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

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Abbreviations: AUC = Area under the time-versus-concentration curve, BCO2 = β -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 (-FeSO₄) and with ferrous sulfate (160 mg, +FeSO₄).

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 +FeSO₄ vs. the test meal -FeSO₄ ($P = 0.02$). Apo-
53 lycopenals were detected in later TRL fractions. No apo-lycopenols or apo-lycopenoic acids
54 were observed in any samples.

55 *Conclusions:* FeSO₄ 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^[1], 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.^[2, 3]
63 Low solubility resulting from its physical-chemical properties reduces absorption,^[4] 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,^[5-9] but not all,^[10]
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.^[11, 12] In studies showing
70 oxidation under digestive conditions, the LYC is ultimately consumed by oxidative reactions,^{[8,}
71 ^{13, 14]} 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.^[15] Likewise, animal studies of colitis have demonstrated a reduction in
76 myeloperoxidase, a marker of colon inflammation, after lycopene administration both with^[16] or
77 without^[17] 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^[18, 19] in the intestinal tract which confer protection.^[20, 21] Recombinant studies have
80 demonstrated that recombinant β -carotene oxygenase 1 (BCO1) obtained from humans cleaves

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

86 *In vitro* studies reveal ~20% loss of LYC over the digestive period,^[10, 29] 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^[30]) 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.^[31] 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 (FeSO₄) 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,^[32] with LYC replacing ¹³C β-carotene for this study. For test meals containing iron
124 (i.e. +FeSO₄), just before serving, an ampoule of powdered Tardyferon® (delivering 160 mg
125 Fe⁺²) 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, study #NCT03492593. Baseline characteristics of the
135 subjects are provided in **Table 2**. Criteria of inclusion and exclusion match those published
136 previously,^[32] 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.^[33, 34]

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. -FeSO₄) or liquid test meal +FeSO₄. Likewise, control meal subjects
148 consumed a liquid control meal -FeSO₄, or a liquid control meal +FeSO₄. 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^[32]
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.^[32] Samples were transported on dry ice between facilities, and held at -80 °C until
156 extraction. Subjects consumed the same meal -FeSO₄ twice, and the same meal +FeSO₄ 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 ≥ 2 weeks between the consumption of each
159 meal, the total study period for each subject was ≥ 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.^[32] Extracts were immediately dried under argon and stored for ≤ 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.^[35] 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, α -tocopherol, and
185 phosphatidylcholines, as previously described.^[36] The mixed micelles were diluted 1:4 with
186 DMEM containing 500 μ mol/L phenol red, and the solution immediately incubated with Caco-2
187 cells (2 mL/well), as previously described.^[36]

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% CO₂ 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.^[37] 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.^[26]

205 *Statistics*

206 Analyses were performed using R, version 3.1.0.^[38] 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 -FeSO₄ vs. the test meal +FeSO₄ 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.^[10] 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^[39] 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 (+FeSO₄
222 or -FeSO₄) 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 -FeSO₄ vs. the test meal +FeSO₄ 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 -FeSO₄ and
240 remained the same in the test meal + FeSO₄, 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 +FeSO₄ 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 -FeSO₄ (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 +FeSO₄ 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 -FeSO₄ as compared to
249 the test meal +FeSO₄ (**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 ± 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 -FeSO₄
285 consumption was on average $118 \pm 58.7 \text{ nmol}\cdot\text{h/L}$ plasma, and after test meal +FeSO₄
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 +FeSO₄ 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 -FeSO₄ vs. the
289 meal + FeSO₄ 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 -FeSO₄. 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 ≥ 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,^[40] 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.^[41]
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.^[42]

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 β -carotene,^[32]
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,^[43] 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.^[33] 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^[33] as compared to LYC already dissolved in oil. Duodenal digesta
330 LYC concentration also increased with time, similar to a previous study with ^{13}C β -carotene,^[32]
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 +FeSO₄ led to a significant decrease in digesta total LYC (and
334 each isomer individually) relative to the test meal –FeSO₄. 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 Fe^{2+} .^[10, 29] 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 Fe^{2+} , or co-oxidation with meal lipid, did occur in the
339 human system. Besides oxidation, the 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 Fe^{2+} /L digesta.^[44] Significant reductions were also observed in a human study when LYC
343 was fed with Ca^{2+} , another divalent metal, plausibly due to change in zeta-potential absolute
344 charge.^[45] Similarly, complexation of iron with free fatty acids and/or bile salts could also reduce
345 micelle formation, and ultimately LYC uptake.^[44, 46] 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.^[2, 3, 47, 48]

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.^[11, 12] 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.^[6] Thus, apo-lycopenals may be transient metabolites of LYC when iron
366 is present, as noted by their increase in the test meal prepared +FeSO₄ vs. -FeSO₄ (**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.^[26] 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,^[26] 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 -FeSO₄ or +FeSO₄. These results were surprising considering the lack of ¹³C apo-
375 lycopenal reported in blood plasma after the feeding of a single 12 mg dose of ¹³C LYC in oil
376 (no ¹³C apo-lycopenals were reported in the dose).^[2] 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.^[49] 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,^[50] and has not been reported to
386 express BCO2. Likewise, mammals do not synthesize lycopene (it can only be obtained from the
387 diet).^[51] 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 ≥ 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.^[27, 28]

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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418 M.N., C.C., J.P.B., S.M., B.G. conducted research, R.E.K. analyzed data and performed
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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 meals¹

Carotenoid	-FeSO ₄ (mg/meal)	+FeSO ₄ (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 ²	0.012 ± 0.002	0.023 ± 0.002
Apo-12'-lycopenal ²	0.017 ± 0.002	0.027 ± 0.002
Apo-14'-lycopenal ²	0.029 ± 0.006	0.078 ± 0.007
Apo-15'-lycopenal ³	0.009 ± 0.004	0.019 ± 0.005
Apo-13'-lycopenone ^{2,3}	0.032 ± 0.010	0.080 ± 0.013

¹Values 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.

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

³Provided as apo-14'-lycopenal equivalents.

Table 2. Characteristics of the subjects¹

	Age (years)	Weight (kg)	BMI (kg/m ²)	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 ²	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 ²	83 ± 4.2	15.8 ± 0.21

¹Values 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 -FeSO₄, the other with lycopene oleoresin +FeSO₄, $n = 2$ for the control meals, i.e. one -FeSO₄ and one +FeSO₄.

²Characteristics 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-lycopenone¹

Analyte	Coefficient (± SE)	<i>Time</i>		Coefficient (± SE)	<i>Digesta</i>		Coefficient (± SEM)	<i>Iron</i>		Coefficient (± SEM)	<i>Time*Iron</i>	
		χ^2	<i>P</i> - value ²		χ^2	<i>P</i> - value ²		χ^2	<i>P</i> - value ²		χ^2	<i>P</i> - value ²
total lycopene ²	1.0E+5(±1.8E+4)	28.1	1.2E-7 ³	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 ³
all- <i>trans</i> - lycopene ²	1.0E+5(±1.8E+4)	21.1	4.3E-6 ³	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 ³
5- <i>cis</i> - lycopene ²	2.3E+4(±4.6E+3)	22.9	1.7E-6 ³	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 ³
other- <i>cis</i> - lycopene ²	2.9E+4(±5.0E+3)	31.2	2.3E-8 ³	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 ³
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 ³	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 ³
apo-10'- lycopenal	1.8E+4(±5.4E+3)	11.6	6.6E-4 ³	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 ³
apo-12'- lycopenal	3.9E+4(±9.8E+3)	15.3	9.0E-5 ³	3.5E+4(±2.0E+4)	3.13	0.08	6.2E+4(±3.0E+4)	6.25	0.012 ³	-4.0E+4(±1.3E+4)	9.10	2.5E-3 ³
apo-14'- lycopenal	1.5E+5(±3.2E+4)	20.0	7.8E-6 ³	1.6E+4(±6.4E+4)	0.06	0.81	2.5E+5(±9.7E+4)	6.38	0.012 ³	-1.7E+5(±4.3E+4)	14.3	1.5E-4 ³
apo-15- lycopenal ⁴	8.0E+4(±1.8E+4)	18.5	1.7E-5 ³	1.3E+4(±3.7E+4)	0.12	0.73	1.1E+5(±5.6E+4)	4.08	4.3E-2 ³	-7.3E+4(±2.5E+4)	8.73	3.1E-3 ³
apo-13- lycopenone ⁴	1.5E+5(±3.7E+4)	16.7	4.3E-5 ³	1.3E+5(±7.5E+4)	3.08	0.08	3.3E+5(±1.1E+5)	8.35	3.8E-3 ³	-1.9E+5(±5.0E+4)	14.1	1.7E-4 ³

¹All test subject-samples at were included in determining the mixed model coefficients provided above ($n=7$ gastric -FeSO₄, $n=7$ gastric +FeSO₄, $n=4$ duodenal -FeSO₄, and $n=6$ duodenal +FeSO₄ subject samples at $t = 0, 0.5, 1, 2, 3, 4$)

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

³ $P < 0.05$ was considered statistically significant.

⁴Provided as apo-14'-lycopenal equivalents

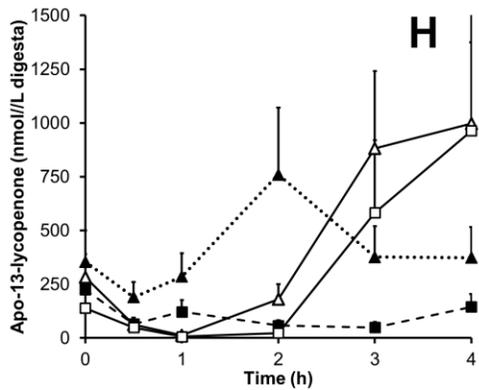
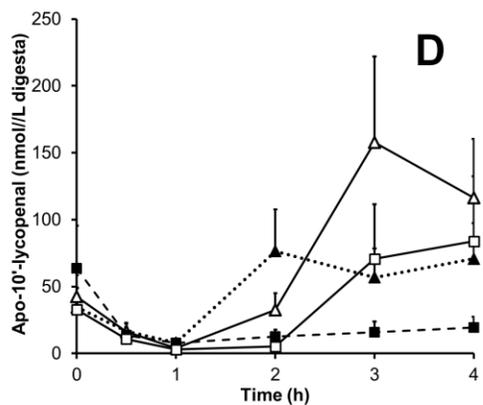
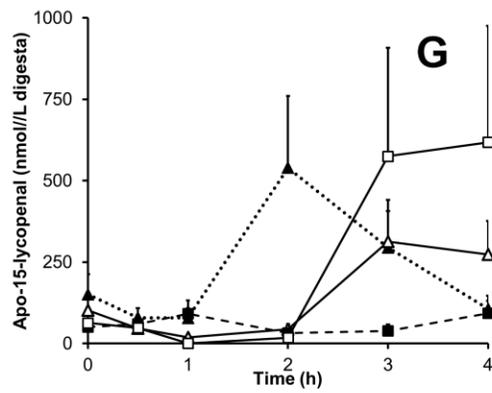
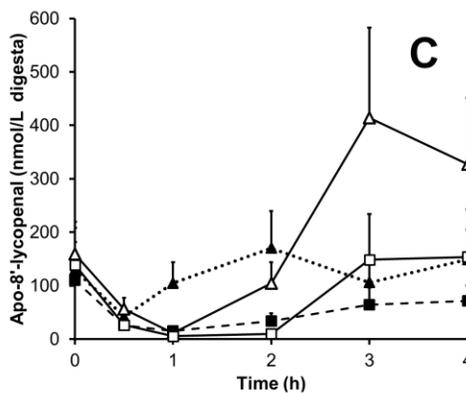
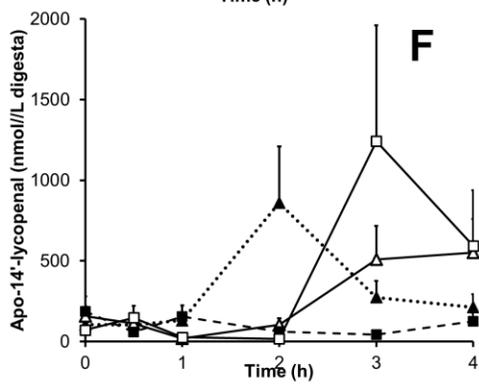
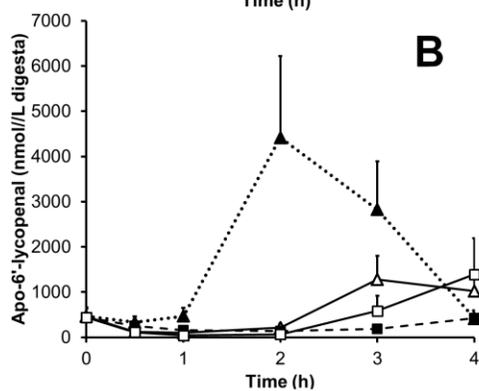
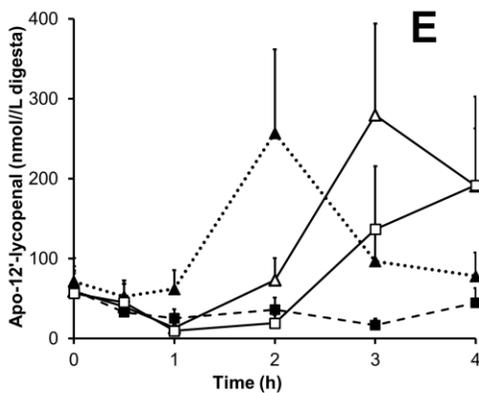
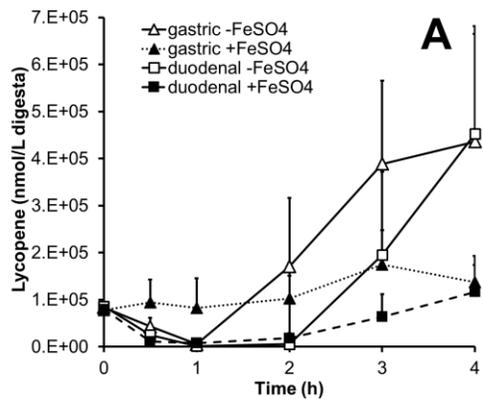


Figure 1. The concentrations (nmol/L) of lycopene and apo-lycopenals/lycopenone in digesta of subjects over 4 h. Sampling from gastric $-FeSO_4$ (Δ), gastric $+FeSO_4$ (\blacktriangle), duodenal $-FeSO_4$ (\square) and duodenal $+FeSO_4$ (\blacksquare) compartments are shown. Concentrations of lycopene 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.

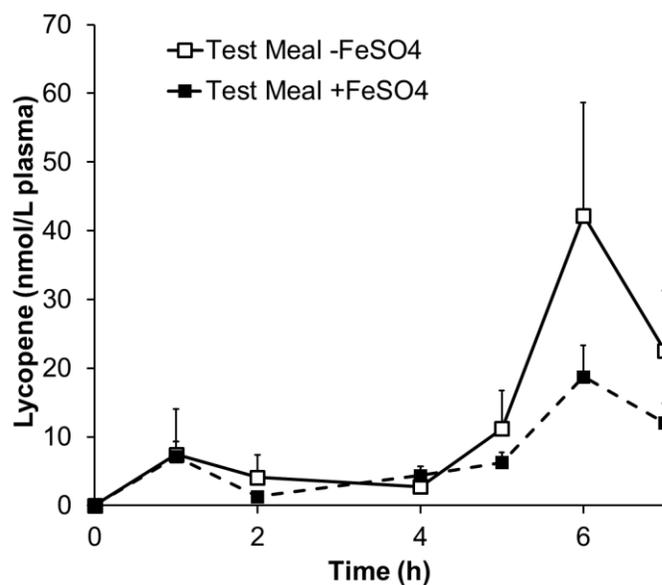


Figure 2. Average baseline-corrected plasma triglyceride-rich lipoprotein (TRL) concentrations of total lycopene over the course of 7 h after test meal consumption $-FeSO_4$ (\square) and test meal $+FeSO_4$ (\blacksquare). Values represent the average of 7 subjects at each time point \pm SEM. The test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

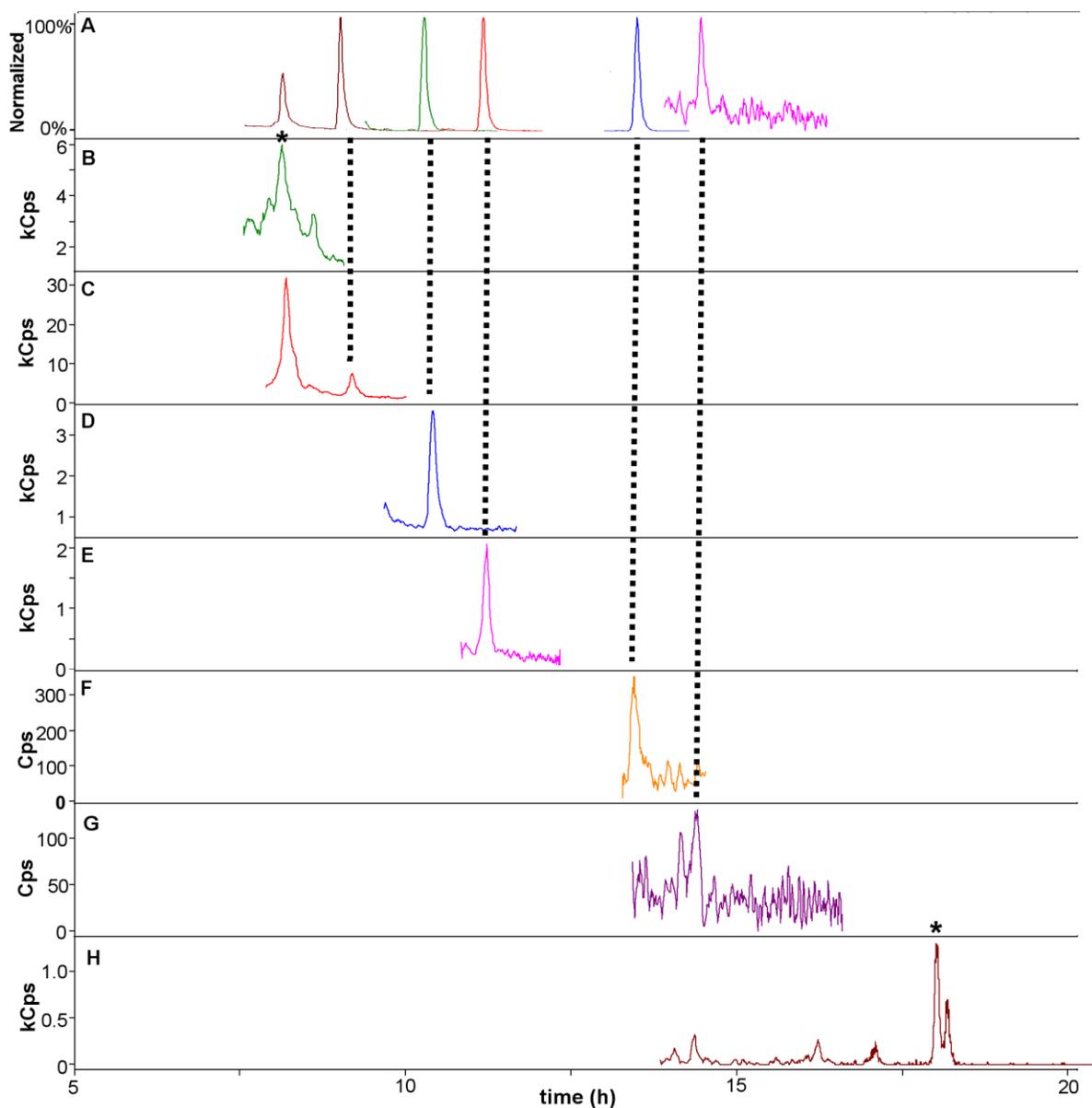


Figure 3. A representative LC-MS/MS chromatogram depicting **A)** overlaid MRMs of apo-lycopenals from the standards mixture normalized to the peak of highest intensity in each SRM and **B-H)** metabolites observed in the TRL fraction of a subject 6 h after meal consumption, shown in ion counts per second,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