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Production of asymmetric oxidative metabolites of  $^{13}\text{C}$   $\beta$ -carotene during digestion in the gastrointestinal lumen of healthy men

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**Running title:**  $^{13}\text{C}$   $\beta$ -carotene metabolism in the GI lumen

**Keywords:**  $^{13}\text{C}$   $\beta$ -carotene, metabolism, asymmetric metabolites, digestion, stomach, duodenum, gastrointestinal lumen

**Abbreviations:** AUC = Area under the time-versus-concentration curve, BCO1 =  $\beta$ -carotene oxygenase 1, BCO2 =  $\beta$ -carotene oxygenase 2, DCM = dichloromethane, EDTA = ethylenediaminetetraacetic acid, LC-MS/MS = high performance liquid chromatography-tandem mass spectrometry, methanol = MeOH, methyl *tert*-butyl ether = MTBE, TRL = triglyceride-rich lipoprotein fraction

This trial was registered at ClinicalTrials.gov as NCT03492593

## ABSTRACT

*Background:* Asymmetric  $\beta$ -apo-carotenoids (non-vitamin A active metabolites) of provitamin A carotenoids have been observed in humans, but no study has investigated their formation during digestion.

*Objective:* To follow the formation and absorption of asymmetric  $\beta$ -apo-carotenoids during digestion.

*Design:* Healthy men were intragastrically and intraduodenally intubated, and randomly assigned to consume a lipid-rich control meal (n=3) or a lipid-rich test meal containing 20 mg  $^{13}\text{C}$ -10  $\beta$ -carotene (n=7). Digesta samples were collected over 5 hours, and blood collected over 7 hours. The triglyceride-rich lipoprotein (TRL) fractions of plasma were also isolated. Lipophilic extracts of digesta, plasma, and TRL were analyzed via an LC-MS/MS method developed to identify  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone,  $^{13}\text{C}$   $\beta$ -apo-carotenols, and  $^{13}\text{C}$   $\beta$ -apo-carotenoic acids.

*Results:* Relative to  $^{13}\text{C}$   $\beta$ -carotene,  $^{13}\text{C}$   $\beta$ -apo-carotenol levels remained ~3 orders of magnitude lower throughout digestion (no  $^{13}\text{C}$   $\beta$ -apo-carotenols, or  $^{13}\text{C}$   $\beta$ -apo-carotenoic acids were observed). A mixed model determined relative influence of digesta type and time on digesta metabolite level. Increasing time significantly increased the model levels of digesta  $^{13}\text{C}$   $\beta$ -apo-10', -12', -14', -15-carotenol and  $^{13}\text{C}$   $\beta$ -apo-13-carotenone ( $P < 0.05$ ) and trended toward decreased  $^{13}\text{C}$   $\beta$ -apo-8'-carotenol ( $P = 0.0876$ ). Gastric digesta was associated with a significantly higher level of  $^{13}\text{C}$   $\beta$ -apo-8'-carotenol ( $P = 0.0289$ ), and lower levels of  $^{13}\text{C}$   $\beta$ -apo-12', -14', -15-carotenol ( $P < 0.05$ ), relative to duodenal digesta. Anticipated retinoids, but no asymmetric  $^{13}\text{C}$   $\beta$ -apo-carotenals,  $^{13}\text{C}$   $\beta$ -apo-carotenols, or  $^{13}\text{C}$   $\beta$ -apo-carotenoic acids, were observed in the blood or TRL samples.

*Conclusions:*  $\beta$ -carotene appears to be robust to digestion, with minor amounts of  $\beta$ -apo-carotenals/carotenone formed. Absence of asymmetric  $^{13}\text{C}$   $\beta$ -apo-carotenals in plasma and TRL suggests lack of absorption, levels below the limit of detection, lack of stability, or further conversion during the digestive process to as yet unidentified products. Lack of asymmetric  $^{13}\text{C}$   $\beta$ -apo-carotenals in plasma also suggests a lack of post-prandial intestinal BCO2 activity in healthy humans.

## INTRODUCTION

Asymmetric metabolites of  $\beta$ -carotene, i.e. asymmetric  $\beta$ -apo-carotenoids, were first proposed as a putative pathway to the formation of vitamin A *in vivo*. The  $\beta$ -ionone ring of  $\beta$ -carotene was postulated to be cleaved with a series of 2-5 carbon losses, producing long-chain  $\beta$ -apo-carotenals as intermediates in the synthesis of retinal (1–3). While it is now appreciated that the bulk of  $\beta$ -carotene-derived retinol comes from symmetric cleavage via  $\beta$ -carotene oxygenase 1 (BCO1) (4,5), *in vitro* work reveals it is possible to obtain vitamin A from longer chain  $\beta$ -apo-carotenals (6). These asymmetric products could be produced by cleavage via  $\beta$ -carotene oxygenase 2 (BCO2) (7,8) or be derived from other enzymatic or oxidative sources.

Studies of cancer risk have also elicited interest into asymmetric  $\beta$ -apo-carotenoids, following the landmark Peto et al. publication in 1981 suggesting an inverse correlation with  $\beta$ -carotene consumption (9). This led to *in vitro* studies implicating an antioxidant mechanism of action (10–12), followed by the recruitment of subjects with known exposure to high oxidative stress (i.e. smokers and former asbestos workers) for prospective studies. Subjects were dosed with highly bioavailable  $\beta$ -carotene ( $\geq 30$  mg in oil per day), which actually increased lung cancer risk (13–15). While studies in healthy individuals have observed a positive effect (16,17), or no effect (18,19), interest in better understanding the effects of  $\beta$ -carotene on smokers was further pursued in *in vitro* (20,21), *ex vivo* (22), and in animal studies (23,24), to identify the scope of metabolites that might result from both chemical and enzymatic  $\beta$ -carotene oxidation. Some of the products identified included  $\beta$ -apo-carotenoids.

Non-provitamin A metabolites of  $\beta$ -carotene are ubiquitous in the food supply (25–30), both as products of carotenoid cleavage oxygenases in plants (31) well as through the addition of certain foods colorants (29,32). They may also be produced during digestion, likely via co-oxidation with

lipids (33–35). In addition, BCO2 is expressed in the intestine (sub-localized to the inner mitochondrial membrane(36)), and could produce  $\beta$ -apo-carotenoids upon uptake (4,7).

It's surprising that with the plethora of  $\beta$ -apo-carotenoid sources, only a few reports of apo-carotenoids have surfaced in humans (37,38). However, in both of these reports, the origin(s) of the  $\beta$ -apo-carotenoids (from digestion of a meal, from enzymatic cleavage, or from chemical oxidation) was not clear.

To date, no study has investigated the formation of asymmetric  $\beta$ -apo-carotenoids during digestion in healthy humans. The principal objective of this study was to follow the post-prandial metabolism of isotopically labeled  $\beta$ -carotene in the digestive tract of healthy men a) to determine whether the digestion of  $\beta$ -carotene can produce asymmetric  $\beta$ -apo-carotenoids (via oxidation in the digestive lumen or BCO2 in the intestinal enterocyte) and b) to determine whether these  $\beta$ -apo-carotenoids can be absorbed into the human body.

## SUBJECTS & METHODS

### *Chemicals*

$^{13}\text{C}$ -10  $\beta$ -carotene (**Figure 1**) was purchased from Euriso-top (Saint Aubin, France) with a chemical purity of > 97% (and a 12,12',13,13',14,14',15,15',20,20'  $^{13}\text{C}$ -10 labeled purity of 99%). Of the dose, 91% of the  $\beta$ -carotene was present as the all-*trans* isomer and 9% as *cis*-isomers. The product contained trace levels of the  $\beta$ -apo-carotenoids of interest (shown in **Figure 1**). LC grade methyl tert-butyl ether (MTBE) and MS grade methanol were purchased from Fisher Scientific (Illkirch, France). A Millipore Q-Plus produced the double deionized water used in the LC mobile phase. MS grade formic acid (>98% pure), and Sigma brand  $\beta$ -carotene (>98% pure), retinal ( $\beta$ -apo-15'-carotenal), retinol ( $\beta$ -apo-15'-carotenol), retinoic acid ( $\beta$ -apo-15'-carotenoic acid), and Fluka brand  $\beta$ -apo-8'-carotenal standards were purchased from Sigma-

Aldrich (Saint-Quentin-Fallavier, France). The glycerol phosphate oxidase assay was purchased from Abliance (Compiègne, France).

Products obtained from local supermarkets for the test meals include sunflower oil and white granular sugar (Auchan, Avignon, France), and demineralized water (Casino, Marseille, France). Ovolife IF 50 phospholipid was kindly donated by Lecico, Inc. (Hamburg, Germany) and contained 50% w/w phospholipid derived from egg yolk and a maltodextrin excipient.

#### *Preparation of $^{13}\text{C}$ $\beta$ -carotene test meal*

All experimental procedures were carried out in the absence of natural light. To ensure consistency and stability of  $^{13}\text{C}$   $\beta$ -carotene dose over the duration of the study, a carotene-in oil preparation was made by mixing  $^{13}\text{C}$   $\beta$ -carotene overnight in sunflower oil under argon, to distribute the carotene. The following day, the carotene in oil was aliquoted into individual amber glass ampoules, which were backfilled with argon and sealed under a flame (with each ampoule containing 10 mg isotopically labeled  $\beta$ -carotene in 2.5 g oil). The sealed ampoules were stored at 4° C until test meal preparation. Each test meal containing  $\beta$ -carotene used two ampoules, as described below.

Demineralized water (400 mL) was measured into a blender, to which sucrose (100 g) was added and mixed until dissolved, then Ovolife IF 50 phospholipid (2.5 g) was added and dissolved over 1 min of blending. Sunflower oil (50 g) was slowly added to the blender while mixing to create an emulsion, followed by the  $\beta$ -carotene in oil (for the test meal) or 5 g sunflower oil alone (for the control meal), blending for an additional 2.5 min over both additions. The meal was either consumed immediately, or stored in the dark at 4° C for not more than 30 min. before consumption (in which case the meal was blended for an additional 30 s just before



serving). Test meal samples (0.5 mL) were taken from each meal just before serving, to be analyzed for  $^{13}\text{C}$   $\beta$ -carotene and  $^{13}\text{C}$   $\beta$ -apo-carotenoid content.

### *Clinical Subjects and Experimental Design*

The clinical portion of the study was conducted at the Clinical Investigation Center of the Hôpital de la Conception in Marseille, France. Characteristics of subjects who completed the study are provided in **Table 1**. Inclusion criteria: male gender, 18-60 years of age, total fasting cholesterol < 220 mg/dL, total fasting triglycerides < 150 mg/dL, fasting blood glucose < 110 mg/dL, hemoglobin < 1.3 g/L. Exclusion criteria: medical treatment, smoking, consumption of  $\beta$ -carotene containing supplements in the previous 3 mo, > 14 servings of alcohol per week, eating disorders, disorders of metabolism, cholesterol altering medication, > 4 ½ hr per wk of intense physical activity, hepatitis B and C, and HIV diagnosis. All subjects provided written, informed consent before participating in the protocol. The study screened 13 healthy male subjects who met the study criteria, with the aim of 9 subjects completing the entire protocol (n=7 test meal subjects, n=2 control meal subjects). Sample size was chosen based on preliminary data obtained in a similarly designed study with lycopene (not yet published). Subjects were randomized to control or test meal by the study coordinator based upon order of enrollment.

After an overnight fast, subjects arrived at the clinic, and the nasoduodenal tube was inserted and positioned either in the gastric compartment or in the duodenum. An X-ray confirmed proper tube placement. Afterward, a 0 h blood sample was drawn, and subjects were asked to consume either the liquid test meal or liquid control meal (delivering 0 g of protein, 100 g of carbohydrate, and 55 g of lipid) within 5 min. An additional 50 mL of demineralized water was used to rinse the liquid meal preparatory glassware, and also given to the subject to drink. Digesta samples (either gastric or duodenal) were taken at 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr,

and 5 hr by aspiration with a syringe, as previously described (39). Samples (~8 mL) were measured for exact volume, and immediately treated with an aq. solution (half the volume of measured digesta, ~4 mL) containing 0.5% pyrogallol and 0.7% EDTA (to prevent oxidation and to chelate free metals, respectively). Samples were immediately vortexed, aliquoted, and frozen at -80 °C. Postprandial blood samples were taken at 1, 2, 4, 5, 6, and 7 hours, and plasma isolated and frozen at -80 °C. TRL fractions were also isolated from 6 mL aliquots of fresh plasma, as previously described (40). All samples were transported to the analysis lab on dry ice, and held at -80 °C until extraction. After the first daylong study visit, the subjects returned home for 3 weeks before returning for a second daylong study visit, to receive the same meal and procedures as previously, and collect samples from the other digestive compartment. The study was approved by the regional Institutional Review Board Comité de Protection des Personnes Sud Méditerranée I, France (Protocol #2013-A01398-37), and the feeding of the <sup>13</sup>C β-carotene was further approved by Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM). Procedures were followed in accordance with the Declaration of Helsinki of 1975 as revised in 1983. The study was registered with ClinicalTrials.gov (NCT03492593).

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

Aliquots of the gastric or duodenal digesta mixture (0.5 mL) and TRL rich fractions (0.5 mL), as well as plasma (1 mL) and test meals (1 mL) were extracted as previously described, with minor modifications as noted (41). Briefly, the samples were mixed with an equal volume of MeOH and vortexed for 1 min. Hexane (equivalent to 4x original sample volume) was added and samples then vortexed for 5 min., followed by centrifugation (2 min at 3,000 rpm using a Jouan MR1822, ThermoFisher Scientific, Walham, MA, U.S.A.). The upper hexane layer was removed and DCM (equivalent to 4x original sample volume) was added and the samples again

vortexed for 5 min. and centrifuged for 2 min. The lower DCM phase was then combined with the first hexane extract, and the samples dried under argon at 30 °C. Dried extracts were stored for no more than 2 days at -80 °C before analysis.

*Production of Unlabelled and Labelled  $\beta$ -Apo-Carotenoids:* The methods detailed previously to generate lutein metabolites (42) were first applied to non-labeled  $\beta$ -carotene, and then to the  $^{13}\text{C}$   $\beta$ -carotene to produce  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone,  $^{13}\text{C}$   $\beta$ -apo-carotenols, and  $^{13}\text{C}$   $\beta$ -apo-carotenoid acids. Confirmation of product identities of  $\beta$ -apo-8'-carotenal, retinal, retinol, and retinoic acid included retention time, UV-visible spectra and parent m/z coincident with authentic standards. The  $^{13}\text{C}$ - $\beta$ -apo-8'-carotenal,  $^{13}\text{C}$ -retinal,  $^{13}\text{C}$ -retinol, and  $^{13}\text{C}$ -retinoic acid had identical UV-Vis spectra, but a slightly shorter retention time ( $\leq 0.2$  min earlier), and a parent m/z increased to reflect of the number of  $^{13}\text{C}$  carbons present in the respective metabolite (**Figure 1**). All remaining species were identified based upon expected retention order and time relative to the confirmed standards, expected UV-Vis spectra (using published values for previously identified products, and estimated values for the remaining), and expected in-source parent m/z (following previously published work, and the behavior of the tested retinoid species) (25,37,43). In addition to all-*trans*  $^{13}\text{C}$   $\beta$ -carotene, an isomer eluted just afterward was presumed to be 9-*cis*, while the isomer eluting prior was assumed to be 13-*cis*, based upon retention order on a C30 column (44). Mixtures were qualitatively evaluated using an Acquity UPLC system (Waters Corp.) interfaced with an HCT Ultra ion trap mass spectrometer (Bruker Daltonics) via an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode (for UPLC conditions, see below; for MS source conditions, see reference (42)). The UPLC system included an in-line photodiode array detector (PDA).

*HPLC-MS/MS analyses*

Labeled and unlabeled  $\beta$ -carotene metabolite mixtures were used for the development of a method for identification and quantitation of metabolites in samples. Parent >daughter transitions monitored in the final method are provided in **Supplemental Tables 1, 2, and 3**, and chromatograms of the separated  $^{13}\text{C}$   $\beta$ -apo-carotenals,  $^{13}\text{C}$   $\beta$ -apo-carotenols, and  $^{13}\text{C}$   $\beta$ -apo-carotenoic acids are shown in **Supplemental Figure 1**.

Digesta, plasma, and TRL extracts were redissolved in 200  $\mu\text{L}$ , while test meal samples were redissolved in 800  $\mu\text{L}$  of a 1:1 MTBE/MeOH added sequentially, as described previously (41). Samples were analyzed using a CTC Pal autosampler (Bruker) with an Advance UHPLC (Agilent Technologies, Santa Clara, CA, U.S.A.), interfaced with an EVOQ Triple Quadrupole mass spectrometer with an APCI probe operated in positive ion mode. The samples were separated using a YMC C30 column (150 mm x 2.0 mm, 3  $\mu\text{m}$  particle size). The gradient method employed solvent A (80:18:2 MeOH/water/0.1% aq. formic acid) and solvent B (20:78:2 MeOH/MTBE/0.1% aq. formic acid), beginning with 0% B, holding 0% B for 0.5 min, increasing to 40% B over 9.5 min, increasing to 90% B over 4.5 min., increasing to 100% B over 2.5 min, holding at 100% B for 3 min, and returning and holding 0% B for 3 min. The flow rate used was 0.8 mL/min, the column temperature was held at 40  $^{\circ}\text{C}$ , and an injection of 20  $\mu\text{L}$  used. The source parameters were as follows: spray current = +10  $\mu\text{A}$ , cone temperature = 300  $^{\circ}\text{C}$ , cone gas flow = 20 (arbitrary units), heated probe temperature = 325  $^{\circ}\text{C}$ , heated probe gas flow = 12 (arbitrary units), nebulizer gas flow = 45 (arbitrary units), with the exhaust gas turned off.

### *Statistics*

Statistical analyses were performed using R software, version 3.1.0 (45). Residual plots were visually inspected to confirm assumptions of homoscedasticity and normality.

Bioavailability of the  $^{13}\text{C}$   $\beta$ -carotene in the TRL fraction was baseline-corrected, and area under

the time-concentration curve (AUC) calculated using trapezoidal approximation. To ensure the same level of  $^{13}\text{C}$   $\beta$ -carotene was absorbed by the same subject during both gastric and duodenal sampling, a paired t-test of  $^{13}\text{C}$   $\beta$ -carotene AUC in TRL was performed ( $P < 0.05$  was considered statistically significant). The relationship between time and digesta type on digesta analyte concentration ( $^{13}\text{C}$   $\beta$ -carotene itself, or the ratio of the  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone to  $^{13}\text{C}$   $\beta$ -carotene in the same sample), was modeled with mixed-effects regression using the lme4 package (46). Fixed effects included sample time (0, 0.25, 0.5, 1, 2, 3, 4, 5 h), digesta type (gastric or duodenal), and meal period (1<sup>st</sup> or 2<sup>nd</sup> meal) were considered. Subject was included as a random effect with individual intercepts. To understand the influence of the fixed effects on the goodness of fit of the model, the likelihood ratio test (LRT) of the full model was compared to the model minus the effect in question.  $P < 0.05$  was considered statistically significant.

## RESULTS

### *Subject Recruitment and Sample Collection*

See flow diagram of study design in **Supplemental Figure 2**. Of the 7 subjects who completed both test meal visits, gastric samples were collected from all 7 subjects, and duodenal samples from 6 subjects (in the seventh subject, the nasoduodenal tube would not pass the pyloric sphincter during the duodenal sampling visit). Of the 3 subjects who consumed the control meal, gastric and duodenal samples were collected from one subject, and only gastric and only duodenal samples collected from the second and third subjects, respectively. Because the gastric and duodenal control profiles were very similar, and control samples were strictly used for analytical method refinement (see *LC-MS/MS method development* below), it was determined that the gastric and duodenal control samples from two different subjects would suffice for the intended purpose.

Duodenal digesta samples were more challenging to obtain than gastric samples. In all but two of the subjects, at least one of the eight duodenal sampling time points was not able to be obtained. Because these “absent” samples were at differing time points in different subjects, a mixed model was a more appropriate statistical approach to understand the influence of time and digesta type on metabolite formation than a repeated measures design (which employs listwise deletion in these instances).

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

The standard mixtures composed of the analytes depicted in **Figure 1** facilitated the development of a combined, targeted LC-MS/MS method with which to search for compounds of interest. Qualitative measures of analysis (**Supplemental Tables 1-3**) matched those previously reported (25,37,43). Chromatograms of the final method are shown in the supplemental material (**Supplemental Figure 2**). The purpose of the samples collected from subjects consuming the control meal was to ensure parent>daughter fragments chosen were unique to the metabolites of interest formed from the isotopically labeled  $\beta$ -carotene. Multiple parent>daughter transitions for which matrix suppression and/or false peaks were observed in the control subject plasma, TRL and digesta were removed from the final method. It is also important to note that  $\beta$ -apo-carotenol could produce the same in-source parent>daughter(s) as the  $\beta$ -apo-carotenol of the same chain length (although the signal intensity was significantly lower). Thus, differing chromatographic retention times for the aldehyde and alcohol species ensured no overlap. An external standard curve of authentic  $\beta$ -apo-8'-carotenol was used to quantitate the asymmetric  $\beta$ -apo-carotenol/carotenone species, and values are provided in  $\beta$ -apo-8'-carotenol equivalents. The  $^{13}\text{C}$ - $\beta$ -apo-15-carotenol (i.e. retinal) was quantitated using unlabeled authentic standard.

*Absorption of  $^{13}\text{C}$   $\beta$ -carotene, and plasma levels of  $^{13}\text{C}$  retinol and  $^{13}\text{C}$  retinyl esters*

No significant difference between the AUC's of TRL  $^{13}\text{C}$   $\beta$ -carotene was observed when the digesta was aspirated from the gastric site as compared to the duodenal site, **Figure 2A** (paired t-test,  $P = 0.30$ ). Likewise, no differences were observed between the AUC's of plasma  $^{13}\text{C}$  retinol or  $^{13}\text{C}$  retinyl esters (paired t-test,  $P = 0.44$  and  $P = 0.54$ , respectively; **Supplemental Figures 3-4**) when the meal was aspirated from the gastric site as compared to the duodenal site. Thus, nasoduodenal tube placement, as well as small changes in sampling volume, had no material impact on total  $\beta$ -carotene absorption or conversion to vitamin A metabolites.

*Digesta levels of  $^{13}\text{C}$   $\beta$ -carotene and  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone*

The test meal delivering 20 mg of  $^{13}\text{C}$   $\beta$ -carotene also provided ~0.01-0.02 mg of each  $\beta$ -apo-carotenal/carotenone (in  $\beta$ -apo-8'-carotenal equivalents). Both the  $^{13}\text{C}$   $\beta$ -carotene dose, as well as the test meal preparation, contributed to the  $\beta$ -apo-carotenals/carotenone in the test meal. The results of the post-prandial digesta levels of the sum of  $^{13}\text{C}$   $\beta$ -carotene isomers are shown in **Figure 2B**. No significant change in  $^{13}\text{C}$   $\beta$ -carotene isomer profile was observed between the test meal and the digesta over the course of 5 hours. The transitions used for quantitation and the retention time alignment of the standard mixture (**Figure 3A**) is shown relative to a representative digesta sample containing  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone and  $^{13}\text{C}$   $\beta$ -apo-carotenone (**Figure 3B-I**). The digesta levels of  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone are shown in **Figure 4**, and have been normalized to  $^{13}\text{C}$   $\beta$ -carotene levels due to dilution effects which occur during digestion (additional detail in Results). Underlying this normalization are three assumptions, 1) that the level of  $^{13}\text{C}$   $\beta$ -carotene in the digesta is orders of magnitude greater than the level of  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone, 2) that  $^{13}\text{C}$   $\beta$ -carotene and  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone are absorbed at approximately the same rate, and 3)  $^{13}\text{C}$   $\beta$ -carotene and  $^{13}\text{C}$

$\beta$ -apo-carotenals/carotenone have approximately the same stability in the gastrointestinal tract. Based on levels of  $^{13}\text{C}$   $\beta$ -apo-carotenals in test meal, we felt this was a safe assumption, and indeed, the fact that great changes in  $^{13}\text{C}$   $\beta$ -apo-carotenal amount relative to  $^{13}\text{C}$   $\beta$ -carotene were not observed further supports this assumption. As shown in **Figure 4**, the level of each  $^{13}\text{C}$   $\beta$ -apo-carotenal/carotenone in the digesta (0.5-90 nmol/L) remained ~3 orders of magnitude lower than the level of  $^{13}\text{C}$   $\beta$ -carotene (2 – 22  $\mu\text{mol/L}$  digesta) throughout digestion.

The full results of the mixed modeling of the digesta are provided in **Table 2**. Meal period (1<sup>st</sup> or 2<sup>nd</sup> meal) had no significant influence on  $^{13}\text{C}$   $\beta$ -apo-carotenal/carotenone levels and was excluded from the final model. Because of the large decrease in  $^{13}\text{C}$   $\beta$ -carotene concentration between 0 and 15 min., the model was tested both including and excluding 0 min. When including 0 min, time was negatively associated with the level of  $^{13}\text{C}$   $\beta$ -carotene ( $P = 7.1 \times 10^{-4}$ ), and no influence of digesta type was observed. When excluding 0 min, increasing time (coefficient =  $1.22 \times 10^6$ ,  $P < 1.0 \times 10^{-5}$ ) and duodenal digesta type (coefficient =  $1.22 \times 10^6$ ,  $P = 0.027$ ) were positively associated with increased  $^{13}\text{C}$   $\beta$ -carotene levels. A negative trend was observed with time and  $^{13}\text{C}$   $\beta$ -apo-8'-carotenal ( $P = 0.0876$ ), while time was positively associated with relative levels of digesta  $^{13}\text{C}$   $\beta$ -apo-10', -12', -14', -15-carotenal and  $^{13}\text{C}$   $\beta$ -apo-13-carotenone ( $P < 0.001$ ). Time was not associated with the relative level of digesta  $^{13}\text{C}$   $\beta$ -apo-11'-carotenal. Gastric digesta was associated with higher levels of  $^{13}\text{C}$   $\beta$ -apo-8'-carotenal ( $P = 0.0289$ ), and lower levels of  $^{13}\text{C}$   $\beta$ -apo-12', -14', -15-carotenal ( $P < 0.05$ ), relative to duodenal digesta. No influence of digesta type was observed on levels of  $^{13}\text{C}$   $\beta$ -apo-10' or -11-carotenal,  $^{13}\text{C}$   $\beta$ -apo-13-carotenone. In the digesta samples, no  $^{13}\text{C}$   $\beta$ -apo-carotenols, or  $^{13}\text{C}$   $\beta$ -apo-carotenoic acids were observed.

*TRL and Plasma Levels of  $^{13}\text{C}$   $\beta$ -apo-carotenoids and Unlabeled  $\beta$ -apo-carotenoids*



Vitamin A derivatives of  $^{13}\text{C}$   $\beta$ -apo-carotene (i.e. labeled retinol, retinyl palmitate, and retinal) as well as unlabeled derivatives (i.e. retinol, retinyl palmitate, retinal, and retinoic acid) were observed in the plasma of the subjects consuming  $^{13}\text{C}$   $\beta$ -carotene. However, no asymmetric  $^{13}\text{C}$   $\beta$ -apo-carotenals,  $^{13}\text{C}$   $\beta$ -apo-carotenols, or  $^{13}\text{C}$   $\beta$ -apo-carotenoic acids were observed in the plasma or TRL samples of treatment subjects.

Native  $\beta$ -apo-13-carotenone (presumably from previous meals), but no other native  $\beta$ -apo-carotenals, were observed in both the fasting and postprandial plasma samples from both control and treatment groups. A false-positive  $^{13}\text{C}$   $\beta$ -apo-13-carotenone peak appeared at the proper retention time and with the same parent>daughter transitions, however the relative intensities of the daughter ions did not match that of the authentic standard, the peak area remained constant over 7 hours, and it was also observed in the plasma of subjects consuming the control meal.

A subset of plasma and TRL samples were also tested for symmetric  $\beta$ -apo-carotenyl esters. The authentic unlabeled retinyl palmitate standard provided an estimated window of retention time where putative asymmetric  $\beta$ -apo-carotenal esters would be expected to elute. It was also presumed that the ester would behave as retinyl palmitate in-source, with the loss of the fatty acid resulting in the charged alcohol species of  $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ . A search of the anticipated alcohol parent>daughters transitions (**Supplemental Table 2**) during the anticipated retention time window found no  $\beta$ -apo-carotenyl esters.

## DISCUSSION

The study presented herein was conducted to assess the species and levels of  $\beta$ -apo-carotenoids that might arise after the consumption of a  $\beta$ -carotene containing meal. Labeled  $\beta$ -carotene was

used to differentiate between  $\beta$ -carotene fed during the study, and  $\beta$ -carotene consumed from previous meals.

The  $^{13}\text{C}$   $\beta$ -apo-carotenoid profile and content of the test meal was characterized, and a series of labeled  $^{13}\text{C}$   $\beta$ -apo-carotenals was noted. Indeed,  $\beta$ -apo-carotenals are not foreign to the food supply. Both fresh fruits (25,27,47), as well as processed fruit juices (48), contain various  $\beta$ -apo-carotenals. A recent comprehensive survey of Philipino plant foods observed  $\beta$ -apo-carotenals in a majority of foods tested (28). Their presence in plant foods is expected, as carotene serves as substrate for a number of plant carotenoid cleavage dioxygenases (31). Apo-carotenoids may also be chemically derived, as the conjugated double bonds of the polyene chain are highly susceptible to oxidation (48,49).  $\beta$ -Apo-carotenoids like  $\beta$ -apo-8'-carotenal (used to color lipid-based margarines and cheeses in the US (32) and EU (50)) and  $\beta$ -apo-8'-carotenoic acid ethyl ester (approved for egg yolk and poultry skin coloration in the EU (30)) are intentionally added to foods. The  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone in our test meal were likely derived via chemical oxidation via exposure to ambient oxygen after synthesis, and also co-oxidation with lipid during test meal blending.

Presuming  $\beta$ -apo-carotenals/carotenone are the primary oxidative metabolites formed,  $\beta$ -carotene appears to be robust to digestion, as the levels of  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone remained quite low relative to  $^{13}\text{C}$   $\beta$ -carotene in digesta. Time was negatively associated with the concentration of  $^{13}\text{C}$   $\beta$ -carotene observed in digesta due to the large drop after the first 15 min. post-consumption (**Figure 2B**) when  $t=0$  was included. The initial drop in  $\beta$ -carotene level is likely due to the dilution effect observed as fluid enters the enteral cavity to aid in the digestive process (39). Over time, a portion of this fluid is reabsorbed, and a portion of the carotene absorbed. This “dilution effect” also influences measured digesta levels of  $^{13}\text{C}$   $\beta$ -apo-

carotenals/carotenone. It is also possible that the larger oil droplets containing  $^{13}\text{C}$   $\beta$ -carotene and  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone floated in the stomach and thus were evacuated at a slower rate than the aqueous digesta. These results contrast with those of Tyssandier et al. (51) where a steady decrease in gastric  $\beta$ -carotene was observed over time after the feeding of carrot puree. This disparity is likely due to differences in  $\beta$ -carotene source (food matrix vs. oil) and differences in digesta sampling size (100-200 mL vs. 8 mL).

Regardless of the source(s) of the initial dilution, it was necessary to account for this effect when assessing changes in  $\beta$ -apo-carotenal/carotenone levels over time, so as to not make erroneous assumptions about increases or decreases actually due to fluid volume changes. Glycerol, formed from the cleavage of tri-, di-, and mono-glycerides, was postulated to serve as a normalization factor, but ultimately did not correct for the known drop in  $\beta$ -carotene digesta levels. Instead,  $\beta$ -carotene levels are presented in the absence of a corrective factor for volume changes, while  $\beta$ -apo-carotenals/carotenone digesta levels have been normalized to digesta  $\beta$ -carotene, assuming the hypothesis that there is no significant difference in absorption efficiency of  $\beta$ -carotene and its metabolites.

Digesta  $^{13}\text{C}$   $\beta$ -apo-8'-carotenal trended toward a decrease in level with time, relative to  $\beta$ -carotene. In contrast, relative levels of digesta  $^{13}\text{C}$   $\beta$ -apo-10', -12', -14', -15-carotenal and  $^{13}\text{C}$   $\beta$ -apo-13-carotenone increased with time (**Figure 4B-F, Table 2**). One possible hypothesis is that the  $^{13}\text{C}$   $\beta$ -carotene and  $^{13}\text{C}$   $\beta$ -apo-8'-carotenal were oxidized (either chemically or enzymatically) into shorter-chain  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone over time. This theory is further supported by the fact that the gastric digesta was associated with higher levels of  $^{13}\text{C}$   $\beta$ -apo-8'-carotenal and lower levels of  $^{13}\text{C}$   $\beta$ -apo-12', -14', -15-carotenal, relative to duodenal digesta. However, other possible explanations exist.

Studies employing an *in vitro* digestion model report a 0-30% loss of  $\beta$ -carotene through the digestive process (41,52,53), with the bulk of loss occurring during the gastric phase (i.e. after the first 30 min. of incubation) (41). Similarly, only slight “spontaneous” oxidation was reported for  $\beta$ -carotene incorporated into artificial micelles and incubated at 37 °C for 2 h (34). In contrast, the co-presence of metmyoglobin resulted in dramatic losses of  $\beta$ -carotene of up to 80% (34,41), but only in the micelle experiments did  $\beta$ -apo-carotenal metabolites accumulate (34). It can be observed from the human digesta data that while some products increased and others decreased over time, none of the  $\beta$ -apo-carotenals/carotenone increased or decreased dramatically, with all changes less than one order of magnitude in level (**Figure 4**). If these  $\beta$ -apo-carotenals/carotenone are presumed to be the primary metabolites formed during digestion, then  $\beta$ -carotene appears to be fairly robust to human digestive conditions, with total losses <2%. The overall lack of  $\beta$ -carotene isomerization in the stomach and duodenal digesta, concurrent with previous work (51), further supports this conclusion.

In our study, only native  $\beta$ -apo-13-carotenone was found circulating in human blood. No other labeled  $\beta$ -apo-carotenals/carotenone observed in the digesta were found in the plasma or TRL fraction. Similarly, neither native nor labeled  $\beta$ -apo-carotenols or  $\beta$ -apo-carotenoic acids (the presumed reduction and oxidation products, respectively) were observed. These results are in line with a previously published report of  $\beta$ -apo-13-carotenone, but no other  $\beta$ -apo-carotenals, observed in the plasma of free-living humans (37). A previous study feeding labeled  $\beta$ -carotene observed  $\beta$ -apo-8'-carotenal in plasma, but only after 3 days post  $\beta$ -carotene consumption (38). It is possible that  $\beta$ -apo-carotenals/carotenone formation *in vivo* may occur after absorption (i.e. not in the digestive tract), and only over longer periods of time (i.e. >> 7 h).

The presence of labeled asymmetric  $\beta$ -apo-carotenals in digesta but not in plasma supports a few hypotheses: a) these compounds are not absorbed into the plasma, b) they are absorbed into the plasma but at levels below our method limit-of-detection (estimated to be 50 fmol on column for  $\beta$ -apo-8'-carotenal), or c) these compounds are absorbed but they are converted or broken down into other metabolites including phase II metabolites (e.g. glucuronidated species). This last idea is further supported by *in vitro* studies where  $\beta$ -apo-8'-carotenal,  $\beta$ -apo-10'-carotenal, and  $\beta$ -apo-13-carotenone were applied to Caco-2 cells (54). After absorption by the cells, all of the original  $\beta$ -apo-carotenoids were transformed into other metabolite(s), most of whose identities are still under investigation. Also, the study presented herein was performed in healthy young men, and different results may be obtained from cohorts with different demographics (e.g. females, smokers, diseased populations).

Collectively, these results demonstrate that  $\beta$ -apo-carotenals/carotenone are observed in the gastric and duodenal digesta. Levels of the shorter-chain  $\beta$ -apo-carotenoids increase in digesta over time but still remain in minor quantities, relative to  $\beta$ -carotene levels.  $^{13}\text{C}$   $\beta$ -Carotene,  $^{13}\text{C}$  retinol, and  $^{13}\text{C}$  retinyl palmitate were observed in the plasma post-test dose feeding, but no asymmetric  $\beta$ -apo-carotenals/carotenone nor  $\beta$ -apo-carotenols or  $\beta$ -apo-carotenoid acids were observed up to 7 hours post-prandially. The absence of newly formed asymmetric  $^{13}\text{C}$   $\beta$ -apo-carotenoids in plasma suggests a lack of absorption and/or conversion to non-predicted metabolites. Furthermore, these results suggest a lack of post-prandial BCO2 activity towards  $\beta$ -carotene in the healthy human intestine, and continue to support the role of BCO1 as the primary cleavage enzyme. Further work would be needed to identify the fate of the  $\beta$ -apo-carotenals/carotenone observed in the digesta.

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**Table 1.** Characteristics of Study Subjects<sup>1</sup>

|              | Age<br>(years) | Weight<br>(kg) | BMI<br>(kg/m <sup>2</sup> ) | Plasma total<br>cholesterol<br>level<br>(mg/dL) | Plasma<br>triacylglycerol<br>level<br>(mg/dL) | Plasma<br>glucose<br>(mg/dL) | Hemoglobin<br>(g/dL) |
|--------------|----------------|----------------|-----------------------------|---|---|------------------------------|----------------------|
| Test Meal    | 24.6 ± 1.1     | 68 ± 8.7       | 21.6 ± 2.1                  | 157 ± 76.6                                      | 70.1 ± 32.7                                   | 76 ± 9.5                     | 14.1 ± 1.19          |
| Control Meal | 24.0 ± 3.0     | 77 ± 16        | 24.0 ± 4.3                  | 150 ± 100                                       | 59.0 ± 16.5                                   | 74 ± 4.5                     | 14.9 ± 1.85          |

<sup>1</sup>Values are means ± standard deviation at the screening visit of subjects who completed the study,  $n = 7$  for test meal,  $n = 3$  for control meal

BMI = Body mass index

Note that characteristics between meal types are not statistically different from one another using a two-tailed unpaired Student's t-test ( $P < 0.05$ ).

**Table 2.** Mixed Model Results for Digesta Concentrations of  $^{13}\text{C}$   $\beta$ -carotene and the ratios of  $^{13}\text{C}$  ( $\beta$ -apo-carotenals or carotenone)/ $^{13}\text{C}$   $\beta$ -carotene.

| Analyte   | Coefficient<br>( $\pm$ SE) | <i>Digesta</i> |      |                      | Coefficient<br>( $\pm$ SEM) | <i>Time</i> |      |                      |
|---|----------------------------|----------------|------|----------------------|-----------------------------|-------------|------|----------------------|
|   |                            | $\chi^2$       | D.F. | P-value <sup>2</sup> |                             | $\chi^2$    | D.F. | P-value <sup>2</sup> |
| $^{13}\text{C}$ $\beta$ -carotene                       | 2.7E+6( $\pm$ 5.1E+6)      | 0.28           | 1    | 0.60                 | 8.7E+4( $\pm$ 2.5E+4)       | 11.46       | 1    | 7.0E-4 <sup>3</sup>  |
| $^{13}\text{C}$ $\beta$ -apo-8'-carotenal <sup>1</sup>  | 1.0E-3( $\pm$ 4.6E-4)      | 4.77           | 1    | 2.9E-2 <sup>3</sup>  | -3.8E-6( $\pm$ 2.3E-6)      | 2.92        | 1    | 8.8E-2               |
| $^{13}\text{C}$ $\beta$ -apo-10'-carotenal <sup>1</sup> | 3.3E-4( $\pm$ 7.1E-4)      | 0.22           | 1    | 0.64                 | 1.1E-5( $\pm$ 3.4E-6)       | 9.64        | 1    | 1.9E-3 <sup>3</sup>  |
| $^{13}\text{C}$ $\beta$ -apo-12'-carotenal <sup>1</sup> | -9.5E-4( $\pm$ 4.1E-4)     | 5.32           | 1    | 2.1E-2 <sup>3</sup>  | 8.2E-6( $\pm$ 2.0E-6)       | 15.85       | 1    | 6.9E-5 <sup>3</sup>  |
| $^{13}\text{C}$ $\beta$ -apo-14'-carotenal <sup>1</sup> | -3.9E-3( $\pm$ 1.7E-3)     | 5.53           | 1    | 1.8E-2 <sup>3</sup>  | 4.5E-5( $\pm$ 8.0E-6)       | 27.93       | 1    | 1.3E-7 <sup>3</sup>  |
| $^{13}\text{C}$ $\beta$ -apo-15'-carotenal <sup>1</sup> | -1.4E-2( $\pm$ 6.8E-3)     | 4.49           | 1    | 3.4E-2 <sup>3</sup>  | 1.4E-4( $\pm$ 3.4E-5)       | 16.57       | 1    | 4.7E-5 <sup>3</sup>  |
| $^{13}\text{C}$ $\beta$ -apo-13-carotenone <sup>1</sup> | 9.2E-4( $\pm$ 1.0E-3)      | 0.83           | 1    | 0.36                 | 1.5E-5( $\pm$ 5.1E-6)       | 8.09        | 1    | 4.5E-3 <sup>3</sup>  |
| $\beta$ -apo-11-carotenal <sup>1,4</sup>                | -8.7E-4( $\pm$ 1.5E-3)     | 0.39           | 1    | 0.53                 | 9.3E-6( $\pm$ 7.2E-6)       | 1.72        | 1    | 0.19                 |

<sup>1</sup>All test subject-samples at were included in determining the mixed model coefficients provided above ( $n=7$  gastric and  $n=6$  duodenal subject samples at  $t = 0, 0.25, 0.5, 1, 2, 3, 4, 5$ )

<sup>2</sup>As determined by the likelihood test ratio

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

<sup>4</sup>Although no  $^{13}\text{C}$  label remains, the only source of this product in the test meal is  $^{13}\text{C}$   $\beta$ -carotene and  $^{13}\text{C}$   $\beta$ -apo-carotenals/carotenone

**Figure 1.** Chemical structures of the isotopically labeled  $^{13}\text{C}$   $\beta$ -carotene and resulting derivatives synthesized and analyzed via LC-MS/MS.

**Figure 2. A)** Average baseline-corrected plasma triglyceride-rich lipoprotein (TRL) concentrations of  $^{13}\text{C}$   $\beta$ -carotene over the course of 7 h after meal consumption during gastric sampling ( $\Delta$ ) and duodenal sampling ( $\blacksquare$ ). Concentrations of  $^{13}\text{C}$   $\beta$ -carotene are represented as means  $\pm$  SEM,  $n = 7$  for gastric,  $n = 6$  for duodenal **B)** The concentration of  $^{13}\text{C}$   $\beta$ -carotene present in gastric ( $\Delta$ ) and duodenal ( $\blacksquare$ ) digesta over 5 h after test meal consumption. Concentrations of  $^{13}\text{C}$   $\beta$ -carotene are represented as means  $\pm$  SEM,  $n = 7$  for gastric,  $n = 6$  for duodenal. Note that levels are baseline corrected as  $^{13}\text{C}$   $\beta$ -carotene was observed in 0 h TRL of some of the subjects before the consumption of the second meal (presumably from VLDL). No statistically significant difference was observed between  $^{13}\text{C}$   $\beta$ -carotene concentrations measured at the same time point regardless of sampling location (paired t-test,  $P < 0.05$ ).

**Figure 3.** A representative LC-MS/MS chromatogram of **A)** the  $^{13}\text{C}$   $\beta$ -apo-carotenal standard mixture and **B-I)** metabolites observed in the duodenal digesta of a representative treatment meal subject at 3 h (**B** =  $\beta$ -apo-11-carotenal, **C** =  $^{13}\text{C}$   $\beta$ -apo-13-carotenone, **D** =  $^{13}\text{C}$   $\beta$ -apo-14'-carotenal, **E** =  $^{13}\text{C}$   $\beta$ -apo-15'-carotenal, **F** =  $^{13}\text{C}$   $\beta$ -apo-12'-carotenal, **G** =  $^{13}\text{C}$   $\beta$ -apo-10'-carotenal, **H** =  $^{13}\text{C}$   $\beta$ -apo-8'-carotenal, **I** =  $^{13}\text{C}$   $\beta$ -carotene). The parent>daughter transition used for quantitation was selected to display (see **Supplemental Table 2** for further detail).

**Figure 4.** The ratio of  $^{13}\text{C}$   $\beta$ -apo-carotenal or -carotenone/ $^{13}\text{C}$   $\beta$ -carotene present in gastric ( $\Delta$ ) and duodenal ( $\blacksquare$ ) digesta over 5 h after test meal consumption. Concentrations of analytes are

represented as means  $\pm$  SEM,  $n = 7$  for gastric,  $n = 6$  for duodenal. **A)**  $^{13}\text{C}$   $\beta$ -apo-8'-carotenal/ $^{13}\text{C}$   $\beta$ -carotene, **B)**  $^{13}\text{C}$   $\beta$ -apo-10'-carotenal/ $^{13}\text{C}$   $\beta$ -carotene, **C)**  $^{13}\text{C}$   $\beta$ -apo-12'-carotenal/ $^{13}\text{C}$   $\beta$ -carotene, **D)**  $^{13}\text{C}$   $\beta$ -apo-14'-carotenal/ $^{13}\text{C}$   $\beta$ -carotene, **E)**  $^{13}\text{C}$   $\beta$ -apo-15-carotenal i.e. retinal/ $^{13}\text{C}$   $\beta$ -carotene (none detected in duodenal digesta at 0.5h, 1h), **F)**  $^{13}\text{C}$   $\beta$ -apo-13-carotenone/ $^{13}\text{C}$   $\beta$ -carotene. Note that the results for  $\beta$ -apo-11-carotenal/ $^{13}\text{C}$   $\beta$ -carotene can be found in **Supplemental Figure 5**.