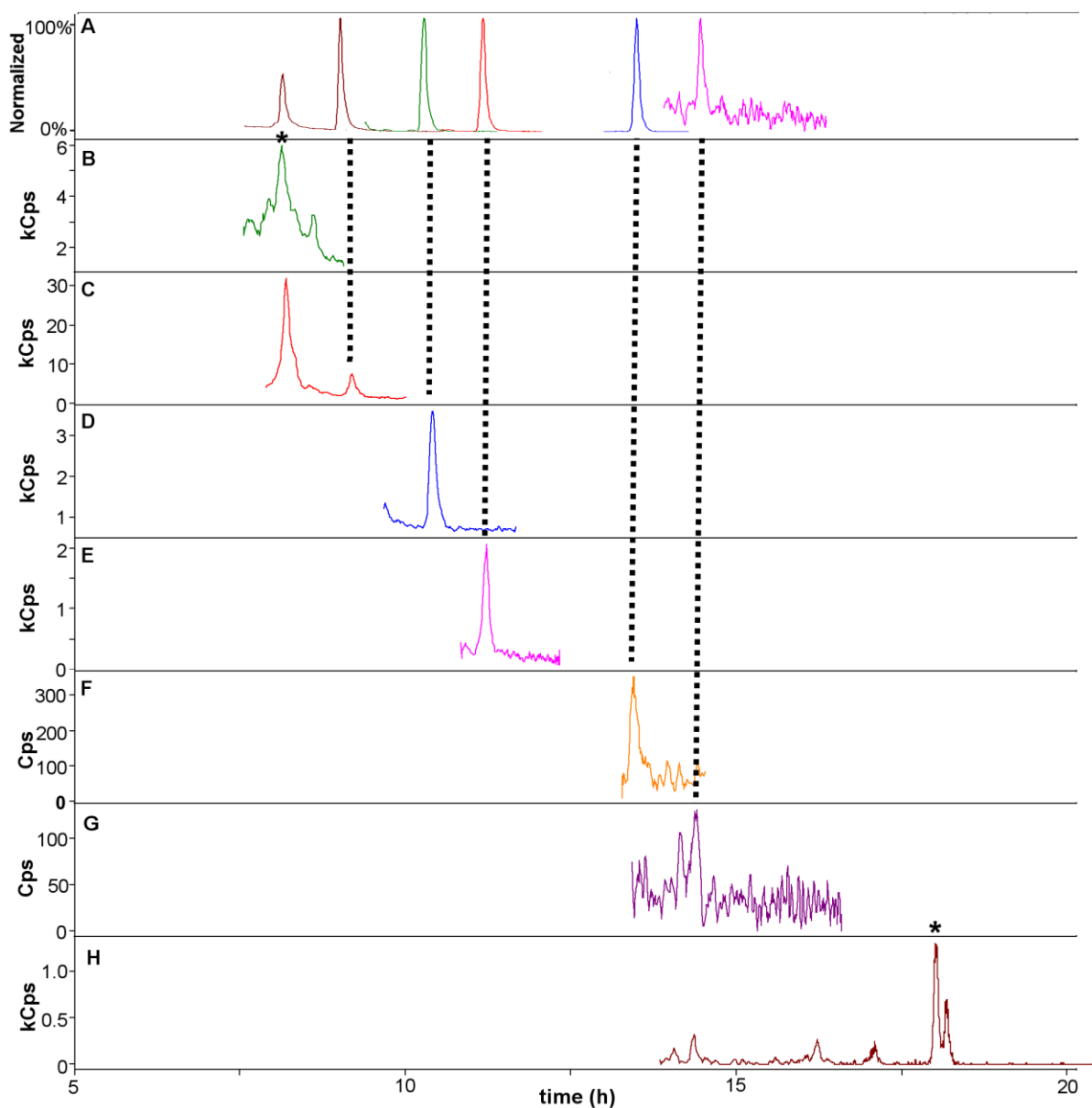
<sup>4</sup>Provided as apo-14'-lycopenal equivalents



**Figure 1.** The concentrations (nmol/L) of lycopene and apo-lycopenals/lycopenone in digesta of subjects over 4 h. Sampling from gastric  $-FeSO_4$  ( $\Delta$ ), gastric  $+FeSO_4$  ( $\blacktriangle$ ), duodenal  $-FeSO_4$  ( $\square$ ) and duodenal  $+FeSO_4$  ( $\blacksquare$ ) compartments are shown. Concentrations of lycopene and apo-lycopenals/lycopenone are represented as means  $\pm$  SEM,  $n = 7$  for gastric  $-FeSO_4$ ,  $n = 7$  for gastric  $+FeSO_4$ ,  $n = 4$  for duodenal  $-FeSO_4$ ,  $n = 6$  for duodenal  $+FeSO_4$ . **A)** total lycopene, **B)** apo-6'-lycopenal, **C)** apo-8'-lycopenal, **D)** apo-10'-lycopenal, **E)** apo-12'-lycopenal, **F)** apo-14'-lycopenal, **G)** apo-15-lycopenal i.e. acyclo-retinal, **H)** apo-13-lycopenone. The relative impact of digesta type, time, iron, and time\*iron on the mixed model describing the concentration of each isomer are provided in Table 3.



**Figure 2.** Average baseline-corrected plasma triglyceride-rich lipoprotein (TRL) concentrations of total lycopene over the course of 7 h after test meal consumption  $-FeSO_4$  ( $\square$ ) and test meal  $+FeSO_4$  ( $\blacksquare$ ). Values represent the average of 7 subjects at each time point  $\pm$  SEM. The test meal  $+FeSO_4$  resulted in a statistically significant decrease in AUC relative to the test meal  $-FeSO_4$  (paired t-test,  $P = 0.0218$ ).



**Figure 3.** A representative LC-MS/MS chromatogram depicting **A)** overlaid MRMs of apolycopenals from the standards mixture normalized to the peak of highest intensity in each SRM and **B-H)** metabolites observed in the TRL fraction of a subject 6 h after meal consumption, shown in ion counts per second, Cps or kilo-ion counts per second, kCps (**B** = apo-13-

lycopenone denoted with asterisk, **C** = apo-14'-lycopenal, **D** = apo-12'-lycopenal, **E** = apo-10'-lycopenal, **F** = apo-8'-lycopenal, **G** = apo-6'-lycopenal, **H** = lycopene, all-*trans*-lycopene denoted with an asterisk). The precursor > product ion transition used for quantitation was selected to display (see **Supplemental Table 1** for further details).