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Lutein does not affect the bioefficacy of beta-carotene measured using gas chromatography-combustion interfaced-isotope ratio mass spectrometry (GC-C-IRMS)

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Lutein does not affect the bioefficacy of beta-carotene measured using gas chromatography-
combustion interfaced-isotope ratio mass spectrometry (GC-C-IRMS)

by

Xixuan Hu Collins

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in partial fulfillment of the requirements for the degree of
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Ames, Iowa

2003

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LIST OF ABBREVIATIONS

- AMD Age-related macular degeneration
- AMS Accelerator mass spectrometry
- ANOVA Analysis of variance
- AP Atom percent
- APE Atom percent excess
- APCI LC-MS Atmospheric pressure chemical ionization liquid gas chromatogram mass spectrometry
- AUC Area under the concentration vs. time curve
- BMI Body mass index
- β -Carotene- d_8 11, 11', 19, 19, 19, 19', 19', 19'- 2H_8 - β -carotene
- CV Coefficient of variance
- CYP Cytochrome P₄₅₀
- EA Elemental analyzer
- EDTA Ethylenediamine tetra-acetic acid
- EI Electron impact
- EST Expressed sequence tags
- FI/APCI-MS Flow-injection/atmospheric pressure chemical ionization-mass spectrometry
- GC Gas chromatography
- GC-C-IRMS Gas chromatography-combustion interfaced-isotope ratio mass spectrometry
- GC/EC-NCI-MS Gas chromatography/electron capture negative chemical ionization mass spectrometry
- HPLC High performance liquid chromatography
- IRMS Isotope ratio mass spectrometry
- LC-MS Liquid chromatography-mass spectrometry
- LC/PB-MS Liquid chromatography/particle beam-mass spectrometry
- LDL Low density lipoprotein
- LDH-C Lactate dehydrogenase
- MTBE Methyl *tert*-butyl ether

RA Retinyl acetate

RAR Retinoic acid receptor

RPE Retinal pigment epithelium

RT-PCR Reverse transcriptase polymerase chain reaction

SIS-ITMS Selected ion storage-ion trap mass spectrometry

SEM Standard error of mean

TRL Triacylglycerol-rich lipoprotein

VLDL Very low density lipoprotein

ABSTRACT

Lutein could be a factor responsible for the low bioavailability and low bioconversion of β -carotene from dark green leafy vegetables. The high sensitivity of gas chromatography-combustion interfaced-isotope ratio mass spectrometry (GC-C-IRMS) and the availability of highly-enriched ^{13}C -labeled tracer doses of β -carotene and lutein make it possible to administer β -carotene and lutein at levels typical of dietary intake and to follow the fate of these carotenoids after their administration. Using this approach, we compared in female subjects the appearance of ^{13}C - β -carotene and its cleavage product, ^{13}C -retinol in the plasma after subjects ingested 1 mg of ^{13}C - β -carotene with or without 3 mg of ^{13}C -lutein. Women ($n = 7$) each ingested both the single dose of ^{13}C - β -carotene and the combined dose of ^{13}C - β -carotene and ^{13}C -lutein in random order. Blood samples were collected at selected intervals until 16 h post dosing. Additional blood samples were collected at selected hours until 528 h post dosing. Lutein, β -carotene and retinol were extracted from plasma and purified by using HPLC. Retinol samples were directly analyzed by using GC-C-IRMS; lutein and β -carotene were derivatized to thermally stable compounds and the derivatives were analyzed by using GC-C-IRMS. The appearance of ^{13}C - β -carotene in the plasma was not different after the two doses; nor was the appearance of ^{13}C -retinol in the plasma. The ratio of 0-96 h area under the concentration vs. time curve (AUC) for ^{13}C -retinol to 0-528 h AUC for ^{13}C - β -carotene was used as an index for conversion of ^{13}C - β -carotene to ^{13}C -retinol, and there was no difference in the ratios after the two doses. Plasma β -carotene concentration at baseline was negatively correlated with the 0-96 h AUC for ^{13}C -retinol after the combined dose ($r = -0.84$, $P = 0.02$). Plasma lutein concentration at baseline was positively correlated with 0-528 h AUC for ^{13}C -lutein ($r = 0.82$, $P = 0.02$), and positively correlated with 0-528 h AUC for ^{13}C - β -carotene after the ^{13}C - β -carotene dose alone ($r = 0.84$, $P = 0.02$). The responses from the subjects after the two doses were highly variable. The interindividual coefficient of variance (CV) of 0-528 h AUC for ^{13}C - β -carotene was 59% after the single dose and 36% after the combined dose. The CV of 0-96 h AUC for ^{13}C -retinol was 64% after the single dose and 53% after the combined dose. The CV of 0-528 h AUC for lutein was 44%. We concluded

that a physiological dose of lutein does not inhibit the bioavailability of β -carotene and bioconversion of β -carotene to vitamin A in young women. This is the first study to investigate the interaction of β -carotene and lutein in humans using dose levels typical of dietary intake and a weight ratio of 3:1 that is common in vegetables.

GENERAL INTRODUCTION

Thesis organization

The dissertation is organized into three chapters, including a literature review, a manuscript prepared for submission to the American Journal of Clinical Nutrition, and general conclusions. The literature review consists of three parts. Part I reviews studies regarding to the interaction of β -carotene and lutein. Part II reviews application of stable isotope tracer methods in studies of β -carotene absorption and conversion. Part III reviews the recent molecular characterization of β -carotene cleavage enzymes. The manuscript presents an investigation of the effects of a physiological dose of lutein on the absorption and bioconversion of β -carotene in young women. The dissertation ends with general conclusions of the study.

Literature Review

Part I. Interactions of β -carotene and lutein

Introduction

In developing countries, provitamin A carotenoids in dark green leafy vegetables and yellow orange vegetables and fruits account for 70-90% of total vitamin A intake (WHO/UNICEF/IVACG Task Force 1997). Beta-carotene is abundant in dark green leafy vegetables but these vegetables are not effective in correcting vitamin A deficiency (de Pee et al 1995, Takyi 1999). The underlying mechanism may involve an inhibitory effect of lutein (β,ϵ -carotene-3,3'-diol), a nonprovitamin A oxycarotenoid and structural analog of β -carotene (**Figure 1**). Lutein is a characteristic matrix component in dark green leafy vegetables. The bioavailability of lutein from dark green leafy vegetables is considerably higher than that of β -carotene (van het Hof et al 1999a, van het Hof et al 1999b). Orange-colored fruits and vegetables, which have little or no lutein, are more effective than dark green leafy vegetables in increasing serum concentrations of retinol and β -carotene in children (de Pee et al 1999, van Lieshout et al 2001). A high lutein content could adversely

affect the bioavailability of β -carotene in spinach and other dark green leafy vegetables by having a competitive or antagonistic effect on the intestinal absorption of β -carotene (Kostic et al 1995, van den Berg and van Vliet 1998), and/or by inhibiting the cleavage of β -carotene to vitamin A (van Vliet et al 1996a).

The growing interest in mechanisms of lutein and β -carotene interaction is also generated from the interest in the putative health benefits of lutein. Consumption of lutein and zeaxanthin rich vegetables is negatively correlated with deterioration of the retina in the form of age-related macular degeneration (AMD) (Snodderly 1995). Epidemiological data from the Los Angeles Atherosclerosis Study have shown that increased dietary intake of lutein is protective against the development of early atherosclerosis (Dwyer et al 2001).

Studies regarding to interaction of lutein and β -carotene, and interaction of other carotenoids, were reviewed a few years ago (van den Berg 1998, White and Paetau 1998, van den Berg 1999).

In vitro studies

In an in vitro measurement of β -carotene cleavage activity, rat and hamster intestinal homogenate were prepared. The $9,000 \times g$ supernatant from the homogenate was used as the source of β -carotene cleavage enzyme. Addition of $9 \mu g$ of lutein to an incubation of $3 \mu g$ β -carotene and the cleavage enzyme reduced retinal formation (van Vliet et al 1995a). Interaction of rabbit small intestine mucosa β -carotene cleavage enzyme and lutein showed that lutein competitively inhibited the β -carotene cleavage reaction (Ershov et al 1993). These studies seem to suggest that lutein inhibits the cleavage of β -carotene by inhibiting the enzymatic activity of β -carotene cleavage enzyme. However, another incubation study using $9,000 \times g$ supernatant prepared from rat intestine as the enzyme source did not find inhibitory effect of lutein on β -carotene cleavage (Grolier et al 1997).

Animal studies

A series of studies carried in the late 40's and early 50's investigated the effects of lutein on the utilization of β -carotene in rats (Kemmerer et al 1947, Kelley and Day 1948,

Johnson and Baumann 1948, High and Day 1951). Relatively large amounts of lutein (2.5 to 20 times the amount of β -carotene) impaired the utilization of β -carotene in rats, whereas small amounts of lutein seemed to promote the utilization of β -carotene. The deposition of vitamin A in the liver was significantly decreased in rats fed more lutein (at β -carotene:lutein ratios < 1) (High and Day 1951). Recent study from our lab showed inhibitory effects of lutein on β -carotene utilization in gerbils. Gerbils fed with a vitamin A-free diet supplemented with the leaves from a lutein-free (*lut2*) *Arabidopsis* mutant had 48% more vitamin A stores in the liver than those fed with a vitamin A-free diet supplemented with the leaves from a wild type of *Arabidopsis* (Yan et al 2003). Therefore in general, animal data seem to support the conclusion that lutein inhibits the bioavailability and/or bioconversion of β -carotene when ingested at higher amounts.

Human Studies

Human studies used pharmacological doses of β -carotene and lutein to investigate their interactions and the results have been inconsistent. In one study (Kostic et al 1995), 8 subjects were given a β -carotene dose and an equimolar mixture of β -carotene and lutein. The dose level was 0.5 $\mu\text{mol/kg}$ body weight. Lutein reduced the area under the concentration vs. time curve (AUC) value for β -carotene in subjects who had higher AUC value when ingested β -carotene alone, but enhanced AUC value for β -carotene in subjects who had lower AUC value when ingested β -carotene alone. In another study, a combined dose of 15 mg β -carotene and 15 mg of lutein decreased AUC for β -carotene in the triacylglycerol-rich lipoprotein (TRL) fraction, but had no effect on the AUC for retinyl esters in TRL (van de Berg and van Vliet 1998). This suggests that lutein negatively affected β -carotene absorption when given simultaneously, but had no effect on β -carotene cleavage. The inhibitory effect of lutein was most marked when lutein was the predominant carotenoid (at a lutein to β -carotene ratio of 2:1) (van den Berg 1998). One possible reason why lutein may inhibit the absorption of β -carotene is the preferential incorporation of lutein into chylomicrons compared with β -carotene. Gartnet et al (1996) reported that there was a preferential increase in chylomicron levels of lutein and zeaxanthin compared with β -

carotene in humans after ingestion of a single dose of Betatene, a natural carotenoid source which contains β -carotene 14-fold higher than lutein. More recently, Tyssandier et al (2002) reported that adding a second carotenoid from a vegetable source to a meal that provided a first carotenoid diminished the chylomicron response to the first carotenoid. Fibers in vegetables have more impact on β -carotene absorption than on lutein absorption (Castenmiller et al 1999). Lutein is more efficiently transferred to micelles than β -carotene in the duodenum in humans (Tyssandier et al 2003).

In these studies, β -carotene and lutein were ingested in doses that greatly exceed effective (absorbed) doses typically derived from fruits and vegetables (Swanson et al 1996). High dose of β -carotene down-regulates the enzymatic activity of β -carotene cleavage enzyme (Brubacher and Weiser 1985, van Vliet et al 1995a). The equimolar doses of β -carotene and lutein that were administered do not extrapolate to the disproportionate content of lutein in dark green leaves (Adewusi and Bradbury 1993, USDA-NCC 1998, van den Berg et al 1998). Therefore there is a need to use physiological doses, and a lutein to β -carotene ratio typically found in vegetables, to investigate the interaction of lutein and β -carotene in our normal dietary intake.

Part I. Application of stable isotopic tracing methods in β -carotene absorption and conversion studies

Introduction

As the major vitamin A precursor in foods, β -carotene is a nutrient of importance. The absorption and bioconversion of β -carotene has been the subject of investigation for over 50 years (Moore 1957). However, there is a lack of characterized animal models which resemble humans with respect to β -carotene metabolism, and a lack of appropriate methodological tools which can be applied to study β -carotene metabolism in humans at levels typical of daily dietary intake. Therefore there remains considerable uncertainty with regard to quantification of β -carotene absorption and bioconversion in humans.

Application of stable isotopic tracing methodology has been a fairly recent approach to the study of β -carotene metabolism in humans. Previous approaches involved radioactive forms of β -carotene. Beta-carotene labeled with either ^{14}C or ^3H was employed in two earlier studies of β -carotene absorption and intestinal metabolism in a small number of surgical patients with catheterized thoracic lymph ducts (Goodman et al 1966; Blomstrand and Werner 1967). These studies indicated that β -carotene absorption efficiency is probably low to moderate and highly variable between individuals.

A stable isotope tracer approach eliminates the exposure of humans to radiation and associated handling and disposal costs of radioactive materials (Dueker et al 1998b; Parker 2000). Other advantages include simultaneous determination of tracer/tracee molar ratio (% enrichment), specific detection of analyte according to its mass and mass fragmentation profile and known chromatographic behavior (hyphenated GC and LC/MS systems), and potential for administration of several different tracers both simultaneously and repeatedly so that more information can be obtained from a single experiment. For study of β -carotene metabolism, a stable isotope tracer approach provides a specific benefit, which is the option to administer a small, physiological dose that is similar to the daily dietary intake. A physiological dose will not disrupt the endogenous pools of β -carotene and its vitamin A metabolites, nor down-regulate the activity of intestinal β,β' -carotene-monooxygenase (Dueker et al 1998b; Parker 2000).

Until now, there have only been a few studies of β -carotene absorption and conversion in humans using stable isotope tracer approaches (**Table 1**). The doses were labeled with ^2H and ^{13}C . There were also two studies using ^{14}C - β -carotene that was not considered as radioactive because of the very small dose (Dueker et al, 2000, Hickenbottom et al 2002). The doses ranged from 1 mg to 126 mg. Except for one study that used kale that was grown in a $^{13}\text{CO}_2$ -enriched atmosphere as the source of β -carotene dose (Kelm et al 2001), all the other studies used purified β -carotene as the dose. The results from these studies indicate that the absorption rate and conversion rate of β -carotene to vitamin A are low, and there is high individual and gender variability (**Table 1**).

Double tracer method for measuring β -carotene absorption and conversion

The use of a double-tracer method makes it possible to directly compare the metabolism of an oral dose of labeled β -carotene with that of an approximately equimolar dose of labeled vitamin A.

In one study (Lin et al 2000), 11 healthy women ingested 30 μ mol hexadeuterated (D_6) retinyl acetate orally, followed with 37 μ mol D_6 β -carotene after one week. The results showed that the mean absorption of D_6 β -carotene was $3.3 \pm 1.3\%$ and the mean conversion ratio was 0.81 ± 0.34 mol of trideuterated (D_3) retinol to 1 mol of D_6 β -carotene. Only 6 of the 11 subjects had measurable plasma D_6 β -carotene and D_3 retinol. The mean absorption of D_6 β -carotene in these 6 subjects was $6.1 \pm 0.02\%$ and their mean conversion ratio was 1.47 ± 0.49 mol of D_3 retinol to 1 mol of D_6 β -carotene. The remaining 5 subjects were low responders with $\leq 0.01\%$ absorption and a mean conversion ratio of 0.014 ± 0.004 mol D_3 retinol to 1 mol D_6 β -carotene. The mean absorption value of β -carotene was lower than values previously published (9-22%). This difference could be due to the fact that in previously published studies, β -carotene was dissolved in oil or dissolved in oil and emulsified, whereas in their study the doses were not solublized and emulsified. The plasma D_6 retinol concentration increased promptly and peaked around 21 hours after dosing. The plasma D_3 retinol concentration also increased promptly but peaked at around 10 hours after dosing with D_6 β -carotene, and this delay suggested that the plasma D_3 retinol might consist of D_3 retinyl esters generated in enterocytes. A positive correlation was found between the AUCs for plasma D_3 retinol and those for its parent D_6 β -carotene, which suggested that intestinal absorption and release of both β -carotene and vitamin A might be controlled by the same or similar factors. The authors concluded 1) there were no biochemical markers in their small group of subjects that were associated with low response; 2) nutritional status, as reflected by baseline plasma β -carotene and retinol concentrations, did not predict a subject's propensity to utilize β -carotene for vitamin A; and 3) both absorption of β -carotene and conversion of β -carotene to vitamin A contributed to the variable plasma response to ingested β -carotene.

In another study with a similar study design (Hickenbottom et al 2002a), 11 healthy men were orally dosed with 30 μmol D₆ retinyl acetate and then one week later with 37 μmol D₆ β -carotene. The results showed a mean absorption of $2.235 \pm 0.925\%$ for all subjects, and a mean conversion ratio of 0.0296 ± 0.0108 mol retinol to 1 mol β -carotene. Only 6 of 11 subjects were responders who had measurable plasma concentrations of D₆ β -carotene and D₃ retinol. The mean absorption of D₆ β -carotene in these 6 subjects was $4.097 \pm 1.208\%$, and the mean conversion ratio was 0.054 ± 0.0128 mol retinol to 1 mol β -carotene. Unlike the findings in the first study, the responder group had significantly lower baseline concentrations of both retinol and β -carotene. Similar to the first study, there was a strong correlation between the AUCs for plasma D₃ retinol and those for its parent compound D₆ β -carotene. The authors also concluded that the low-responder trait was not due to a greater conversion of ingested β -carotene to vitamin A, because the low responders to D₆ β -carotene also had low plasma D₃ retinol response. This conclusion is different from that suggested by Kostic et al (1995).

The third study used three tracer doses: 30 μmol D₆ retinyl acetate, 30 μmol D₆ β -carotene, and 0.27 μmol ¹⁴C- β -carotene (Hickenbottom et al 2002b). The D₆/D₃ ratio of the two retinols in the plasma was not constant during the absorption/distribution phase (3-11 h), ranging between a value of 3 and 16. However between 11 and 98 h postdosing the ratio was relatively constant with a mean value of 8.5 h. They suggested that a single sample taken between 11-98 h postdosing may provide a reliable estimation for the relative dose of the two forms of vitamin A. In their subject, 8.5 mol of β -carotene would provide a retinol activity equivalent to 1 mol of preformed vitamin A. On a mass base, 15.9 μg of β -carotene was equivalent to 1 μg of retinol. Cumulative 24-h stool and urine samples were collected for two weeks to follow the absorption and excretion of ¹⁴C- β -carotene. According to accelerator mass spectrometry (AMS) analysis of ¹⁴C in the cumulative stool, 53% of the total administered β -carotene was absorbed.

Stable isotope reference method

Tang et al (1997) developed a method that used flow-injection atmospheric pressure chemical ionization-mass spectrometry (FI/APCI-MS) to measure the plasma enrichment of β -carotene- d_8 (11, 11', 19, 19, 19, 19', 19', 19'- 2H_8 - β -carotene) in a subject orally supplemented with β -carotene- d_8 . They also investigated the kinetics of the metabolism of β -carotene- d_8 to retinol- d_4 by using gas chromatography-mass spectrometry with electron capture negative chemical ionization (GC/EC-NCI-MS) to measure the plasma enrichment of retinol- d_4 derivatives. By applying these methods the same research group studied the vitamin A equivalence of β -carotene in a woman (Tang et al 2000) and in a group of 22 adults (Tang et al 2003).

In the first study (Tang et al 2000), two dosage levels of β -carotene- d_8 (a pharmacological dose, 126.0 mg, and a physiological dose, 6.0 mg) were used 2.5 year apart in a female adult, and multiple blood samples were collected after each dose over a 21-day period. Retinyl acetate- d_8 (9.0 mg) was used as the vitamin A reference. Serum β -carotene concentrations did not change after the 6-mg β -carotene dose. However, serum β -carotene concentration was about three times the baseline level at 24 h after the 126-mg dose. The retinol- d_4 response in the circulation peaked at 24 hours after the β -carotene- d_8 dose with a higher percent enrichment after the pharmacological dose than the physiological dose. The retinol- d_4 formed from a 6-mg dose of β -carotene- d_8 was calculated to be equivalent to 1.6 mg of retinol (conversion rate: 3.8:1), and the retinol- d_4 formed from a 126-mg of β -carotene- d_8 was equivalent to 2.3 mg of retinol (conversion rate: 55:1). These results indicate that the size of the dose affects the conversion rate of β -carotene to vitamin A, and a higher dose results in lower conversion.

In the second study (Tang et al, 2003), 10 men and 12 women ingested 6 mg β -carotene- d_8 , and three days later, 3 mg of retinol- d_8 as reference dose. Multiple blood samples were collected over 56 d. The average conversion factor was 9.1 to 1 by wt or 4.8 to 1 by mol. The conversion efficiency was positively correlated with body mass index. The postabsorptive conversion of β -carotene was estimated as 7.8%, 13.6%, 16.4%, and 19% of the total converted retinol at 6, 14, 21, or 53 d after the β -carotene- d_8 dose, respectively.

Since at 53 d after dosing the conversion of β -carotene was 19% of the total converted retinol, therefore of the total retinol converted from a β -carotene dose, 81% was from intestinal cleavage, and 19% was from extraintestinal cleavage.

Postprandial TRL approach and extrinsic stable isotope reference method

van Vliet et al (1995b) showed that the plasma triacylglycerol-rich lipoprotein fraction (TRL, $d < 1.006 \text{ kg/L}$) may be useful in the assessment of β -carotene uptake and intestinal metabolism to retinyl esters after an oral dose of β -carotene. The advantage of the TRL approach is the use of the chylomicron-rich fraction, which contains newly absorbed β -carotene. However, interpretation of TRL data is limited by the lack of means to control for inter-individual variation in clearance kinetics or variation in chylomicron recovery during TRL preparation and analysis (Parker 2000). Edwards et al (2001, 2002) adapted the TRL-model by co-administering a small quantity of 10,19,19,19- $^2\text{H}_4$ -retinyl acetate (d_4 -RA) in a maximally bioavailable form (oil solution) together with carotenoids from food sources. The authors argued that this extrinsic reference dose controlled for variation in chylomicron kinetics *in vivo*, and for retinyl ester recovery during TRL preparation and analysis. The extrinsic dose was also a reference. The mass of unlabeled vitamin A derived from intestinal cleavage of provitamin A carotene from a test meal could be estimated by comparison with the mass of the extrinsic reference dose.

The investigators applied the extrinsic stable isotope reference method to study the vitamin A potency of β -carotene from various food sources, including raw carrots, spinach, carrot puree (commercial baby food), and boiled-mashed carrots. In the first study (Edwards et al, 2001), three adults consumed a standardized test meal containing 6 mg β -carotene as either raw carrot or spinach, with either 1 g or 20 g added fat, and 6.0 μmol d_4 -retinyl acetate as the extrinsic reference. To calculate masses of absorbed d_0 -retinol and carotene, the authors assumed a 80% absorption of the d_4 -RA reference dose, based on previous studies of retinol absorption in humans (Olson 1987, Sivakumar and Reddy 1972, Goodman et al 1966). Plasma concentrations of d_4 -retinyl ester, d_0 -retinyl ester, and β -carotene peaked between 4.5 and 5.5 h after all high-fat trials, regardless of the food sources of β -carotene, and returned to near baseline around 7 h. In contrast, after the low-fat test meals, the

absorption profile was attenuated and prolonged. The results showed that with 20 g fat and assuming central cleavage, $7 \pm 5\%$ (range: 3%-16%) of the 6 mg β -carotene ingested was taken up (total absorption) with 0.3 ± 0.1 mg as retinol (conversion). If a stoichiometry of conversion of 1:1 was assumed, the calculated percentage of β -carotene taken up from the high fat vegetable meals was $11 \pm 7\%$. In the high-fat trials, the estimated mass of retinol derived from 6 mg of β -carotene in raw carrot or spinach ranged from 0.14 to 0.51 mg. The mean observed equivalency ratio for these raw vegetables under ample fat condition was 27:1 or 23:1 respectively, if the potential contribution of α -carotene in the carrots was ignored. This study showed that the bioefficacy of β -carotene from raw carrots and spinach was low, and the fat content of a meal had impact on the uptake of β -carotene from the meal.

In a second study (Edwards et al, 2002), 9 healthy adults consumed test meals of either carrot puree (commercial baby food) or boiled-mashed carrots on separate days. Of the 9 subjects, 6 also consumed a test meal of raw grated carrot. Test meals supplied $34.7 \mu\text{mol}$ (18.6 mg) β -carotene and $6 \mu\text{mol}$ deuterium-labeled retinyl acetate (d_4 -RA) in oil solution. The results showed that absorption of β -carotene and α -carotene was approximately 2-fold greater from carrot puree than from boiled-mashed carrots, whereas the retinol yield was only marginally influenced by treatment. Carotene and retinyl ester absorption from raw-grated carrot was intermediate to, and did not differ significantly from, cooked preparations. Assuming 80% absorption efficiency of the d_4 -RA reference, 0.44 ± 0.15 mg of β -carotene ($2.4 \pm 0.8\%$) was absorbed intact from carrot puree, which was significantly higher than the 0.16 ± 0.11 mg β -carotene ($0.9 \pm 0.6\%$) absorbed from boiled-mashed carrot. An estimated 0.38 ± 0.28 mg β -carotene ($1.7 \pm 1.3\%$) was absorbed intact from raw carrots. The carrot puree yielded 0.53 ± 0.2 mg vitamin A, the boiled-mashed carrot yielded 0.44 ± 0.17 mg of vitamin A, and the raw carrot meal yielded 0.43 ± 0.27 mg vitamin A. The apparent β -carotene conversion efficiency, defined as the proportion of β -carotene taken up by the intestine that was metabolized to retinol, was $44 \pm 11\%$ (range: 24-58%) for carrot puree, significantly lower than $59 \pm 12\%$ (range: 36-72%) for boiled-mashed carrots. For raw-carrots, the apparent conversion efficiency was $63 \pm 10\%$ and was not different from either of the cooked carrot treatments. These results showed that food

processing and cooking methods had impact on the bioavailability and bioconversion of β -carotene to vitamin A in carrots.

Long-term kinetic study of β -carotene using accelerator mass spectrometry

In general, the studies reviewed above used relatively large doses of β -carotene. Accelerator mass spectrometry (AMS) detection and mass balance were used to study the concentration-time course of a physiological (306 μ g, 200 nCi) oral dose of ^{14}C β -carotene for 209 days in plasma in 1 subject. AMS is an isotope ratio instrument that measures $^{14}\text{C}/^{12}\text{C}$ ratio to parts per quadrillion (10^{-15}), quantifying labeled biochemicals to attomolar (10^{-18}) levels in milligram-sized samples. At attomolar levels of sensitivity, the radiation exposure is decreased to negligible levels.

In two studies (Dueker et al 2000, Hickenbottom et al 2002), ^{14}C - β -carotene, ^{14}C -retinyl esters, ^{14}C -retinol, and several other ^{14}C -retinoic acids were analyzed. ^{14}C analytes appeared 5.5 h after dosing. Labeled β -carotene and its cleavage product ^{14}C retinyl esters showed nearly identical kinetic profiles over the first 24-h period. Cumulative urine and stool samples were collected for 17 and 10 days, respectively. In the stool 57.4% of the dose was recovered within 48 hours postdosing. The stool was the major excretion route for the absorbed dose. The turnover time for β -carotene and retinol were 58 and 302 days, respectively. Area under the curve (AUC) analysis of the plasma response curves suggested a molar vitamin A value of 0.53 for β -carotene, with a minimum of 62% of the absorbed β -carotene cleaved to vitamin A. The data also suggested that retinyl esters derived from β -carotene may undergo hepatic resecretion in VLDL in a process similar to that for β -carotene, because retinyl ester and β -carotene concentration-time courses were parallel for 24 h postdosing.

Study of β -carotene metabolism by using ^{13}C - β -carotene and high-precision isotope ratio mass spectrometry

Introduction to high-precision isotope ratio mass spectrometry

Isotope ratio mass spectrometers (IRMS) are highly specialized mass spectrometers designed for the high-precision determination of isotope ratios for C, H, N, O, or S (Brenna et al 1997). Unlike organic mass spectrometers, samples must be converted to one of several gases prior to introduction to the IRMS. For C, samples are usually combusted to yield CO_2 , with an isotope ratio representative of the material of interest. Carbon dioxide is admitted to a tight electron impact (EI) ion source and produces molecular ions at M/Z 44, 45, and 46, which are monitored continuously using three Faraday cup detectors. The beams are comprised primarily of $^{12}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{16}\text{O}_2 + ^{12}\text{C}^{17}\text{O}^{16}\text{O}$, and $^{12}\text{C}^{18}\text{O}^{16}\text{O}$. The M/Z 46 signal is used to adjust M/Z 45 for the contribution of ^{17}O , yielding a ratio of $^{13}\text{C}/^{12}\text{C}$. For tracer applications employing a baseline correction, the ^{17}O correction is negligible and can be ignored (Brenna et al 1997).

The distinguishing characteristic of IRMS is measurement at high precision, which is defined as a standard deviation in the range of 4-6 significant figures (Brenna 1994, Brenna et al 1997). High precision is accomplished at the expense of flexibility. The electron impact ion source is tight so as to maximize ionization probability, which sets a requirement for gases of high volatility and low reactivity. This limits IRMS to analysis of the pure gases of CO_2 , H_2 , N_2 , N_2O , NO , O_2 , CO , and SO_2 , or SF_6 . $^{13}\text{C}/^{12}\text{C}$, $^2\text{H}/^1\text{H}$, $^{15}\text{N}/^{14}\text{N}$, $^{18}\text{O}/^{16}\text{O}$, and $^{34}\text{S}/^{32}\text{S}$ analyses are performed through analysis of these gases. For continuous flow applications, these gases will usually be analyzed in helium carrier gas (Brenna 1994, Brenna et al 1997).

Since 1990, gas chromatography (GC) interfaced to IRMS by means of an inline microcombustion furnace (GC-C-IRMS) has been available commercially and has facilitated high-precision determination of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ from mixtures separated by GC. Components of commercial instruments include metal oxide combustion furnaces and either NafionTM or cryogenic water traps. The GC is a standard capillary system with split/splitless or on-column injectors. The GC effluent is directed to a combustion reactor, which is normally a ceramic or quartz tube with capillary connections at the entrance and exit. The

reactor is typically loaded with a metal oxide derived from oxidizing metal wire, such as Cu, sometimes along with Pt catalyst to promote completion of the oxidation reaction. CuO is a source of O₂ under 850°C. The replenishing or recharging of Cu to CuO can be accomplished automatically by including a small amount of O₂ in the stream of carrier gas flowing through the furnace between samples, or more often it can be completed by introducing a stream of O₂ or air into the furnace for several hours or overnight at 500°C. Solvent peaks are diverted from the furnace by a backflush switching of flow pressure under computer control, or an automatic rotary valve, to avoid prematurely depleting this reagent.

CO₂ and H₂O are the products after organic analytes are combusted in the furnace. Water is removed prior to admission to the ion source by either a NafionTM tube or a cryogenic trap, because water reacts with CO₂ to produce HCO₂⁺, which interferes with analysis at m/z 45. As analyte CO₂ passes through the ion source, three traces, representing masses 44, 45, and 46, are recorded. For CO₂, the relative gains in Finnigan MAT 252 are 3 to 200 to 1000 for m/z 44, 45, and 46 channels. A commonly observed phenomenon is that the 45 peak elutes prior to the 44 peak, causing a “positive-negative-going” ratio trace. This reversal is thought to be due to differences in intermolecular interactions between the isotopically substituted peaks and all ¹²C peaks. These traces are used to generate integrated areas by applying either the summation or curve-fitting algorithms. Isotopic calibration is accomplished if one of the peaks in the chromatograms is an internal standard, or more commonly and conveniently, a pulse of calibrated CO₂ from a gas bottle is admitted to the ion source through a separate inlet during a period when no peaks elute from the GC. For carbon, the minimum sample size required to yield a precision of SD (δ¹³C) less than 0.3‰ is about one nmol carbon.

The dynamic range over which enrichments can be accurately and precisely measured by GC-C-IRMS is limited. Brenna et al (1997) suggested that carbon isotope ratios much above δ¹³C = 1000‰ will compromise precision and accuracy, partly due to the large difference in isotope ratio as compared with natural abundance standards. Since the amplification among the channels are set to produce natural abundance signals of equal magnitude and expected to measure simultaneously the ratio anticipated for the enriched samples and for the

baseline samples, this limitation problem cannot easily be solved even by using enriched standards.

Study of β -carotene metabolism by using GC-C-IRMS

Parker et al (1993) first applied GC-C-IRMS to the study of β -carotene metabolism. In their preliminary study, they successfully administered an oral dose as low as 0.5 mg to a subject and measured the metabolism and plasma biokinetics of ^{13}C - β -carotene from algae using GC-C-IRMS. Later in a study (Parker et al 1997), they extracted and purified per-labeled ^{13}C - β -carotene (> 95% ^{13}C) from a crude hexane extract of unicellular green algae that were grown with ^{13}C -labeled CO_2 as the sole carbon source. The results showed that at the times corresponding to the maximum concentration of each ^{13}C -labeled analyte during the 22-h period, the proportion of total analyte existing in the plasma as the labeled species was approximately 1.2% for β -carotene, 0.8% for retinol, and 9.2% for retinyl ester. Most of the absorbed dose was converted to vitamin A, with retinyl esters predominating. Assuming that all three analytes were removed from the plasma at the same rate, about 64% (a molar basis) of absorbed ^{13}C from ^{13}C - β -carotene entered the plasma as retinyl esters, 21% as retinol, and 14% as intact β -carotene.

They applied the same method to study ^{13}C - β -carotene metabolism in a group of male subjects ranging in age from 27 to 41 years (Swanson et al 1996). The subjects were given 1 mg ^{13}C - β -carotene. Short-term (0 - 50 h) and long-term (0 - 600 h) kinetics of plasma ^{13}C -labeled β -carotene, retinyl ester, and retinol were studied. The labeled β -carotene and retinyl ester peaked 5h after dosing, corresponding to the known kinetics of chylomicrons, and represented absorption of unmetabolized ^{13}C -labeled β -carotene and its major intestinal metabolite, ^{13}C -retinyl esters. The second broad peak of labeled β -carotene between 24 and 48h reflected hepatic secretion of β -carotene in very low density lipoproteins (VLDLs) and subsequent lipolysis of VLDL to lower density lipoproteins. ^{13}C -retinol, a minor intestinal metabolite of ^{13}C - β -carotene, exhibited a single peak at about 12 h, reflecting hepatic secretion of the retinol-binding protein-transthyretin complex.

You et al (1996) applied this method to study the isomerization of 9-*cis*- β -carotene during absorption in humans. Three subjects were given 1.0 mg ^{13}C - β -carotene of which 99.4% as in the 9-*cis* form. The results showed a significant proportion of the ^{13}C -9-*cis*- β -carotene dose was isomerized to the all-*trans* form before entering the blood stream.

Study of vitamin A status by using GC-C-IRMS

Vitamin A status assessment is not as simple as determining plasma or serum concentrations of retinol, because serum concentrations of retinol are homeostatically controlled and do not begin to decline until liver reserves are dangerously low (Underwood 1984). Direct assessment of liver storage of vitamin A can be achieved by liver biopsies but this method is only justified under unusual circumstances. Indirect measurements of liver reserves are more practical and useful. Vitamin A assessment methods that will accurately determine the liver reserve of populations using a minimum number of blood samples are still in development (Tanumihardjo 2000). The deuterated vitamin A assay has been successfully applied in several population groups, but a large dose of vitamin A (35-140 μmol for adults) must be used due to the low sensitivity of GC-MS analysis (Furr et al 1989, Haskel et al 1998, Ribaya-Mercado et al 1999). Tanumihardjo (2000) developed an isotope dilution method in a rat model by using ^{13}C -labeled retinol and GC-C-IRMS. This method requires only physiological doses of retinol and routine venipuncture techniques. The calculated and measured values of total body reserves of vitamin A were 7% of each other overall, and the relationship was linear. The serum retinol concentrations did not show differences among different groups. The author concluded that this method offers more sensitivity than traditional methods and may be applicable to human vitamin A status assessment when total body reserve estimations are desired.

Other applications of GC-C-IRMS in carotenoid studies

Liang et al (1998) used GC-C-IRMS to determine the natural abundance ^{13}C in lutein isolated from C_3 and C_4 plant sources. The C_3 and C_4 photosynthetic pathways differ in the isotopic discrimination of the initial carboxylating enzyme that incorporates CO_2 from the atmosphere. As a result the ^{13}C content of C_4 plants is higher than that of C_3 plants. Carbon

isotope fractionation in plants is potentially useful for studies of the bioavailability and metabolism of ^{13}C -enriched lutein derived from C_4 plant foods in animal models or humans. In their study, they used GC-C-IRMS to determine the stable carbon isotope ratios of lutein isolated from a C_3 (marigold flower) and a C_4 (corn gluten meal) plant source. The $\delta^{13}\text{C}$ value was $-19.77 \pm 0.27\text{‰}$ for lutein isolated from corn gluten meal, which was significantly different from the $\delta^{13}\text{C}$ value of $-29.90 \pm 0.20\text{‰}$ for lutein isolated from marigold flower. This is the first study that developed a method to analyze a carotenoid other than β -carotene by using GC-C-IRMS.

Yao et al (2000) used a ^{13}C tracer and GC-C-IRMS to quantify the plasma appearance of a physiological dose of lutein in humans. Biosynthetic per-labeled ($>99\%$ ^{13}C) lutein was purified from a commercially-available extract of algal biomass. Subjects ($n = 4$) ingested 3 mg of ^{13}C lutein with a standardized low-carotenoid breakfast. Blood samples were collected at baseline and then hourly for 12h; additional blood samples were drawn at 16, 24, 48, 72, 96, 192, 360, and 528 h. Plasma lutein was purified by using HPLC and hydrogenated on palladium-on-carbon catalyst with acid-catalyzed hydrogenolysis. The hydrogenation product, perhydro- β -carotene, was injected into the GC-C-IRMS and the stable isotope ($^{13}\text{C}/^{12}\text{C}$) ratio was measured. Plasma concentrations of ^{13}C -lutein were calculated based on the $^{13}\text{C}/^{12}\text{C}$ ratio and the total plasma lutein concentration. The results showed a rapid increase in ^{13}C lutein in plasma until 16 h after dosing, followed by a decline until 24 h. A small increase in ^{13}C -enrichment from baseline could still be measured in plasma lutein at 528 h. This study is a successful adaptation of the ^{13}C -tracer approach developed by Parker et al (1993, 1997). In this study, a tracer approach and GC-C-IRMS were used to study the metabolism of another predominant dietary carotenoid, lutein. The tracer method and use of GC-C-IRMS offers a sensitive and selective experimental approach to study the absorption and metabolism of lutein when ingested in physiologically relevant amounts by humans.

Study of β -carotene metabolism by using LC-MS

Liquid chromatography-mass spectrometry (LC-MS) has been used to study the metabolism of β -carotene. LC-MS methods are sensitive and do not involve extensive sample preparation. Therefore they could potentially be useful for large-scale comprehensive

studies. Pawlosky et al (2000) used a combination of liquid chromatography/particle beam-mass spectrometry (LC/PB-MS) to study the metabolism of β -carotene- d_8 in humans. In this study, one subject ingested a 5 mg dose of β -carotene- d_8 , and blood samples were collected at 0 h, 8 h, 8 d, 15 d, and 29 d. The results showed a detection limit of about 0.6 pmol for β -carotene. Using β -carotene- $^{13}C_{40}$ as an internal standard, β -carotene- d_8 could be quantified over a concentration range of two orders of magnitude. The concentration of β -carotene- d_8 appeared to reach the maximum at 8 h. Using the same approach this group determined ^{13}C -labeled and endogenous β -carotene, lutein, and vitamin A in the plasma of a subject who consumed kale that has been grown in a $^{13}CO_2$ -enriched atmosphere (Kelm et al 2001). Internal standards including 2H_8 - β -carotene, β -apo-8'-carotenal and 2H_4 -retinol were used to quantify the $^{13}C/^{12}C$ isotopomers of β -carotene, lutein, and retinol, respectively.

High-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (APCI LC-MS) was developed to quantify the bioavailability of retinyl palmitate and β -carotene and the bioconversion of β -carotene to retinol in humans (Wang et al 2000, van Lieshout et al, 2001). In their study, subjects were given physiological doses of [8,9,10,11,12,13,14,15,19,20- $^{13}C_{10}$]-retinyl palmitate and [12,13,14,15,20,12',13',14',15',20'- $^{13}C_{10}$]- β -carotene. Plasma retinol and β -carotene were extracted analyzed with reverse-phase HPLC interfaced to an APCI mass spectrometer. No further purification or derivatization of retinol or β -carotene was necessary. APCI LC-MS showed a linear detector response for β -carotene over 4 orders of magnitude. APCI LC-MS is a simple, straightforward analytical approach that is sensitive and specific. It does not require the high resolution of tandem mass spectrometry. The detection limit for β -carotene was determined to be 0.5 pmol injected on-column, which makes it suitable for studies using small doses of labeled β -carotene doses.

APCI LC-MS was applied to study the bioefficacy of β -carotene dissolved in oil when ingested by Indonesian children (van Lieshout et al, 2001). Children aged 8-11 y ($n = 35$) consumed 160 μg of [12,13,14,15,2,12',13',14',15',20'- $^{13}C_{10}$]- β -carotene (with a *trans* to *cis* ratio of 3:1) and 160 μg of [8,9,10,11,12,13,14,15,19,20- $^{13}C_{10}$]-retinyl palmitate, in the form of capsules, seven days a week for ten weeks or less. Different study durations were chosen to assess the time required for the isotopic enrichment of retinol and β -carotene in

serum to reach a plateau. A total of three blood samples were drawn over this period. Isotopic enrichment in serum retinol with $^{13}\text{C}_5$ and $^{13}\text{C}_{10}$ and in β -carotene with $^{13}\text{C}_{10}$ were measured using APCI LC-MS. The results showed that isotopic enrichment in both plasma retinol and β -carotene reached plateau by day 21. The amount of β -carotene in oil required to form 1 μg retinol was 2.4 μg with the trans:cis ratio of 3:1 in the dose. This number is superior to the 1 μg retinol from 3.3 μg β -carotene previously estimated. Bioefficacy is a new term introduced in this paper. It is defined as the amount of ingested provitamin A required to yield 1 μg retinol in the body.

Besides having the advantages shared by all stable isotope tracer approaches, this method offers several special advantages. The use of multiple doses enables the isotopic enrichment to reach a plateau, which makes analysis of data and subsequent calculation and interpretation of results easier than in single-dose studies. This method does not require extensive purification and derivatization of β -carotene and retinol. In their editorial, Solomons and Russell (2001) regard this stable-isotope approach as “appropriate technology” for vitamin A field research. This approach might be extended to address the question of vitamin A potency of β -carotene in fruits and vegetables.

Summary

Stable isotope tracer techniques are relatively new tools for studying in humans the bioavailability and bioconversion of dietary carotenoids, particularly β -carotene. Use of such techniques have several advantages, including the possibility of administering β -carotene doses that are similar to dietary intake, therefore providing more reliable and representative data about the bioavailability and bioconversion of β -carotene from food source. This information can help the scientific community to better understand food-based approaches to eliminating vitamin A deficiency worldwide. A recent review (Lieshout et al 2003) provides a step-by-step discussion of aspects related to the isotopic techniques for studying of bioavailability and bioefficacy of dietary carotenoids, including study design, choice of isotopic tracers, dosing regimen, collection of samples, chemical analysis of samples, and data analysis.

Part III. Molecular characterization of β -carotene cleavage enzymes

Introduction

When doses are low, the majority of the ingested β -carotene is cleaved at the central carbon 15,15'-double bond to form two molecules of retinal (Olson and Hayaishi 1965, Bloomstrand and Werner 1967; Goodman et al 1965). The enzyme that catalyzes this reaction was named β -carotene 15,15'-dioxygenase (Goodman et al 1965, Olson and Hayaishi 1965). The enzyme was first described about 50 years ago (Moore 1957). Subsequent biochemical characterization suggested the enzyme is a soluble, cytosolic enzyme (Goodman et al 1966a, 1966b, Fidge et al 1969) and its enzymatic activity depends on an iron-containing cofactor (Fidge et al 1969, Lakshmanan et al 1972). The enzyme is associated with a high-molecular-mass lipid protein aggregate fraction (Sklan 1983). In mammals, the highest carotene oxygenase activity is found in intestinal mucosa, and more specifically, jejunum enterocytes, with a gradient of decreasing activity from duodenum/jejunum to colon (Duszka et al 1996, van Vliet et al 1996). The enzymatic activity has also been found in liver, lung, kidney, and brain (During et al 1996). Assuming an optimal β -carotene/retinal cleavage ratio of 1:2, the maximum capacity of β -carotene cleavage in an adult was estimated to be 12 mg/day, one fifth by small intestine and four fifths by liver (During et al 2001).

This enzyme was regarded as a dioxygenase until recently, when Leuenberger et al (2001) showed that the reaction mechanism of the cleavage of the central carbon 15,15'-double bond in β -carotene involves a monooxygenase-type mechanism. In their experiments using labeled oxygen molecules, only one ^{17}O atom of molecular oxygen was incorporated in the reaction and the other O atom was ^{18}O from labeled water. Thus, the enzyme was renamed β -carotene 15,15'-monooxygenase. This monooxygenase mechanism resembled the mechanism proposed by the same research group for a supramolecular enzyme model catalyzing the regioselective cleavage of β -carotene (French et al 2000).

The reaction mechanism of β -carotene monooxygenase had been controversial (Wolf 1995). Central cleavage leading to the formation of two retinal molecules was accepted as

the major metabolic pathway for retinoid formation from β -carotene. Eccentric cleavage of β -carotene leading to the formation of apocarotenals was first proposed by Glover et al in 1960, and is supported by some recent *in vivo* and *in vitro* studies. Wang et al (1991) found retinoic acid as the only metabolite after incubation of β -carotene with intestinal homogenates of humans, monkeys, ferrets, and rats. Yeum et al (2000) suggested that β -carotene was converted exclusively to retinal by central cleavage in the presence of an antioxidant such as α -tocopherol, but cleaved randomly by enzyme-related radicals to produce β -apo-carotenoids in the absence of an antioxidant. Recently, Wirtz et al (2001) used a partially-purified chicken intestine enzyme preparation and isomerically pure α -carotene as substrate to investigate the substrate specificity of β , β -carotene 15,15'-monooxygenase. The results suggest that this enzyme has a rather rigid binding site in which the substrate-protein interaction determines the absolute preference to cleave the central carbon 15,15'-double bond of the carotenoids that are at least "half-identical" to β , β -carotene, with one "natural", unsubstituted β -ionone ring (Wirtz et al 2001). Kiefer et al (2001) identified and characterized a second type of carotene monooxygenase that catalyze exclusively the asymmetric oxidative cleavage of β -carotene at the 9', 10' double bond, resulting in formation of β -apo-10'-carotenal (C_{27}) and β -ionone (C_{13}). Therefore new studies seem to provide evidence to bring an end to the controversy (Wolf 2001). The central and eccentric cleavage of β -carotene both exist but are controlled by different enzymes.

The enzymatic activity of the β -carotene cleavage enzyme is regulated by nutritional status and dietary factors. The activity of β -carotene 15,15'-monooxygenase is positively correlated with iron concentration and negatively correlated with copper concentration in the small intestine in rats (During et al 1999). Its activity is iron-dependent (During et al 2001). Protein deficiency lowers the level of enzymatic activity in rats (Parvin and Sivakumar 2000). Vitamin A intake affects the enzymatic activity of β -carotene 15,15'-monooxygenase. Without β -carotene supplementation, the intestinal monooxygenase activity was 90% higher in rats fed with a low vitamin A diet than in rats fed with a high vitamin A diet (van Vliet et al 1996). Supplementation of β -carotene significantly lowered the enzymatic activity of β -carotene monooxygenase. A diet rich in polyunsaturated triacylglycerols enhanced the β -

carotene monooxygenase activity in rat intestine (During et al 1998). Dietary flavonoids inhibited β -carotene 15,15'-monooxygenase activity *in vitro* using a pig intestinal homogenate as the enzyme source. The inhibitory effect of flavonoids was also confirmed in cultured Caco-2 cells (Nagao et al 2000).

Lutein and other carotenoids affect the enzymatic activity of β -carotene 15,15'-monooxygenase in *in vitro* studies. Canthaxanthin inhibited β -carotene monooxygenase in rat intestine both *in vivo* and *in vitro* (Grolier et al 1997). An *in vitro* study measured β -carotene cleavage activity with the $9,000 \times g$ -supernatant from rat and hamster intestinal homogenates. Addition of 9 μg lutein to the incubation with 3 μg of β -carotene reduced retinal formation, whereas lycopene had no effect (van Vliet et al 1995). Lycopene, lutein, and astaxanthin competitively inhibit the enzymatic activity of β -carotene 15,15'-monooxygenase isolated from rabbit small intestinal mucosa (Ershov et al 1993).

The activity of β , β -carotene 15,15'-monooxygenase is regulated by its direct metabolites in rats and chickens (Bachmann et al 2002). There were dose-dependent decreases in intestinal β , β -carotene 15,15'-monooxygenase activity after oral administration of retinyl acetate, β -carotene, apo-8'-carotene, all-*trans* retinoic acid, and 9-*cis* retinoic acid. The activity of this enzyme in the liver was not affected. Apo-12'-carotenal and a retinoic acid receptor (RAR) α -antagonist significantly increased the intestinal enzymatic activity by 55% and 94% respectively. Two cytochrome P₄₅₀ (CYP) inducers increased the enzymatic activity in rat intestine. In chickens, a transcriptional study showed that treatment with retinoic acid resulted in low expression of the intestinal enzyme.

The studies reviewed above have provided accumulated knowledge about the nature and characteristics of β -carotene 15,15'-monooxygenase. However the enzyme had not been purified and molecular data on this key step in vitamin A formation had been missing until the recent molecular identification and cDNA cloning of β -carotene 15,15'-monooxygenase from fruit fly (von Lintig and Vogt 2000) and chicken (Wyss et al 2000). Since then, β -carotene 15,15'-monooxygenase has also been cloned in mouse and humans (Table 2). A mammalian enzyme that catalyzes the asymmetric oxidative cleavage of provitamin A carotenoids was also identified and characterized (Kiefer et al 2001). These findings opened

new perspectives to further study of the metabolism of β -carotene. Two different approaches, *in vivo* studies such as stable isotope tracer approaches, and *in vitro* incubation studies which characterize cloned and purified β -carotene 15,15'-monooxygenase, will complement each other and provide new information about absorption and bioconversion of β -carotene.

Molecular identification of a β -carotene monooxygenase from *Drosophila melanogaster*

von Lintig and Vogt (2000) cloned and identified, for the first time, a β -carotene-cleaving enzyme in fruit flies. They used a novel test system consisting of a β -carotene synthesizing and accumulating *Escherichia coli* strain. The expression of a β -carotene 15,15'-monooxygenase in the genetic background of this *E. coli* strain results in the formation of retinoids and can be monitored by a color shift of the *E. coli* from orange (β -carotene) to almost colorless due to the cleavage of β -carotene to retinoids. In plants, *vp14* has been cloned and identified as the gene that encodes a protein that catalyzes the oxidative cleavage of 9-*cis* epoxy carotenoids at the 11-12 carbon double bond, to form xanthoxin, the direct precursor of abscisic acid (Schwartz et al 1997). A homologues of this plant carotenoid-cleaving enzyme was found in *Drosophila melanogaster*. A full-length cDNA was cloned and used to directly test β -carotene monooxygenase activity. The results demonstrated that the cloned cDNA encodes a β -carotene monooxygenase (named β -diox), and the cleavage products of β -carotene were exclusively retinoids, showing a central cleavage pattern. The sequence analyses revealed that the cDNA encoded a protein of 620 amino acids, with a calculated molecular mass of 69.9 kDa. The deduced amino acid sequence shares sequence homology to the plant carotenoid monooxygenase *vp14*, to lignostilbene synthase from *Pseudomonas paucimobilis*, and to several proteins of unknown function in the *Cyanobacterium Synechocystis*. However, the highest sequence homology was to RPE65, a protein from the retinal pigment epithelium (RPE) in vertebrates. RPE 65 and β -carotene 15,15'-monooxygenase share 36.7% overall sequence identity. The sequence comparison revealed that β -carotene 15,15'-monooxygenase belongs to a new class of monooxygenases described only in bacteria and plants, and RPE65 could be the vertebrate

equivalent to β -carotene 15,15'-monooxygenase. The 15,15'-monooxygenase mRNA was restricted exclusively to the head, whereas in the thorax and abdomen, no mRNA was detected. This protein is soluble and is not tightly associated with membranes. Its activity depends on iron, as previously reported. The apparent K_m value for β -carotene was estimated to be 5 μ M. This enzyme was not able to use zeaxanthin as a substrate to directly form hydroxylated retinoids under the experimental conditions.

The 15,15'-monooxygenase gene is located at position 87F on chromosome 3 in the *Drosophila* genome. A *Drosophila* mutant, *ninaB*, has been mapped in this region. The mutant phenotype has a reduced rhodopsin content in all photoreceptor classes. The mutant phenotype can be rescued by the dietary supplementation of retinol but not by β -carotene (von Lintig et al 2000). In two independent *ninaB* alleles, mutations in the gene encoding 15,15'-monooxygenase were indeed found (von Lintig et al 2000). These mutations lead to a defect in vitamin A formation and are responsible for the blindness of these flies.

Cloning and expression of β , β -carotene 15, 15'-monooxygenase in chicken

Wyss et al (2000) cloned the first vertebrate β , β -carotene 15,15'-monooxygenase that symmetrically cleaves β -carotene at the 15,15'-double bond. The isolated full-length cDNA for β , β -carotene 15,15'-monooxygenase has a length of 3.1 kb. The coding sequence spans 1578 bp and leads to a protein of 526 amino acids. The cDNA shows high homology to RPE65. When the enzyme was expressed in *E. coli*, retinal was the sole reaction product. No apocarotenals were detected, indicating that the enzyme specifically cleaves β -carotene at the 15,15'-double bond. When the enzyme was expressed in baby hamster kidney cells, enzymatic activity was found in the whole-cell and in the cytosolic extract, but not in the membrane fraction. The authors concluded that the chicken β , β -carotene 15,15'-monooxygenase is a cytoplasmic enzyme of 60.3 kDa and is expressed primarily in duodenum.

The expression pattern and localization of β , β -carotene 15,15'-monooxygenase in different tissues in chicken and the homology between chicken, mouse and *drosophila* enzymes were studied (Wyss et al 2001). The regions of amino acids 97-108 and 132-155 of the enzyme were found almost identical between mouse and chicken, showing high

conservation during evolution. Overall, the chicken and mouse β,β -carotene 15,15'-monooxygenases show 81% homology at the amino acid level, whereas the drosophila β,β -carotene 15,15'-monooxygenase show 50% homology with the chicken and 48% with the mouse.

Northern blotting showed that β,β -carotene 15,15'-monooxygenase was expressed primarily in the digestive tract of chicken, with the highest expression being in the duodenum but remarkably lower expression in the ileum. In mouse, there was a strong expression of mRNA in liver, marginal expression in kidney, and no expression in lung. In mouse testis, there were three distinct bands, which may represent alternative splicing products of a single gene or three distinct β,β -carotene 15,15'-monooxygenase genes.

In situ hybridization was used to analyze the expression pattern of β,β -carotene 15,15'-monooxygenase in duodenum, liver, lung, ileum, and skin in chicken. In duodenum, the epithelial cells of crypts and villi were positively stained. Some stromal cells were also stained, hypothetically representing T- or B-cells, which suggests β -carotene may be converted to vitamin A in immune cells.

Identification and expression of mouse enzymes that catalyze the symmetric and asymmetric cleavage of β -carotene

Redmond et al identified the first mammalian β,β -carotene 15,15'-monooxygenase in mouse (2001). The mouse cDNA has 2120 bp and contains an open reading frame that is 1698 bp in length. The cDNA encodes a protein of 566 amino acids, with a calculated molecular mass of 63.859 kDa. The deduced polypeptide does not have an obvious signal peptide, predicted transmembrane domains, or potential sites for N-linked glycosylation.

The deduced mouse polypeptide showed 70%, 30% and 85% identities respectively with chicken, drosophila β,β -carotene 15,15'-monooxygenase and a predicted product of a human cDNA without known function. The mouse β,β -carotene 15,15'-monooxygenase has only 37% amino acid identity to mouse RPE65. There are 10 residues, including 4 histidine residues and 6 acidic residues, that are absolutely conserved in these sequences. There is also a well conserved region at residues 469-480 of mouse sequence.

Expression of mouse β,β -carotene 15,15'-monooxygenase in β -carotene, ϵ -carotene, and lycopene-accumulating *E. coli* resulted in bleaching as a consequence of cleavage of the endogenous carotenoids. Purified recombinant β,β -carotene 15,15'-monooxygenase cleaves β -carotene *in vitro* with a C_{max} of 36 pmol of retinal/mg of protein and a K_m of 6 μ M. At levels of lycopene concentrations comparable to the K_m for β -carotene, no acyclic retinal was detected. However, trace amounts were seen at 2.5-times the β -carotene K_m .

In several tissues that are active sites of vitamin A metabolism, there was β,β -carotene 15,15'-monooxygenase mRNA accumulation. The major mRNA, seen in liver, kidney, testis, and small intestine, was 2.4 kb. The 2.4 kb message had low abundance in small intestine, which was explained by the authors as the results of dilution of mRNA caused by the outer muscular layers of the gut. Smaller messages were seen in testis and skin, particularly a 1.8 kb message is abundant in testis. Analysis of a mouse development blot showed a strong signal for the 2.4 kb message at 7 days post-conception, which declined as the development age increased.

Paik et al (2001) also identified a cDNA encoding a mouse homolog of a carotene cleavage enzyme. The cDNA generated from a mouse kidney library showed 48% amino acid sequence identity to the *Drosophila* sequence. The full length cDNA includes polyadenylation sites and an open reading frame consisting of 566 amino acids. The protein deduced from this sequence has a molecular mass of 64,000. The sequences of chicken, mouse, and human have an identity of 65%. The sequences of mouse and human have 85% identity.

When the cDNA was expressed in either *E. coli* or Chinese hamster ovary cells, expression conferred upon bacterial and Chinese hamster ovary cell homogenates the ability to cleave β -carotene to retinal. All *trans*-retinal and some 13-*cis*-retinal (<10% of all *trans*-retinal) were sole products that could be detected upon incubation of all-*trans*- β -carotene with the recombinant protein, and no apocarotenals were observed. The dependence of reaction velocity showed a sigmoidal relationship with β -carotene concentration. Analysis of the kinetic data gave rise to a $K_{0.5}$ for β -carotene of 0.95 μ M, a V_{max} of 368 pmol/mg/h, and a hill coefficient of 0.659. Addition of iron chelating agents decreased the activity of this enzyme.

Kinetic analyses of the recombinant enzyme suggested that this enzyme interacts with other proteins present within cell or tissue homogenates. An atypical testis-specific isoform of lactate dehydrogenase (LDH-C) is associated with recombinant carotene cleavage enzyme. The authors suggested that if LDH-C could catalyze either retinal reduction to retinol or retinal oxidation to retinoic acid, the association between the recombinant enzyme and LDH-C could mean that β,β -carotene 15,15'-monooxygenase acts physiologically in concert with other proteins involved in retinoid metabolism.

The mRNA of 15,15'-monooxygenase is expressed most highly in the testis followed by the liver and kidney. Surprisingly, no expression was detected in the small intestine using Northern blot analysis. However, RT-PCR analysis using total RNA could detect the expression in small intestine. The authors attributed this observation to species differences, or the use of the entire small intestine instead of only the proximal portion of the small intestine. The testis expressed two transcripts, 2.6 kb and 1.7 kb. The smaller sized transcript is missing a portion of 5'-region of the cDNA.

The expression pattern of carotene 15,15'-monooxygenase during early stages of mouse embryogenesis was also investigated. *In situ* hybridization showed that at embryonic days 7.5 and 8.5, mRNA for this enzyme is highly expressed in maternal tissue surrounding the embryo but is not present at detectable levels in embryonic tissues.

In all the studies mentioned above, the sole cleavage product of the carotene 15,15'-monooxygenases identified is all-*trans*-retinal, with small amount of 13-*cis*-retinal as addition. Asymmetric cleavage product has not been observed in any of these studies, which showed that carotene 15,15'-monooxygenase only cleaves the double bond at the 15,15' position. If asymmetric oxidative cleavage of carotene does exist, this reaction must be catalyzed by an enzyme or enzymes other than carotene 15,15'-monooxygenase. Kiefer et al (2001) identified and characterized in mouse, human, and zebrafish cDNAs that encode a second type of carotene monooxygenase that catalyze exclusively the asymmetric oxidative cleavage of β -carotene at the 9',10' double bond, resulting in formation of β -apo-10'-carotenal (C₂₇) and β -ionone (C₁₃). Besides β -carotene, lycopene is also oxidatively cleaved by this enzyme.

The cDNA encoding the new type of carotene monooxygenase encoded a protein (named β -diox-II by the authors) of 532 amino acids. The amino acid sequence shared 39% identity with the mouse β,β' -carotene 15,15'-monooxygenase. Several highly conserved stretches of amino acids and six conserved histidines probably involved in binding of the cofactor Fe^{2+} were found, indicating that the encoded proteins belong to the same class of enzymes as β,β' -carotene 15,15'-monooxygenase.

To test its functional characterization, the authors expressed β -diox-II as a recombinant protein in *E. Coli* and performed both *in vitro* and *in vivo* analyses. The cleavage product was identified to be β -apo-10'-carotenal by using HPLC and LC-MS. The HPLC method did not detect β -ionone, which the authors explained was due to its volatility and/or its partitioning to the medium. The bacterial growth medium was analyzed after solid phase extraction of lipophilic compounds by GC-MS and significant amount of β -ionone was detected in the medium. Based on these findings the authors named the enzyme β,β -carotene 9',10'-dioxygenase. This enzyme also catalyzes the oxidative cleavage of lycopene in the *E. coli* test system.

To verify the existence of this second type of enzyme in other metazoan organisms, the authors searched for expressed sequence tags (EST) with sequence identity in the data base, and found EST fragments from human and the zebrafish, and cloned the corresponding full-length cDNA. The cDNA cloned from the total RNA derived from human liver encoded a protein of 556 amino acids, whereas the cDNA isolated from the zebrafish encoded a protein of 549 amino acids. The deduced amino acid sequences shared 72% and 49% sequence identity respectively to the mouse β,β -carotene-9',10'-dioxygenase. By performing a phylogenetic tree calculation, the authors concluded that the three groups of polyene chain monooxygenase, the two different β -carotene monooxygenases and REP65, mostly likely emerged from a common ancestor. In contrast, in *Drosophila* and *Caenorhabditis elegans*, only one type of monooxygenase was found in the entire genome.

Total RNA from several mouse tissues were analyzed by RT-PCR to estimate the steady-state mRNA levels of the two carotene cleavage enzymes. Both types of mRNAs are detected in the small intestine, liver, kidney, and testis, whereas low abundance steady-state mRNA of β,β -carotene 9',10'-dioxygenase was additionally present in spleen, brain, and

heart. A 2.2 kb message was found in heart and liver for β,β -carotene 9',10'-dioxygenase, whereas a transcript of 2.4 kb was found mainly in kidney.

Cloning and characterization of human β,β -carotene 15,15'-monooxygenase

Yan et al cloned the first human β,β -carotene 15,15'-monooxygenase in 2001. The open reading frame of the cloned human cDNA encodes a protein of 547 amino acids with a calculated molecular mass of 62 kDa. The predicted amino acid sequence shows 67% identity and 80% similarity to a chicken sequence (Wyss et al 2000). Homology between human and chicken sequences is distributed fairly equally throughout the entire protein, but the human enzyme has an extra 20 amino acid residues at its carboxyl terminus. For comparison, the authors also obtained and sequenced from the GenBank, a murine protein of 566 amino acids that has 83% identity with the human clone. Most of the variations between the human and the murine clones are located at the carboxyl terminus. The human clone has a 24% homology to the *Drosophila* clone. The alignment of the human β,β -carotene 15,15'-monooxygenase and the human RPE65 reveals four areas of increased sequence conservation, two of which have particularly high and specific homology.

The gene structure and chromosomal localization of human β,β -carotene 15,15'-monooxygenase were determined. The gene spans approximately 20 kb and consists of 11 exons and 10 introns. Exons range in size from 95 to 795 bp. Intron sizes range from 660 bp to 3 kb. All the 5'-donor and 3'-acceptor sites are consistent with the GT-AG consensus for premRNA splicing recognition sequences. Several potential initiation sites were identified, with the major site at 216 bp upstream of the initiation methionine, consistent with the existence of a typical TATA box 30 bp upstream of the transcription site.

The chromosomal localization of human β,β -carotene 15,15'-monooxygenase was mapped to chromosome 16q21-q23, close to the BBS2 locus for Bardet-Biedl syndrome, an autosomal recessive disease associated with abnormalities of retinoid metabolism.

Northern analysis indicates that β,β -carotene 15,15'-monooxygenase gene is highly expressed in human RPE as a 2.4 kb transcript, 0.7 kb shorter than the chicken gene transcript. Human β,β -carotene 15,15'-monooxygenase preferentially expressed in the RPE,

but also expressed at lower levels in kidney, testis, liver, and brain, and the level of expression in intestine seems similar to that in kidney but lower than that in RPE.

To study the biochemical characteristics of human β -carotene 15,15'-monooxygenase, these researchers expressed the enzyme using a baculovirus system. All-*trans*-retinal and small amount of 13-*cis*-retinal were identified as products of β , β' -carotene cleavage. The enzymatic activity was significantly reduced by addition of EDTA, an iron chelating agent. The enzyme showed substrate specificity. Lutein and lycopene were not cleaved by this enzyme under the similar conditions used for β , β' -carotene assay.

Recently, Lindqvist and Andersson (2002) also characterized a purified recombinant human β -carotene 15,15'-monooxygenase. A highly active enzyme was expressed and purified to homogeneity from baculovirus-infected *Spodoptera frugiperda* 9 insect cells. The K_m and V_{max} of the enzyme for β -carotene were 7 μM and 10 nmol retinal/mg \times min, respectively. These values corresponded to a turnover number (K_{cat}) of 0.66 min^{-1} and a catalytic efficiency (K_{cat}/K_m) of $\sim 10^5 M^{-1} \cdot min^{-1}$. The specific enzymatic activity is 300-fold higher than that of the partially purified enzyme from chicken intestinal mucosa (Wyss et al 2000), and 300-1600-fold higher than that of the partially expressed and purified mouse enzymes (Redmond et al 2001, Paik et al 2001). The enzyme existed as a tetramer in solution. The enzyme catalyzes the cleavage of β -carotene and β -cryptoxanthin, but not of zeaxanthin or lycopene. This substrate specificity suggested that at least one unsubstituted β -ionone ring half-site was necessary for efficient cleavage of the carbon 15,15'-double bond in carotenoid substrates. The enzyme shows a 4-fold higher K_m when β -cryptoxanthin was used as substrate as compared with β -carotene. Alignment of the amino acid sequences showed that about 8% of the highly hydrophobic amino acids were conserved, and these residues might participate in the formation of the substrate-binding pocket. The enzyme was shown to be sensitive to micromolar concentrations of the metal chelating agents α , α -bipyridyl and *o*-phenanthroline, confirming its dependence on iron. High levels of mRNA were observed along the whole intestinal tract, in the liver, and in the kidney, whereas lower levels were present in the prostate, testis, ovary and skeletal muscle. The expression of the

β,β -carotene 15,15'-monooxygenase gene in nondigestive tissues suggest this enzyme may also play a role in peripheral vitamin A synthesis for plasma-borne provitamin A carotenoids.

Summary

Fifty years after its discovery, new perspectives have been opened for understanding of the molecular structure and mechanism of the enzyme that cleaves β -carotene, due to the cloning and characterization of β,β -carotene 15,15'-monooxygenase in various species. There are differences in gene sequences for this enzyme in different species, but some amino acids are highly conservative. In general, β,β -carotene 15,15'-monooxygenase is a protein with about 560 amino acids and a calculated molecular mass of about 64 kDa. Its activity is iron-dependent. It has selectivity on substrates and only cleaves at the 15,15'-double bond when the substrate has at least one unsubstituted β -ionone ring. Eccentric cleavage of β -carotene is catalyzed by a different enzyme. Application of the cloned and purified β -carotene cleavage enzymes will help answer some fundamental questions at a new level in carotenoid and vitamin A research.

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Table 1 Stable isotope tracer methods for study of β -carotene absorption and conversion

References	Subjects	Doses	Fat contents	Analytical Methods	Results/Conclusions
Dueker et al 2001	One 35-y non-smoking male	^{14}C - β -carotene, 306 μg (200nci)	25g total	Accelerator mass spectrometry (AMS)	*Absorption rate: 46.2% of dose *Conversion rate: 62% of the absorbed dose *Turn-over time: 58 days for β -carotene and 209 days for retinal
Dueker et al. 1994	One 53-y non-smoking male	β -carotene- d_8 , 73 μmol	2g	Tandem mass spectrometry (MS/MS) with electron ionization and HPLC	Establishing a feasible method for investigation of absorption and metabolism of β -carotene- d_8 in humans
Dueker et al. 1998	One 22-y female	β -carotene- d_8 , 56 μmol	N/A	Selected ion storage-ion trap mass spectrometry (SIS-ITMS)	SIS-ITMS approach expands the scope of vitamin A/ β -carotene kinetic studies by permitting the reliable quantitation of labeled vitamin A at low enrichment
Lin et al. 2000	11 healthy female	30 μmol D_6 retinyl acetate; 37 μmol D_6 β -carotene	16g total	Double-tracer; SIS-GC-MS for retinal and reversed phase HPLC for β -carotene	*Absorption rate of D_6 β -carotene: $3.3 \pm 1.3\%$ (6 subjects: $6.1 \pm 0.02\%$; 5 subjects: $\leq 0.01\%$) *Conversion rate: 0.811 ± 0.343 mol D_3 ROL: 1 mol D_6 - β -carotene (6 subjects: 1.47 ± 0.49 mol:1; 5 subjects: 0.014 ± 0.004 :1)

(Continued)

Table 1 (Continued)

References	Subjects	Doses	Fat contents	Analytical Methods	Results/Conclusions
Hickenbottom et al. 2002	11 healthy male	30 μmol D ₆ retinyl acetate, 37 μmol D ₆ β -carotene	16 g total	Double-tracer; SIS-GC-MS for retinal and reversed phase HPLC for β -carotene	*Absorption rate of D ₆ β -carotene: 2.2 \pm 0.9% (6 subjects: 4.1 \pm 1.21%; 5 subjects: 0.0003 \pm 0.0002%) *Conversion rate: 0.029 \pm 0.0108 mol D ₃ ROL: 1 mol D ₆ - β -carotene (6 subjects: 0.0540 \pm 0.0128 mol:1; 5 subjects: 0.0003 \pm 0.0002:1)
Hickenbottom et al. 2002	One male subject	30 μmol D ₆ retinyl acetate, 30 μmol D ₆ β -carotene, 0.27 μmol ¹⁴ C β -carotene	16 g total	Double-tracer; SIS-GC-MS for retinal and reversed phase HPLC for β -carotene, AMS for ¹⁴ C	Conversion rate: 8.5 mol of β -carotene : 1 mol retinol, Absorption rate: 55% of the dose

(Continued)

Table 1 (Continued)

References	Subjects	Doses	Fat contents	Analytical Methods	Results/Conclusions
Tang et al.2000	One female	1) 234.7 μ mol β -carotene- d_8 , 2) 11.2 μ mol β -carotene- d_8 , (two doses are 5-y apart) 3) Reference dose: 30.6 μ mol retinyl-acetate- d_8 , (taken between two doses)	1) 32 g total 2) 30.5 g total 3) 0.5g total	Stable isotope reference method; Electron capture negative chemical ionization	*Conversion rate: 1) 55:1 2) 3.8:1
Tang et al 2003	10 men, 12 women	6 mg β -carotene- d_8 3 mg retinyl-acetate- d_8 (reference dose)	13.5 g, 25% of energy	Stable isotope reference method; LC-atmospheric pressure chemical ionization-MS for β -carotene GC-electron capture negative chemical ionization-MS for retinol	*Conversion factor: 9.1:1 by weight or 4.8:1 by mol (range: 2.4-20.2) *Conversion factor positively correlated with BMI

(Continued)

Table 1 (Continued)

References	Subjects	Doses	Fat contents	Analytical Methods	Results/Conclusions
Tang et al. 1997	One female	235 μ mol β -carotene- d_8	32g total	1. Flow-injection atmospheric pressure chemical ionization-mass spectrometry (FI/APCI-MS) for β -carotene- d_8 absorption 2. Gas chromatography-mass spectrometry with electron capture negative chemical ionization (GC/EC-NCI-MS) for conversion of β -carotene- d_8 to retinal- d_4	*Method development
Edwards et al. 2001	3 adults	11.2 μ mol β -carotene, from carrot or spinach; 6.0 μ mol Retinyl acetate- d_4	20g total	Postprandial chylomicron approach using retinyl-acetate- d_4 as extrinsic reference; GC-MS and HPLC	*Absorption rate: 2.9% of dose (0.5-7.8%) *Conversion rate: 27:1 or 23:1 (when ignoring the effect of α -carotene)

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Table 1 (Continued)

References	Subjects	Doses	Fat contents	Analytical Methods	Results/Conclusions
Edwards et al. 2002	9 adults	34.7 μ mol β -carotene from carrot puree or boiled-mashed carrots, or raw chopped carrot; 6 μ mol d ₄ -retinyl acetate	5g	Postprandial chylomicron approach using retinyl-acetate-d ₄ as extrinsic reference; GC-MS and HPLC	*Absorption rate: 2.4 \pm 0.81% for carrot puree, 0.86 \pm 0.59 % for mashed carrots, 1.7 \pm 1.3% for raw carrots *Conversion rate: 35:1 for puree (4.6% of ingested β -carotene), 42:1 for mashed carrots (2.5% of ingested β -carotene) *Apparent efficiency of conversion: 44 \pm 11% (24-58%) for carrot puree, 59 \pm 12% (36-72%) for boiled-mashed carrots, and 63 \pm 10% for raw chopped carrots
Parker et al. 1993	One 41-y male	1.86 μ mol perlabeled ¹³ C- β -carotene	20g tri-capnylin	High precision isotope ratio mass spectrometry (gas chromatography combustion-gas isotope ratio mass spectrometry (GCC-IRMS))	*A safe and sensitive approach which requires only very small dose of β -carotene typical or less than daily dietary intake *Most of the absorbed does was converted to vitamin A: 64% as retinyl ester, 21% as retinal, and 14% as intact β -carotene

(Continued)

Table 1

References	Subjects	Dose	Fat content	Analytical Methods	Results/Conclusions
van Lieshout et al. 2001	Indonesia children aged 8-11y (n = 35)	[12,13,14,15,20, 12',13',14',15',20' ¹³ C ₁₀]β-carotene in capsules, 80 μg × 2/d, 7 d/wk, for ≤ 10 wk; [8,9,10,11,12,13, 14,15,19,20- ¹³ C ₁₀]retinyl palmitate in capsules, 80 μg × 2/d, 7 d/wk, for ≤ 10 wk.	41g total	Atmospheric pressure chemical ionization liquid chromatography-mass spectrometry (APCI LC-MS)	*Plateau isotopic enrichment was reached by day 21 *The amount of β-carotene in oil required to form 1 μg of retinol was 2.4 μg (95% CI: 2.1, 2.7), with a <i>cis-trans</i> ratio of 3:1 for β-carotene in the capsules
Pawlosky et al 2000	One subject	β-carotene-d8, 5 mg	N/A	Liquid chromatography/particle beam-mass spectrometry (LC/PB-MS)	Method development Detection limit: 0.6 pmol for β-carotene
Kelm et al 2001	One man	¹³ C enriched kale	N/A	Liquid chromatography/particle beam-mass spectrometry (LC/PB-MS)	Method development

Table 2. Molecular characterization of β -carotene cleavage enzymes

References	Species	Molecular Characteristics	Tissue Distribution	Enzymatic Activity and Characteristics	Cleavage Product	Homology
Von Lintig and Vogt 2000	<i>Drosophila melanogaster</i>	*Protein: 620 amino acid residues, with a calculated molecular mass of 69.9 kDa. *Gene: located at position 87F on chromosome 3.	*mRNA distribution restricted exclusively to the head	*Soluble protein not tightly associated to membranes *Enzymatic activity dependent on iron *Apparent Km = 5 μ M for β -carotene	Exclusively retinoids	* 6.7% overall sequence identity to RPE 65 * 5.2% overall sequence identity to VP14
Wyss et al. 2000	Chicken	Protein: 526 amino acid residues with a calculated molecular mass of 60.3 kDa	Primarily in duodenum	A cytoplasmic enzyme	Retinal as the sole product	High homology to RPE 65

(Continued)

Table 2 (continued)

References	Species	Molecular Characteristics	Tissue Distribution	Enzymatic Activity and Characteristics	Cleavage Product	Homology
Redmond et al 2001	Mouse	<p>*Protein: 566 amino acid residues, with a calculated molecular weight of 63 kDa.</p> <p>*Conserved histidines and acidic residues</p>	<p>*Liver, kidney, testis and small intestine (highest in jejunum): 2.4 kb mRNA;</p> <p>*Testis and skin: 1.8 kb mRNA</p> <p>*2.4 kb mRNA at 7 days post-conception in embryonic tissue, declined afterwards</p>	<p>*Km = 6 μM, Vmax = 36 pmol of retinal/mg/min;</p> <p>*A cytosolic enzyme;</p> <p>* Substrate specificity: low activity towards carotenoids other than β-carotene (including lycopene)</p>	All <i>trans</i> retinol	<p>*70% identity to chicken</p> <p>*30% identity to <i>Drosophila</i>,</p> <p>*85% to a predicted human cDNA,</p> <p>*37% to mouse RPE65</p>

(Continued)

Table 2 (continued)

References	Species	Molecular Characteristics	Tissue Distribution	Enzymatic Activity and Characteristics	Cleavage Product	Homology
Paik et al 2001	Mouse	Protein: 566 amino acid residues, with a molecular mass of 64 kDa	*Most highly in the testis, followed by liver and kidney, much lower in small intestine (only the proximal portion of the small intestine including duodenum and part of jejunum) *During embryogenesis: highly expressed in maternal tissue but not in embryonic tissue during embryonic days 7.5 and 8.5	*Apparent Km = 0.52-9.5 μ M for β -carotene, Vmax = 368 pmol/mg/h (23.8-1300) *Activity iron-related	>90% all- <i>trans</i> retinal and <10% 13- <i>cis</i> retinal	*48% identity to <i>D. melanogaster</i> *41% identity to rat and human RPE65 *85% identity to human

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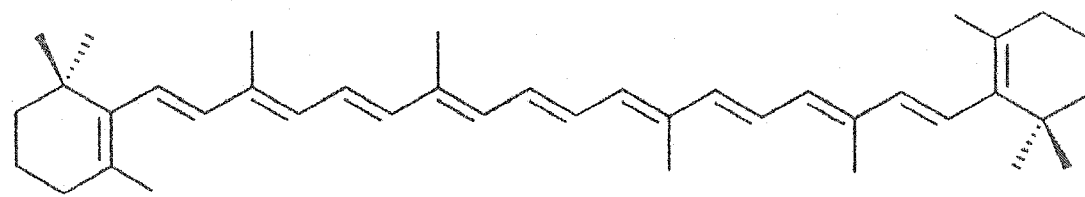
Table 2 (continued)

References	Species	Molecular Characteristics	Tissue Distribution	Enzymatic Activity and Characteristics	Cleavage Product	Homology
Yan et al 2001	Human and mouse	<p>*Human Protein: 547 amino acid residues, with a calculated molecular weight of 62 kDa</p> <p>*Human gene: ~ 20 kb, 11 exons and 10 introns, mapped to chromosome 16q21-q23</p> <p>*Mouse protein: 566 amino acid residues</p>	<p>*Highly expressed in RPE (a 2.4 kb transcript)</p> <p>*Lower level in kidney, intestine, liver, brain, stomach and testis</p>	<p>*No enzymatic activity with lutein or lycopene</p> <p>*Activity significantly reduced by the addition of EDTA</p> <p>*A soluble protein not associated to membrane</p>	<p>*Major product: all-<i>trans</i>-retinal</p> <p>* Minor products: 13-<i>cis</i> retinal</p>	<p>*Human: 68% identity and 80% similarity with Chicken, and 24% identity with <i>Drosophila</i></p> <p>*Mouse: 83% identity with human and 60% with chicken</p>

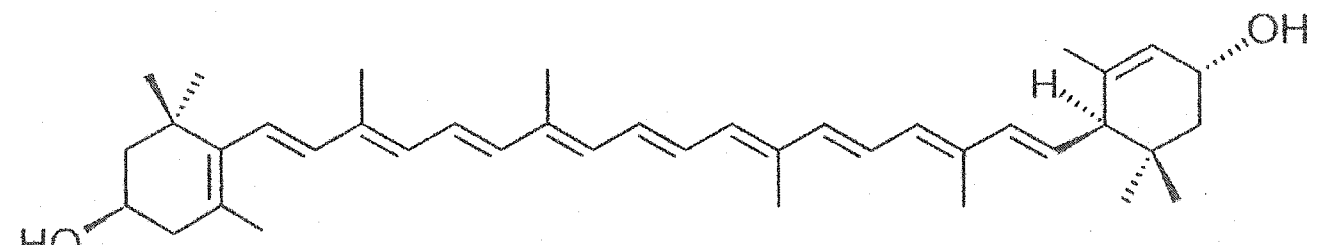
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Table 2

References	Species	Molecular Characteristics	Tissue Distribution	Enzymatic Activity and Characteristics	Cleavage Product	Homology
Lindqvist and Andersson 2002	Human	*~64 kDa protein *8% of the highly hydrophobic amino acids are conserved	*High levels along the whole intestinal tract, liver, kidney *Low levels in the prostate, testis, ovary, and skeletal muscle	*No enzymatic activity with lutein or lycopene *4-fold higher Km with β -cryptoxanthin than with β -carotene *A tetramer in solutions *Sensitive to metal chelating agents *Dependent on reduced sulfhydryl groups for maximal enzymatic activity <i>in vitro</i>	*All- <i>trans</i> -retinol	*22% protein sequence identity to the <i>Drosophila</i> protein



β -Carotene



Lutein

Figure 1. Lutein is a nonprovitamin A dihydroxy structural analog of β -carotene.

**A physiological dose of lutein does not inhibit the bioavailability and bioconversion of
β-carotene in young women as measured using
¹³C tracers and GC-C-IRMS**

A paper to be submitted to the American Journal of Clinical Nutrition

Xixuan H. Collins, Jeanne W. Stewart, Murray L. Kaplan, and Wendy S. White

ABSTRACT

Background: Lutein could be a contributing factor to the low bioavailability and bioconversion of β-carotene from dark green leafy vegetables. The high sensitivity of gas chromatography-combustion interfaced-isotope ratio mass spectrometry (GC-C-IRMS) makes it possible to administer ¹³C-labeled tracer doses of β-carotene and lutein at levels typical of dietary intake and to follow the fate of these carotenoids after their administration.

Objective: We compared in female subjects the appearance of ¹³C-β-carotene and its cleavage product, ¹³C-retinol, in the plasma after ingestion of a 1-mg dose of ¹³C-β-carotene with or without a 3-mg dose of ¹³C-lutein.

Design: Women (n = 7) each ingested both a single dose and a combined dose in random order. Blood samples were collected at selected intervals until 528 h. Lutein, β-carotene, and retinol were extracted from saponified plasma and purified by using HPLC. Stable isotope ratios of samples were analyzed by using GC-C-IRMS.

Results: The appearance of ¹³C-β-carotene in the plasma was not different after ingestion of the two doses, nor was the appearance of ¹³C-retinol. Plasma-β-carotene concentration at baseline was negatively correlated with the 0-96 h area under the concentration vs. time curve (AUC) for ¹³C-retinol after the combined dose (r = -0.84, P = 0.02). Plasma lutein concentration at baseline was positively correlated with the 0-528 h AUC for ¹³C-β-carotene after the ¹³C-β-carotene dose alone (r = 0.84, P = 0.02).

Conclusion: At a level typical of dietary intake and a lutein to β-carotene weight ratio of 3:1 that is common in vegetables, lutein has no significant effects on the absorption and conversion of β-carotene.

KEY WORDS bioavailability, bioconversion, β -carotene, ^{13}C -tracer, GC-C-IRMS, physiological dose, lutein, stable isotope, retinol

INTRODUCTION

In developing countries, provitamin A carotenoids in dark green leafy vegetables and yellow orange vegetables and fruits account for 70-90% of total vitamin A intake (1). Beta-carotene is abundant in dark green leafy vegetables but these vegetables are not effective in correcting vitamin A deficiency (2, 3). The underlying mechanism may involve an inhibitory effect of lutein (β,ϵ -carotene-3,3'-diol), a nonprovitamin A oxycarotenoid and structural analog of β -carotene. Lutein is a characteristic matrix component in dark green leafy vegetables. The bioavailability of lutein from dark green leafy vegetables is considerably higher than that of β -carotene (4). In one study (5), 69 volunteers consumed meals with different vegetables. Consumption of a lutein-rich spinach meal did not increase plasma β -carotene concentrations, whereas consumption of meals with broccoli and green pea introduced significant increase in plasma β -carotene level, despite the much higher (10-fold) β -carotene content in the spinach meal than that in the broccoli and green pea meals (5). In another recent study involving 77 Indonesian children, the bioefficacy and bioavailability of β -carotene in pumpkin were both 1.7 times that of β -carotene in spinach (6). There is a need to identify the matrix component in dark green leafy vegetables that restricts the absorption and conversion of β -carotene to vitamin A. This study focused on lutein as the candidate matrix component because a high lutein content could adversely affect the bioavailability of β -carotene in spinach and other dark green leafy vegetables by having a competitive or antagonistic effect on the intestinal absorption of β -carotene (7; 8), and/or by inhibiting the cleavage of β -carotene to vitamin A (9).

In this study, gas chromatography-combustion interfaced-isotope ratio mass spectrometry (GCC-IRMS) and highly-enriched ($>95\%$ ^{13}C) ^{13}C -labeled tracer doses of β -carotene and lutein (10-12) were used. By using this approach, we had the analytical sensitivity to employ small, physiological tracer doses of β -carotene and lutein, and the specificity to quantify the bioconversion of newly absorbed β -carotene to retinyl esters.

Studies conducted without benefit of stable isotope tracers have had inconsistent findings as to whether lutein inhibits the intestinal absorption and/or cleavage of β -carotene in humans (7, 8). In these studies, β -carotene and lutein were ingested in doses that greatly exceed effective (absorbed) doses typically derived from fruits and vegetables (13). The equimolar doses of β -carotene and lutein that were administered do not extrapolate to the disproportionate content of lutein in dark green leaves. Use of small physiological doses of β -carotene in this project has the advantages of avoiding down-regulation of β -carotene 15,15'-monooxygenase activity (14, 15), and specifically detecting retinoid metabolites of the ingested β -carotene tracer. The high-sensitivity tracer detection provided by gas chromatography-combustion interfaced-isotope ratio mass spectrometry (GC-C-IRMS) is uniquely suited to accomplish our research objectives.

SUBJECTS AND METHODS

Subjects

Healthy Caucasian women ($n = 7$), aged 20-31 y, were selected based on interview, physical examination, and complete blood count and blood biochemistry profile according to our established protocols (12, 16, 17). Criteria for exclusion were current or recent cigarette smoking, chronic disease, lipid malabsorption or gastrointestinal disorders, lactose intolerance, vegetarianism, hyperlipidemia confirmed by blood lipid profile, history of anemia and excessive bleeding, pregnancy, use of oral contraceptive agents or contraceptive implants, use of medications that may affect lipid absorption or transport (including antibiotics), use of vitamin or mineral supplements, and frequent consumption of alcoholic beverages (>1 drink per day). The characteristics of the subjects are presented in **Table 1**. Informed consent was obtained from all subjects, and the study protocol was approved by the Human Subjects Review Committee of Iowa State University.

Treatments

In a crossover design, subjects were assigned to two treatments in random order. For the single-dose treatment, subjects consumed a tracer dose of 1 mg (1.7 μ mol) of ^{13}C - β -

carotene. For the mixed-dose treatment subjects consumed a combined dose of 1 mg of ^{13}C - β -carotene and 3 mg (5.3 μmol) of ^{13}C -lutein. The test doses were ingested in random order and were separated by a washout period of ≥ 4 wk.

Experimental Diets

Subjects were provided a list of carotenoid-rich fruits and vegetables and vitamin A-rich foods and were instructed to avoid consumption of those foods for 4 days before the beginning of each of the 2 study periods. During the study periods, subjects consumed a controlled low-carotenoid, low-vitamin A diet of conventional foods for 2 days before and 4 days after dosing. A single daily menu of weighed food portions was provided. The meals were prepared and consumed in the Human Nutrition Metabolic Unit of the Center for Designing Foods to Improve Nutrition at Iowa State University, except for the carry-out lunches and evening snacks on weekdays. Adherence to the experimental diet was monitored by written self-report and by analysis of fasting plasma carotenoid concentrations using high-performance liquid chromatography (HPLC). Duplicate aliquots of 24-h diet composites from each of the two study periods were analyzed for carotenoid content. The extraction of the carotenoids followed the protocol described previously (18). On average, across two study periods, the daily diet provided (mean \pm SEM): 6.2 \pm 0.1 μg lutein, 7.5 \pm 0.4 μg β -carotene, 0.8 \pm 0.1 μg retinol, and no detectable α -carotene, β -cryptoxanthin, or lycopene. The macronutrient composition of the diet was estimated using Nutrition Data System for Research, Version v4.03/31 (N-Squared Computing Inc., Salem, OR). The daily diet of 9.7 MJ was distributed as 14% of total energy from protein, 59% of total energy from carbohydrate, and 27% of total energy from fat.

Preparation of biosynthetic ^{13}C -labeled tracers

Autotrophic algae grown with $^{13}\text{CO}_2$ as sole carbon source were previously used to economically produce per-labeled ($>98\%$ ^{13}C) β -carotene (10, 11) and per-labeled ($>99\%$ ^{13}C) lutein (12) for use as stable isotopic tracers. In this study, ^{13}C - β -carotene was isolated from a crude lipid extract of the saponifiable fraction of *Dunaliella* sp. as a contaminant from the nonsaponifiable fraction. ^{13}C -Lutein was isolated from a commercially-available

(Martek, Columbia, MD) crude lipid extract of the nonsaponifiable fraction of *Chlorella* sp. All solvents were HPLC grade, and were purchased from Fisher Scientific (Chicago, IL).

To isolate ^{13}C - β -carotene, the saponifiable fraction (50 mg) was extracted repeatedly with 15 mL of a mixture of hexane/ethyl ether (2:1, vol:vol) until the residue was almost colorless. The combined hexane/ethyl ether layers were evaporated to dryness *in vacuo*. Methanol (5 mL) was added to the residue to dissolve lutein and other xanthophylls but to sparingly dissolve β -carotene (19), which was then isolated by filtration. During the filtration step, the yellow residue on the filter paper was washed twice with 5 mL of methanol to remove residual tracers of lutein and other xanthophylls. The β -carotene was then further purified from the yellow residue by crystallization. The yellow residue was dissolved in a minimal amount (4 mL) of warm (40°C) methanol/water (90:10, vol:vol) solution and stored under argon overnight at -20°C to allow crystallization. The β -carotene crystals in methanol/water solution were filtered and washed with methanol (50 mL).

A modified solvent partitioning method based on a protocol established in our laboratory (12) was used to purify per-labeled ^{13}C -lutein. A portion of the crude algal extract (150-200 mg) was added to a 50-mL culture tube with a screwcap. Acetone (25 mL) was added to the crude extract and vortexed for 2 min. The mixture was centrifuged at $1380 \times g$ for 10 min and the acetone layer was removed. This procedure was repeated 3 times and the combined acetone layers were concentrated under argon to a volume of about 50 mL. Ten milliliters of the concentrated acetone solution were partitioned with hexane (1:1, vol:vol) and an equal volume of water. The mixture of acetone and water was discarded and the hexane fraction was saved and combined and washed with an equal volume of water. An equal volume of a methanol-water mixture (95:5, vol:vol) was added to the hexane fraction carefully in a separation funnel, without shaking to avoid formation of an emulsion, and the methanol layer was saved. The methanol fraction was further partitioned by adding an equal volume of ethyl ether. Water was added until two layers were formed. The methanol and water layer was discarded and the ethyl ether layer was transferred to a 50-mL tube with a screwcap. The ethyl ether layer was washed with water to remove the methanol residue, and then dried *in vacuo*. The dried product was dissolved in 4 mL hexane, vortexed, and filtered

(MSI MAGNA nylon disc filter, 0.22 μm , Micron Separations Inc, Westboro, MA.). The filtrate was washed with 100 mL cold hexane.

The crude lutein product was further purified from the yellow residue by crystallization. The yellow residue was dissolved in 1-2 mL of warm (40°C) methanol, and an equal volume of hexane was added drop-wise after the methanol was cooled to room temperature. The mixture was then stored in the dark, under argon, at -20°C overnight for crystallization. The lutein crystals were filtered and washed with 50 mL cold hexane. The final product was left in the dark in a hood for 8 hours to completely evaporate the solvent. The tracers were stored under argon in the dark, at -80°C.

The purity of the ^{13}C - β -carotene tracer was estimated to be 91% on the basis of peak area using HPLC analysis with photodiode array detection; the contaminants were unidentified but there were not detectable lutein or zeaxanthin (**Figure 1A**). The estimated purity of ^{13}C -lutein tracer was 96%; the contaminant was 4% zeaxanthin and there was no detectable β -carotene (**Figure 1B**). The retention times and ultraviolet/visible absorbance spectra of ^{13}C - β -carotene (**Figure 2**) and ^{13}C -lutein (**Figure 3**) tracers were identical to those of commercial β -carotene (Sigma Scientific, St Louis, MO) and lutein (Kemin Industries, Des Moines, IA) standards. The ^{13}C enrichment of β -carotene was $\geq 96.5\%$ and the ^{13}C enrichment of lutein was $\geq 100\%$, as determined by using serial dilution with unlabeled β -carotene (Sigma Scientific, St. Louis, MO) and lutein (Kemin Industries, Des Moines, IA) and stable carbon isotope-ratio analysis by GC-C-IRMS. Confirmatory mass spectra of the purified ^{13}C - β -carotene and ^{13}C -lutein (**Figure 4**) were obtained using a Finnigan (currently Thermoquest, San Jose, CA) TSQ 700 triple quadrupole mass spectrometer in electron ionization mode (70 eV) and were compared with those of standards. The sample was introduced into the ion source by direct insertion of probe at 200°C. Mass spectra were acquired over the range m/z 100-800 in 0.75 s.

The doses were prepared as previously described (10, 12) with slight modification. Specifically, β -carotene or β -carotene and lutein were solubilized in 28 g of high-oleic acid sunflower oil (Abitec Co., Janesville, WI) and emulsified using a hand-held mixer with a puree of 30 g of banana and 70 mL of nonvitamin-fortified nonfat milk (generously donated by Anderson Erickson Dairy Co., Des Moines, IA). The original vial and the mixture were

then rinsed with 30 mL of nonvitamin A-fortified nonfat milk, which was added to the emulsion. After drinking the emulsion, the subjects were instructed to rinse the container with 50 mL of nonvitamin-fortified nonfat milk and consume the rinse. The subjects consumed 94 g of plain bagel immediately after ingestion of the dose.

Study protocol

On the morning of the third day of the low-carotenoid low-vitamin A diet, subjects arrived at the metabolic unit after an overnight (12-h) fast. A baseline blood sample (21 mL) was drawn via a catheter placed in a forearm vein by a registered nurse. After ingestion of the dose, blood samples (14 mL) were drawn at hourly intervals for 12 h via the intravenous catheter into a syringe and transferred to tubes containing heparin as anticoagulant. The patency of the catheter was maintained by flushing with sterile physiological saline, as previously described (12, 16, 17). During the period of hourly blood collection, subjects continued the low-carotenoid, low vitamin A experimental diet, including a breakfast ingested with the ^{13}C -tracer dose, a lunch ingested immediately after the 5-h blood draw, and an evening meal ingested immediately after the 10-h blood draw. Additional blood samples were drawn from the antecubital vein via venipuncture at 16 h and after an overnight (12-h) fast at 24, 48, 72, 96, 192, 360, and 528 h. Blood samples were immediately placed on ice and protected from light. Plasma was separated by centrifugation ($1380 \times g$, 4°C , 20 min) and stored at -80°C in the dark until analyzed.

Purification of plasma β -carotene, lutein and retinol fractions

Beta-carotene, lutein and retinol were purified from individual plasma samples. Samples were not duplicated due to the large volume of plasma needed in order to extract enough analytes that have sufficient masses to be analyzed using GC-C-IRMS. We could not draw more than 14 mL of plasma from each subject at each time point from 1 h until 12 h post dosing, due to the close interval (1 h) between each blood draw. Plasma samples (4 mL) were divided into four 1.0-mL aliquots to ensure efficient extraction. The samples were deproteinized with 1 mL of ethanol containing 0.1g/L butylated hydroxytoluene, and

extracted twice with 8 mL of hexane containing 0.1g/L butylated hydroxytoluene. The combined hexane layers were evaporated to dryness under vacuum. A saponification step was needed to remove lipids that would otherwise interfere with the gas-liquid chromatographic separation of the major products of derivatization of lutein and β -carotene before elution into the combustion interface of the GC-C-IRMS. The dried hexane extracts were dissolved in 0.5 mL of ethanol containing 0.1g/L butylated hydroxytoluene in a 4-mL screw-capped vial. Another 0.5 mL of ethanol containing 5.5 mM pyrogallol was added to the vial followed by 1.0 mL of 5.3 M aqueous potassium hydroxide. The vial was filled with argon and the contents were mixed by vortexing for 1 minute. The samples were saponified at 65°C for 60 minutes in the dark with shaking. After cooling the saponified samples to room temperature over ice, the contents were transferred to a glass culture tube (16 × 100 mm) and extracted twice with 8 mL of hexane containing 0.1 g/L of butylated hydroxytoluene. The combined extracts were washed twice with 5.0 mL of HPLC grade water. Commercially available unlabeled β -carotene standard (Sigma, St. Louis, MO) and lutein standard (generously donated by Kemin Industries Inc, Des Moines, IA) were added to the extracts as carriers to ensure sufficient mass to be analyzed by GC-C-IRMS. The unlabeled standards also diluted the ^{13}C concentrations of the analytes so that they could be measured with high precision within the linear range of GC-C-IRMS (20). The accurate amounts of the added standards were recorded each time (β -carotene: ~ 3.4 nmol, and lutein: ~ 4.7 nmol) and used for back-calculation of the enrichment of the samples. The stable carbon isotope ratio (expressed as Atom Percent vs. the international standard PDB) was 1.08246 for unlabeled β -carotene standard and 1.07710 for unlabeled lutein standard, as measured without derivatization by using a NA 1500 elemental analyzer (EA) (CE Elantech, Lakewood, NJ) interfaced to the Optima isotope ratio mass spectrometer. Lutein and β -carotene were quantitatively combusted to CO_2 and H_2O ; H_2O vapor was removed by a chemical trap, and CO_2 was further purified by GC on a Poropak QS column (2 m × 4 mm i.d.) and administered into the Fisons/VG Isotech Optima IRMS (currently Micromass UK, Manchester, UK).

The combined hexane extracts and added β -carotene and lutein standards were dried under vacuum using a SpeedVac Concentrator (Savant, Farmingdale, NY) in preparation for isolation of retinol, β -carotene and lutein fractions by HPLC.

The dried residue was dissolved in 50 μ L of ethyl ether and 200 μ L of methanol with 1g/L ammonium acetate (mobile phase A) and a 245- μ L aliquot was injected into the HPLC system. The components of the HPLC system were manufactured by Waters Chromatography (Milford MA) and consisted of the 717Plus autosampler with temperature control set at 5°C, two 510 solvent delivery systems, and the 996 photodiode array detector. The system operated with Millennium³² Chromatography Manager software. Solvents were HPLC grade: methanol, methyl *tert*-butyl ether (MTBE), and ammonium acetate were purchased from Fisher Scientific (Chicago, IL). The mobile phase was filtered (nylon membrane filter, 0.2 μ m, Whatman International Ltd., Maidstone, England) and degassed before use. A 5- μ m C₃₀ Carotenoid Column (YMC Inc, Wilmington NC, now Waters Co., Milford MA) (4.6 \times 250 mm) protected by a precolumn packed with the same stationary phase was used to achieve baseline resolution of lutein and the constitutional isomer, zeaxanthin. Carotenoids were eluted by using a linear mobile phase gradient from 100% methanol (with 1g/L ammonium acetate) to 100% MTBE over 30 min, as modified from Sander et al (1994) (21) and Yao et al (2000) (12). The flow rate was 1.0 mL/min. Elution of plasma carotenoids were monitored at 453 nm and elution of retinol was monitored at 325 nm. The fractions of retinol, β -carotene and lutein were collected, dried under vacuum, and re-dissolved in 50 μ L of ethyl ether and 150 μ L of methanol (with 1g/L ammonium acetate) and re-purified by injecting 195 μ L into the HPLC system, using the same reversed-phase HPLC conditions. Before the second purification, the C₃₀ column was washed with a mixture of mobile phase A (methanol with 1g/L ammonium acetate) and 2-propanol (1:1, vol:vol) for 20 minutes with a flow rate of 1 mL/min to minimize the lipid residues left in the column from the initial HPLC purification (22). During the second purification, the retinol fraction was collected, and 100 μ L of methanol with 1g/L butylated hydroxytoluene was added (23). The retinol fraction was then stored under argon in the dark at -20°C until further purification with a normal phase HPLC column before being injected into GC-C

IRMS. The β -carotene and lutein fractions were immediately derivatized for GC-C-IRMS analysis.

The retinol fractions were dried under vacuum, reconstituted with 50 μ L of ethyl ether and 150 μ L of isooctane/ethanol (95:5, vol/vol), and a 195- μ L aliquot was injected into a Betasil Cyano analytical column (Keystone Scientific, Bellefonte, PA) (4.6 \times 150 mm) and eluted using an isocratic mobile phase of isooctane/ethanol (95:5, vol/vol). The flow rate was 1.0 mL/min. The retinol fraction was collected and dried under vacuum and immediately injected into GC-C-IRMS.

To ensure that there was no carry-over of ^{13}C in the HPLC system after purification of samples that could be highly enriched, samples from an unlabeled plasma pool were periodically purified with the same HPLC procedures, derivatized (as follows), and analyzed with GC-C-IRMS. The results showed that there was no ^{13}C contamination in the HPLC column.

Derivatization of β -carotene and lutein for GC-C-IRMS analysis

The conjugated polyene system of carotenoids is thermally labile, therefore the alcohol hydrogenolysis and hydrogenation of lutein (12) and hydrogenation of β -carotene to produce a thermally stable analog (10, 11) were necessary prior to stable carbon isotope analysis by using GC-C-IRMS. Platinum oxide was used as catalyst for hydrogenation of β -carotene and palladium on carbon (5 wt%) was used for lutein (12). The catalysts were purchased from Alfa Aesar (Ward Hill, MA). The hydrogenation tubes were filled with argon, and the catalysts (1 mg of platinum oxide or 10 mg of palladium on carbon) were promptly added to the tubes, followed by addition of 0.5 mL of HPLC-grade cyclohexane and 0.5 mL of glacial acetate acid containing 0.02 M *p*-toluenesulfonic acid monohydrate (Aldrich, Milwaukee, WI). The catalysts were then reduced by hydrogenation for at least 30 min prior to addition of the dried, purified β -carotene and lutein fractions that were suspended in 250 μ L of cyclohexane. The reaction proceeded at 60°C and under 12 psi hydrogen pressure with agitation generated by a micro stir bar for 16 hours. The reaction products were washed with 2 mL of HPLC-grade water and extracted twice with 6 mL of

hexane. The combined hexane layers were washed twice with 4 mL of HPLC-grade water, dried under vacuum, and stored under argon at -20°C until analyzed by using GC-C-IRMS. Hydrogenation of β -carotene and hydrogenolysis and hydrogenation of lutein yielded the same major product, perhydro- β -carotene. Confirmatory mass spectra of perhydro- β -carotene derived from plasma β -carotene and lutein (**Figure 5**) were obtained using a Finnigan TSQ 700 triple quadrupole mass spectrometer in electron ionization mode (70 ev).

GC-C-IRMS analysis

The stable carbon isotope ratios of retinol and the perhydro- β -carotene were determined by using a 5890A Hewlett-Packard (Wilmington, DE) GC fitted with a Fisons/VG Isotech Isochrom GC-combustion interface to the Fison/VG Isotech Optima IRMS. A $15\text{ m} \times 0.25\text{ mm}$ i.d. ($0.25\ \mu\text{m}$ film thickness) HP-1MS (J & W Scientific, Folsom, CA) fused-silica capillary column with on-column injector was used with ultrapure helium as carrier gas at a flow rate of 40 cm/s. The temperature program proceeded from 50°C followed by a gradient of $30^{\circ}\text{C}/\text{min}$ to 150°C followed by a gradient of $15^{\circ}\text{C}/\text{min}$ to 300°C and a 22-min hold at 300°C . The computer-generated stable carbon isotope ratio measurements, expressed in delta (δ) per mil (part per thousand, ‰) units, were used to calculate the atom percent ^{13}C in each plasma retinol sample according to the following formula:

$$\text{Atom}\% \ ^{13}\text{C} = \frac{(100 \times R_{\text{PDB}}) \times (\delta^{13}\text{C}/1000 + 1)}{1 + (R_{\text{PDB}}) \times (\delta^{13}\text{C}/1000 + 1)} \quad [1]$$

in which R_{PDB} represents the $^{13}\text{C}/^{12}\text{C}$ ratio for the international standard for carbon, Pee Dee Belemnite (PDB), with an accepted value $R_{\text{PDB}} = 0.0112372$ (13).

The atom percent (AP) values of β -carotene and lutein in samples were calculated based on the following mass balance equation (20):

$$F_m = (n/n_m)F + (n_l/n_m)F_l \quad [2]$$

and $AP = 100 \times F$ (in %) [3]

in which n is the amount of the unlabeled material; n_l is the amount of labeled material; n_m is the final amount of the mixture ($n_l + n$). F is the atom fraction of unlabeled material, as determined by directly analyzing the isotopic composition of unlabeled β -carotene and lutein standards without hydrogenation by using EA (see above). The F value was 0.0108246 for β -carotene standard and 0.010771 for lutein. F_m is the atom fraction of the mixtures (plasma β -carotene or lutein plus added unlabeled β -carotene or lutein standards), as measured by using GC-C-IRMS.

For each subject, the atom percent excess (APE) ^{13}C in plasma β -carotene, lutein and retinol at each time point was calculated by subtraction of the atom percent ^{13}C in plasma β -carotene, lutein and retinol at baseline from that in plasma β -carotene, lutein, and retinol after ingestion of the ^{13}C - β -carotene and ^{13}C -lutein tracers. The APE controls for variation in natural abundance and reflects the percentage of total plasma β -carotene and lutein that is ^{13}C -labeled, and the conversion product of the ^{13}C - β -carotene to retinol after ingestion of the ^{13}C tracers (11).

Calculation of ^{13}C - β -carotene, lutein and retinol in plasma

The plasma concentrations of ^{13}C - β -carotene, lutein and retinol were calculated from the total (labeled plus unlabeled) plasma concentrations determined by using HPLC and the APE ^{13}C in the plasma fractions determined by using GC-C-IRMS. In order to measure the total plasma concentrations, duplicate 200- μL aliquots of plasma were deproteinized by adding an equal volume of absolute ethanol containing 0.1g/L butylated hydroxytoluene and echinenone (Carotenature GMBH, Lupsingen, Switzerland) as an internal standard. Samples were then extracted twice with 4 mL hexane containing 0.1g/L butylated hydroxytoluene, and the combined hexane layers were evaporated to dryness under vacuum. The residues were reconstituted with 50 μL ethyl ether and 150 μL methanol (with 1g/L ammonium acetate), and 50 μL aliquots were injected into the HPLC system. The reversed-phase HPLC conditions were as previously described for the purification of β -carotene, lutein and retinol fractions. Calibration curves were generated from the ratio of the peak area of the standards

to that of the echinenone internal standard plotted against the injected amounts of the standards. Analyses were performed under yellow light. A quality control sample from an unlabeled plasma pool was analyzed daily by using HPLC to ensure the accuracy and reproducibility of the analyses. The inter-assay coefficient of variance (CV) for retinol, β -carotene, and lutein in the pooled plasma samples were 4.3%, 4.4% and 1.0%, respectively.

The plasma concentrations of ^{13}C - β -carotene, lutein and retinol were calculated as the product of the total plasma concentrations determined by using HPLC and the atom fraction excess of ^{13}C in dose, using the following formula (24):

$$\frac{(\text{Total concentration of X})(\text{APE}^{13}\text{C in X})}{100 (\text{fraction excess } ^{13}\text{C in dose})} \quad (4)$$

in which the fraction excess is 0.965 for ^{13}C β -carotene and 1.00 for ^{13}C -lutein as determined by using serial dilution with unlabeled β -carotene and lutein, and stable carbon isotope-ratio analysis by GC-C-IRMS (see above). Since plasma ^{13}C -retinol originates solely from the ^{13}C - β -carotene dose, 0.965 was used to calculate the plasma concentration of ^{13}C -retinol.

Statistical analyses

The areas under the plasma ^{13}C - β -carotene, lutein and retinol concentrations vs. time curves (AUCs) were calculated by using trapezoidal approximation (25). Concentrations of total retinol (free retinol and retinyl esters) were used because saponification of plasma samples has hydrolyzed retinyl esters. The time intervals used for AUC calculation were chosen based on when the plasma concentrations of ^{13}C analytes came back close to baseline after doing; 0-528 h AUCs were calculated for ^{13}C - β -carotene and ^{13}C -lutein, and 0-96 h AUC was calculated for ^{13}C -retinol. The ratio of AUCs for ^{13}C -retinol to ^{13}C - β -carotene was calculated and used as an index for conversion efficiency (26).

The effects of treatment on the AUCs for ^{13}C - β -carotene and ^{13}C -retinol for each subject were compared using paired student's *t*-test. The effects of treatment on conversion efficiency (ratio of AUCs for ^{13}C -retinol to ^{13}C - β -carotene) were also compared using

student's *t*-test. The plasma appearance and disappearance of ^{13}C - β -carotene and ^{13}C -retinol in the presence and absence of lutein were analyzed by repeated measures ANOVA. The correlation between AUCs for ^{13}C - β -carotene, ^{13}C -retinol, and ^{13}C -lutein, and the correlation between plasma carotenoid concentrations at baseline and AUCs, were analyzed. A P value ≤ 0.05 was regarded as significant.

RESULTS

We modified the purification protocol previously developed in our laboratory for purification of ^{13}C -lutein tracer from crude algal extract (12). With the old protocol, the purity of the lutein tracer was estimated to be 93% on the basis of peak area using HPLC analysis with photodiode array detection, and the contaminants were 2% β -carotene and 5% zeaxanthin. With the modified protocol, the purity of lutein was estimated to be 96% with HPLC analysis, with 4% zeaxanthin as contaminant and no detectable β -carotene. The absence of β -carotene contamination in lutein avoided addition of ^{13}C - β -carotene as part of the ^{13}C -lutein dose, therefore eliminating the extra ^{13}C - β -carotene as a confounding factor.

We also modified published protocols for purification of plasma β -carotene and lutein (11, 12, 22, 24). By adding a wash step during the HPLC purification procedure, we did not need to further purify β -carotene and lutein with a normal phase column after two previous reversed-phase HPLC purifications. Therefore the sample preparation procedures were less time-consuming which facilitated processing of the large number of samples in the current study. This modification also proved to be efficient in reducing sample matrix and preventing contamination by plasma lipid, as shown in the GC-C-IRMS chromatograms of the derivatized plasma β -carotene (**Figure 6**) and lutein (**Figure 7**) products. The plasma retinol fraction was also highly pure by using the current purification protocols (**Figure 8**). In these chromatograms (**Figure 6**, **Figure 7**, and **Figure 8**), there is a peak at the end of the mass spec program, close to the second sets of CO_2 standards. This is not a peak of any analyte; instead it was generated when the hot-split (HS) valve was closed at the end of the mass spec program, and the gas pressure built up during the mass spec program was carried over and released to the flame-ionization detector (FID).

The appearance of ^{13}C - β -carotene in the plasma was biphasic after ingestion of both doses (**Figure 9**). After the single dose, there was a smaller peak at 10 h and a bigger peak at 24 h. After the combined dose, the smaller peak appeared at 4 h and the bigger peak at 24 h. At 24 h, the concentration of ^{13}C - β -carotene was higher after the ingestion of the ^{13}C - β -carotene dose alone as compared with that after ingestion of the combined dose (0.210 ± 0.039 vs. 0.191 ± 0.023 $\mu\text{mol/L}$), but the difference was not statistically significant by paired student's *t*-test. Repeated measures ANOVA showed that there was no significant difference in the appearance of ^{13}C - β -carotene in the plasma after the two treatments.

The appearance of ^{13}C -retinol, the product of ^{13}C - β -carotene cleavage, showed three concentration peaks (**Figure 10**). After the single dose, there was a small peak at 5 h, a higher peak at 9 h, and the highest peak at 12 h. After the combined dose, there was a peak at 5 h, 10 h, and 12 h respectively, with the peak at 10 h being the highest and the one at 5 h being the lowest. At 5 h and 12 h postdosing, the concentrations of ^{13}C -retinol were higher after the ingestion of the ^{13}C - β -carotene dose alone as compared with those after ingestion of the combined dose (5h: 0.263 ± 0.060 vs. 0.254 ± 0.035 $\mu\text{mol/L}$; 12h: 0.446 ± 0.047 vs. 0.431 ± 0.062 $\mu\text{mol/L}$), but the differences were not statistically significant by paired student's *t*-tests. The appearance of ^{13}C -retinol did not differ significantly after the two treatments, as analyzed using repeated measures ANOVA.

There was a rapid increase in the appearance of ^{13}C -lutein in the plasma after dosing, and it reached a plateau from 10h to 48 h postdosing, with an average value of 25.054 ± 0.534 $\mu\text{mol/L}$ (range: 23.391 ± 7.146 to 26.682 ± 10.421 $\mu\text{mol/L}$) (**Figure 11**). There was still detectable amount of ^{13}C -lutein in the plasma at 528 h after dosing, with a value of 2.478 ± 1.504 $\mu\text{mol/L}$, as compared with 0.408 ± 0.336 $\mu\text{mol/L}$ at 2 h after dosing. The plasma area under the concentration-versus-time curve (AUC) values for ^{13}C -lutein for 0-528 h are listed in **Table 2**.

Plasma area under the concentration-versus-time curve (AUC) values for 0-528 h for ^{13}C - β -carotene and the within subject difference after ingestion of the ^{13}C - β -carotene dose alone and combined dose of ^{13}C - β -carotene and ^{13}C -lutein are shown in **Table 3**. Paired student's *t*-tests indicate no significant differences between the AUCs for ^{13}C - β -carotene

after the single dose and those after the combined dose. Addition of lutein to the dose resulted in increased AUC values for β -carotene in three subjects who had lower AUCs ($< 30 \mu\text{mol} \cdot \text{h/L}$.) after ingestion of the single dose alone, decreased AUC values in three subjects who had higher AUCs ($> 45 \mu\text{mol} \cdot \text{h/L}$) after ingestion of the single dose alone, and almost no change in one subject (**Table 3**).

The AUC values for 0-96 h for ^{13}C -retinol and the within-subject difference are shown in **Table 4**. One subject (subject 3) had a negative AUC value after ingestion of the single dose. GC-C-IRMS is very sensitive in measuring small fluctuations of the $^{13}\text{C}/^{12}\text{C}$ ratio in samples, therefore if the enrichment of a sample is close to the natural abundance, the instrument may generate a reading of $^{13}\text{C}/^{12}\text{C}$ ratio that is slightly different from the true value of the sample due to normal sampling errors. If the measured value is lower than the true value, the increment of plasma ^{13}C concentration could be a negative number. The negative number can have a large impact on calculation of the AUC if the time interval is large, which can result in a negative total AUC. The subject who had the negative AUC had negative increment in plasma ^{13}C -retinol concentration over baseline from 48 h to 528 h post dosing. This could mean that this subject had ^{13}C -retinol concentrations close to baseline from 48 h after dosing, which is different from the rest of the subjects who had plasma ^{13}C -retinol concentrations approaching to baseline only from 96 h after dosing.

Paired student's *t*-tests indicate no significant differences between the AUCs for ^{13}C -retinol after the two doses. Addition of lutein to the dose resulted in reduced AUCs for ^{13}C -retinol in three subjects, increased AUCs for the other three, and almost no change in one subject. Unlike the AUCs for ^{13}C - β -carotene, the AUCs for ^{13}C -retinol after the single dose does not seem to predict or related to the AUC values after the combined dose.

The ratios of AUCs for ^{13}C -retinol to AUCs for ^{13}C - β -carotene are showed in **Table 5**, and the comparisons of the appearance of ^{13}C -retinol and ^{13}C - β -carotene are showed in **Figure 12**. The ratio of AUCs for ^{13}C -retinol to AUCs for ^{13}C - β -carotene reflects conversion efficiency (26), and the higher the ratio, the higher the conversion efficiency. After ingestion of the single dose, the subject who had the smallest AUC for ^{13}C - β -carotene showed the highest ratio, but the subject who had the highest AUC for ^{13}C - β -carotene did not show the lowest ratio. With the exception of one subject (subject 4), the subjects who had higher

ratios (> 1) after ingestion of the single dose all had lower ratios after ingestion of the combined dose, whereas those who had lower ratios (< 1) after the single dose had higher ratios after the combined dose (**Table 5**). Paired student's *t*-test did not show significant differences between the ratios after the two treatments, although the average value of the ratio was higher after the single dose (0.52 ± 0.25 vs. 0.29 ± 0.13).

The responses from the subjects to the ^{13}C - β -carotene dose, with or without the addition of ^{13}C -lutein, were highly variable. For ^{13}C β -carotene, the interindividual CV for 0-528 h AUC was 59% (a 2-to 6-fold difference) after the single dose and 36% (a 1.5- to 2-fold difference) after the combined dose. For ^{13}C -retinol, the interindividual CV for 0-96 h AUC was 62% (a 1- to 6-fold difference) after the single dose and 53% (a 1- to 7-fold) after the combined dose. For ^{13}C -lutein, the CV for 0-528 h AUC was 44% (a 3- to 9- fold difference).

There was no correlation between AUC values for β -carotene, retinol, and lutein. Plasma concentration of β -carotene at baseline was negatively correlated with the 0-96 h AUC for ^{13}C -retinol after the combined dose ($r = -0.84$, $P = 0.02$) (**Figure 13A**). Plasma lutein concentration at baseline was positively correlated with the 0-528 h AUC of ^{13}C - β -carotene after the ^{13}C - β -carotene dose alone ($r = 0.84$, $P = 0.03$) (**Figure 13B**), and positively correlated with the 0-528 h AUC of ^{13}C -lutein ($r = 0.82$, $P = 0.02$) (**Figure 13C**). Plasma concentration of lutein at baseline was negatively correlated with BMI ($r = -0.91$, $P = 0.004$) (**Figure 13D**).

DISCUSSION

Parker et al (1993, 1997) first developed the GC-C-IRMS method to study the metabolism of a physiological dose of perlabelled ^{13}C - β -carotene in humans (10, 11). Yao et al (2000) successfully adapted this approach for the study of another prominent dietary carotenoid, lutein (12). The current study is the first to apply this stable isotope tracer method to investigate the effects of lutein on the intestinal absorption and bioconversion of β -carotene. Lutein and β -carotene were in amounts similar to those typically ingested from a single served vegetables (2, 5, 6, 27), and in a lutein to β -carotene ratio typically found in dark green leafy vegetables (27-29). The 1 mg of ^{13}C - β -carotene used is similar to the

average amount in a single serving of dark green leafy vegetables: 1.0 mg in boiled broccoli, 0.5 mg in Brussels sprouts, and 5.5 mg in raw spinach (27). The amount is also similar to what was used in intervention studies in terms of Retinol Activity Equivalent (RAE) (2, 5, 6). The ratio of lutein to β -carotene (3:1) is comparable to the average ratio of lutein to β -carotene in various vegetables: 2.5 in cooked kale (27), 4.9 in cassava leaves (28), 2.0 in broccoli and spinach (29). Previous studies regarding to the effects of lutein on β -carotene absorption and conversion and/or interaction of lutein and β -carotene utilized much higher doses of lutein and β -carotene, often as high as 15 mg, which do not reflect our dietary intake. A high dose of β -carotene also down-regulates the enzymatic activity of β -carotene 15,15'-monooxygenase (14, 15), which is a confounding factor for the study of effects of dietary components on β -carotene bioconversion.

The use of physiological tracer doses was possible due to a metabolic tracer technique using highly enriched substrates uniformly labeled with ^{13}C and analysis by high-precision GC-C-IRMS (30). GC-C-IRMS combines the advantage of the small sample requirement of GC-MS and the high precision of conventional IRMS (12). However it has a limited dynamic range over which enrichments can be accurately and precisely measured (20). In our study, three Faraday cup detectors continuously monitored ion currents at m/z 44 ($^{12}\text{CO}_2$), 45 ($^{13}\text{CO}_2 + ^{12}\text{C}^{17}\text{O}^{16}\text{O}$) and 46 ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$) (12). Amplification of signal at the Faraday cups is accomplished by dedicated circuits mounted close to the detectors, and the feedback amplifiers are usually highly shielded and are made with high performance resistors to yield amplification factors corresponding to the approximate isotopic abundance in nature (20). In the case of C isotope ratio, a delta value much above 1000 will compromise precision and accuracy, partly due to the large difference between the measured values and natural abundance standards (20). In the current study, our samples were highly enriched compared with samples with natural abundance, which required caution when measuring them with GC-C-IRMS. Brenna et al (1997) suggested two possible solutions to this problem: using enriched standards, which still does not eliminate the problem of mismatch in amplifications, or coupling a combustion-IRMS system and an ion trap mass spectrometer (20). In our case, we applied an economical solution to this problem, by spiking the samples with unlabeled commercial β -carotene and lutein standards. We used mass balance (see

SUBJECTS AND METHODS), also referred to as the Master Equation, to determine the relative contribution of the two carbon sources to the samples: ^{13}C from the tracers and from the natural abundance in plasma β -carotene and lutein, and ^{12}C from the plasma β -carotene and lutein, and from the added unlabeled standards. The isotopic ratios of baseline samples (unenriched) and those of the samples from an unlabeled plasma pool (see SUBJECTS AND METHODS) were in the range of natural abundance as measured using GC-C-IRMS, indicating no errors were introduced to the calculation of $^{13}\text{C}/^{12}\text{C}$ ratios by adding unlabeled standards and using mass balance approach.

The appearance of ^{13}C - β -carotene (**Figure 9**) in the plasma was biphasic with a smaller peak at 10 h after the single dose and at 4 h after the combined dose, and a higher, broad peak at 24-48 h. The first peak was somewhat delayed after the single dose compared with previously published data. After the single dose, two subjects showed a small peak at 4 h, three showed a small peak at 5 h, one showed a small peak at 6 h, and one showed a small peak at 8 h. Overall there was no distinct peak at 5 h after dosing. The small peak at 4 h post dosing after ingestion of the combined dose, and the second high, broad peak at 24 h after ingestion of both doses were consistent with previous studies (13, 24), in which there were a smaller peak at 5 h and a second, broad peak between 24 and 48h. Our previous studies (16, 17) and those of others (31, 32) using large, unlabelled doses of β -carotene showed that the initial peak coincides with appearance of β -carotene in postprandial triacylglycerol-rich lipoproteins (TRLs) and the second peak with the delayed appearance of β -carotene in LDL, with the latter reflecting the resecretion of β -carotene from liver.

The appearance of ^{13}C -retinol, the product of ^{13}C β -carotene cleavage, showed three concentration peaks (**Figure 10**). After the single dose, there was a small peak at 5 h, a higher peak at 9 h, and the highest peak at 12 h. After the combined dose, there was a peak at 5 h, 10 h, and 12 h, respectively, with the peak at 10 h being the highest and the one at 5 h being the lowest. As the first peak of β -carotene, the first peak of retinol at 5 h coincides with the secretion and clearance of chylomicrons, as in the case of dosing with unlabeled β -carotene (33), which indicates plasma ^{13}C -retinol at 5 h originates from cleavage of ^{13}C - β -carotene in the enterocytes. Addition of lutein did not affect this process, since there was no significant differences in the concentrations of ^{13}C -retinol at 5 h after the two treatments,

analyzed using paired student's *t*-test. The appearance of the highest ^{13}C -retinol peaks at 12 h and 10 h postdosing may suggest that the majority of the cleavage products originate from the hepatic cleavage of ^{13}C β -carotene. This is similar to the results in the study by Lin et al., in which the plasma D_3 retinol concentration peaked at around 10 hours after dosing with D_6 β -carotene. Addition of lutein did not affect the concentrations of ^{13}C -retinol at 10 h and 12 h post dosing.

The mean plasma concentration of ^{13}C -lutein was highest at 16 h (**Figure 11**), consistent with the plasma appearance of ^{13}C -lutein in our previous study (12), as well as the results in healthy adults who ingested a single pharmacological dose of unlabeled lutein (7). The mean concentrations of ^{13}C -lutein were fairly constant between 10 h and 28 h, ranging from 23.4 ± 7.2 $\mu\text{mol/L}$ to 26.7 ± 10.4 $\mu\text{mol/L}$, with almost identical values at 16 h and 48 h (26.7 mol vs. 26.4 μmol). This broad plateau in plasma ^{13}C -lutein concentration was not reported by Yao et al (12), and it is not clear if it was due to the concurrent ingestion of ^{13}C β -carotene and ^{13}C -lutein.

The responses from the subjects to the ^{13}C β -carotene dose, with or without the addition of ^{13}C -lutein, were highly variable. For ^{13}C β -carotene, the intrerindividual CV for 0-528 h AUC was 59% (a 2-to 6-fold difference) after the single dose and 36% (a 1.5- to 2-fold difference) after the combined dose. For ^{13}C -retinol, the interindividual CV for 0-96 h AUC was 62% (a 1- to 6-fold difference) after the single dose and 53% (a 1- to 7-fold) after the combined dose. For ^{13}C -lutein, the CV for 0-528 h AUC was 44% (a 3- to 9- fold difference). This result is consistent with previous studies, in which high variability of responses to β -carotene doses from different subjects was a commonly observed phenomenon. In a group of 79 healthy men, there was a high variability (CV = 61%) of response to a 120 mg β -carotene dose, as estimated by the appearance of β -carotene in chylomicrons at 3 h postdosing (33). In another study involving 10 male subjects, after an oral dose of 15 mg of β -carotene, the interindividual variation in the triacylglycerol-adjusted response of β -carotene in the triacylglycerol-rich lipoprotein (TRL) fraction was 42%, and the value for retinyl palmitate was 36% (34). O'Neil and Thurnham (1998) also reported that, after ingesting either 40 mg β -carotene, 31.2 mg lutein or 38 mg lycopene, subjects

showed approximately a twofold difference in uptake of β -carotene into the TRL fraction, a two- to threefold difference in uptake of lycopene and a two- to threefold variation in uptake of lutein (35). In more recent studies using isotopic tracer doses, subjects also showed high variability. Lin et al (2000) reported that after ingesting 37 μmol D₆ β -carotene and 30 μmol D₆ retinyl acetate, only 6 of their 11 female subjects had plasma D₆ β -carotene and D₃ retinol concentrations that could be measured. The remaining 5 subjects were low responders with $\leq 0.01\%$ absorption (25). In a separate study with similar design, Hickenbottom et al (2002) reported that after ingestion of 37 μmol of D₆ β -carotene and 30 μmol of D₆ retinol dose, only 6 of 11 men had sufficient plasma concentrations of D₆ β -carotene and D₃ retinol that could be measured (36).

Besides variable responses to a single β -carotene dose, in our study and previous studies, subjects also showed different responses to a combined dose of β -carotene and another carotenoid. In this study, three subjects had higher values of 0-528 h AUC for ¹³C β -carotene after the single dose, whereas three subjects had higher values after the combined dose, and one subject had very similar values after the two doses. The same situation was true for the values of 0-96 h AUC for ¹³C-retinol, with three subjects showing larger values after the single dose and four showing larger values after the combined dose. Addition of lutein to the β -carotene dose enhanced the plasma AUC for ¹³C- β -carotene in subjects who had lower AUCs for ¹³C- β -carotene when ingested the single dose, whereas the trend was not so clear for AUCs for ¹³C-retinol. These results were consistent with previous studies (7, 16). In a study by Kostic et al (1995), 8 subjects were given single β -carotene dose and a combined equimolar doses (0.5 $\mu\text{mol}/\text{kg}$ body wt) of β -carotene and lutein. In 5 subjects lutein decreased the AUC value for β -carotene but enhanced it in three others (7). Paetau et al (1997) reported that ingestion of 25 mg canthaxanthin and 25 mg β -carotene resulted in decreased plasma AUC for β -carotene in 5 of their 9 subjects, but increased AUC for β -carotene in the other 3, although overall the AUC values for β -carotene were not different with or without canthaxanthin. In contrast, ingestion of the combined dose of β -carotene and canthaxanthin resulted in decreased AUC for canthaxanthin in all subjects (16). In these two studies, lutein and canthaxanthin enhanced the serum β -carotene AUC of those subjects who

had low AUC values after ingestion of β -carotene alone, as we have observed in our study (Table 3).

There seems to be no biomarker to predict a subject's response to either a single dose of β -carotene or a combined dose of β -carotene and another carotenoid. In women, nutritional status, as reflected by baseline plasma β -carotene and vitamin A concentration, did not predict a subject's propensity to utilize β -carotene for vitamin A (26); however in men, pre-study intake of vitamin A and β -carotene was observed to modulate the response to a β -carotene dose (36). Most recently, Lemke et al (2003) reported that vitamin A supplementation decreased the cleavage of β -carotene, but increased apparent absorption of β -carotene, resulting in larger molar vitamin A values(37). In the current study, the baseline lutein concentration was significantly and positively associated with AUC for β -carotene after the single dose but not after the combined dose, and was also significantly and positively associated with AUC for lutein. This suggests that subjects with higher baseline carotenoid concentrations could be more responsive to supplementation of one carotenoid without the presence of a second carotenoid. The plasma concentration of β -carotene at baseline was negatively and significantly correlated with 0-96 h AUC for ^{13}C -retinol after the combined dose ($r = -0.84$, $P = 0.02$), but the negative correlation was not significant after the single dose ($r = -0.59$, $P = 0.16$). Although highly speculative, this result could indicate an effect of combined dose on the AUC for retinol. The plasma concentration of β -carotene at baseline was not correlated with 0-528 h AUC for ^{13}C - β -carotene after the single dose ($r = 0.11$, $P = 0.82$), or after the combined dose ($r = 0.45$, $p = 0.31$).

Studies have reported conflicting results regarding to the relationship between plasma carotenoid concentrations and body mass index (BMI). The plasma β -carotene AUC values did not correlate significantly with body weight, BMI, fat mass serum cholesterol or triacylglycerol, total dietary protein, or fat (25, 33, 35). However, the increase in plasma β -carotene concentration in response to β -carotene supplementation was significantly and inversely correlated with BMI and fat-free mass in older men (38), and significantly and inversely correlated with percentage of body fat and fat mass in older women (39). In our study and the study by Lin et al (26), subjects were young women (aged 20 to 31 y in our

study and 19 to 39 y in their study), and there was no significant correlation between BMI and the β -carotene response. This may suggest that age or age-related factors play roles in the uptake of β -carotene supplements. There was no significant correlation between BMI and conversion ratio (as estimated by the ratio of ^{13}C -retinol AUC to ^{13}C - β -carotene AUC). This is consistent with Lin et al (26) and Hickenbottom et al (35) but different from the results from a recent study, in which the conversion of $^2\text{H}_8$ - β -carotene to $^2\text{H}_4$ -retinol was significantly and positively associated with BMI (40). In our study, although BMI is not correlated with the response to doses, it is negatively and significantly correlated with the baseline concentration of lutein. This result is to some extent consistent with the previous studies. In the study by Zhu et al (38), BMI was significantly and negatively correlated with baseline plasma total carotenoid concentration. In Yeum's study (39), BMI was significantly and negatively associated with baseline plasma concentration of carotenoids in older women, but in younger women the negative correlation was only significant between BMI and baseline β -carotene. Serum β -carotene concentration and BMI were negatively and significantly associated in 276 women (41). Higher BMI values generally mean higher body fat percentage (42), and body fat could be a repository or sink for β -carotene, resulting in lower serum concentration of β -carotene (40), which could also be true for lutein.

In our small group of subjects, lutein did not show an inhibitory effect on β -carotene absorption and conversion. The lack of improvement of vitamin A status with increased consumption of dark-green leafy vegetables (2, 3) despite the abundance of β -carotene in these vegetables suggests that some component(s) may inhibit the absorption and conversion of β -carotene. Lutein is an obvious candidate because it is another prominent carotenoid in these vegetables and even more prominent than β -carotene. Lutein is also a nonprovitamin A structural analog to β -carotene, and the structural similarity could interfere with the enzymatic activity of β -carotene cleavage enzyme. The hypothesis that lutein inhibits the absorption and/or conversion of β -carotene seems to be supported by evidence from in vitro studies, and animal data. The bioavailability of lutein from dark green leafy vegetables is 5 times higher than that of β -carotene (4). Orange fruits, which have little or no lutein, are more effective than dark-green, leafy vegetables in increasing serum concentrations of retinol

and β -carotene in children with marginal vitamin A status (43). In an in vitro measurement of β -carotene cleavage activity, addition of 9 μg of lutein to an incubation of 3 μg β -carotene and cleavage enzyme reduced formation of retinal (9). The deposition of vitamin A in liver was significantly decreased in rats fed more lutein (at β -carotene:lutein ratios < 1) (44). Recent study from our lab showed that genetic modification of leaves to decrease lutein content improves β -carotene utilization in gerbils. Gerbils fed with a vitamin A-free diet supplemented with the leaves from a lutein-free (*lut2*) *Arabidopsis* mutant had 48% more vitamin A stores in the liver than those fed with a vitamin A-free diet supplemented with the leaves from a wild type of *Arabidopsis* (45). Lutein was preferentially incorporated into chylomicron compared with β -carotene. Gärtner et al (1996) reported that there was a preferential increase in chylomicron levels of lutein and zeaxanthin compared with β -carotene in humans after ingestion of a single dose of Betatene, a natural carotenoid source that contains 14-fold higher β -carotene than lutein (46). More recently, Tyssandier et al (2002) reported that adding a second carotenoid from a vegetable source to a meal that provided a first carotenoid diminished the chylomicron response to the first carotenoid (47). Despite all the evidence, human studies using high doses of β -carotene and lutein generated inconsistent results as for the interaction of the carotenoids. Kostic et al (1995) reported that lutein reduced β -carotene AUC in subjects who had higher AUC value when ingested β -carotene alone, but enhanced β -carotene AUC in other subjects (7). van den Berg (1998) reported that lutein inhibited β -carotene absorption but not conversion, and the inhibitory effect of lutein was most marked when lutein was the predominant carotenoid (at a lutein to β -carotene ratio of 2:1) (29). In this study we found similar results as reported by Kostic et al (1995) (7). Lutein had mixed effects on the AUC value for β -carotene and its cleavage product retinol in different subjects. Overall lutein did not affect either β -carotene absorption or conversion at a physiological dose and at a 3:1 ratio to β -carotene. Other factors in dark-green leafy vegetables, such as contents and types of fiber, could be responsible for preventing the efficient absorption and/or conversion of β -carotene to vitamin A (48-50). Fibers have different effects on the absorption of β -carotene and lutein (48), which may partly explain the higher uptake of lutein than β -carotene from vegetables. In our study, we

used physiological doses, but both β -carotene and lutein were in their pure forms, not in a food matrix. The interaction of β -carotene and lutein could be very different in a food matrix. Recently Kelm et al (2001) determined ^{13}C labeled and endogenous β -carotene, lutein and vitamin A in a subject who consumed steamed ^{13}C -kale that was grown in a $^{13}\text{CO}_2$ chamber with > 98% enrichment of ^{13}C in β -carotene and lutein (51). Further investigation using stable isotope labeled plant foods may shed light over the question about interaction of β -carotene and lutein in a food matrix.

Simultaneous ingestion of carotenoids may induce an antioxidant-sparing effect in the intestinal tract (47, 52, 53), and thus result in increased levels of uptake of the protected carotenoids. In the case of β -carotene and lutein, lutein could inhibit β -carotene absorption and conversion as shown in *in vitro*, as well as protect β -carotene as an antioxidant, and the overall effect would be a balance between the inhibitory effect and the protective effect. Plasma xanthophyll carotenoids have been found to correlate inversely with indices of oxidative DNA damage and lipid peroxidation (54). In a carotenoid-containing unilamellar liposome model for studying hydrophobic-hydrophilic antioxidant interaction, higher levels of β -carotene (>0.02 nmol/mg phospholipid) and lycopene (>0.06 nmol/mg phospholipid) exhibited prooxidant effects, whereas lutein inhibited lipid peroxidation in a dose-dependent manner between 0.02 and 2.6 nmol/mg phospholipid (55). Lycopene and β -carotene degraded more rapidly than lutein and zeaxanthin upon exposure to various pro-oxidants *in vitro* (56). These experimental results suggest that, if lutein does have an antioxidant sparing effect to protect β -carotene, this effect could potentially be important.

The discrepancy between our results and the data from animal studies, *in vitro* studies, and human studies using pharmacological doses suggests that non-human, *in vitro* data should not be extrapolated to humans without caution. The study has to be done in humans, which makes it important to develop appropriate methods that are not invasive so that they can be used in humans, and sensitive enough that can simulate physiological doses and dietary intake. The ^{13}C tracer and GC-C-IRMS approach used in this study provides one possibility to reach the research goals.

Plant breeding to enhance nutritional values of plant foods has become a new tool for fighting micronutrient malnutrition (57-59). Rice was successfully engineered to produce β -

carotene in the endosperm (60), and tomatoes that have 1.8-fold higher lycopene and 2.2-fold higher β -carotene than wild type have also been produced (61). It will not be surprising if high-lutein plants are bred to capitalize upon the putative health benefits of lutein. Our study has shown that lutein dose not inhibit bioavailability and bioconversion of β -carotene at a normal dietary intake, and this could ease the concerns that high-lutein plant foods could potentially impair the utilization of β -carotene. Our finding could also be important knowledge base for the supplementation industry for promoting the health benefits of lutein without compromising the importance of β -carotene in human nutrition.

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TABLE 1Subject characteristics at baseline¹

Characteristics	Value
Age	23.6 ± 1.6 (20-31)
BMI (in kg/m ²)	23.8 ± 1.3 (20.6-28.8)
Fasting plasma concentrations at baseline (μmol/L)	
Retinol	1.407 ± 0.093 (1.153-1.778)
β-Carotene	0.222 ± 0.035 (0.105-0.375)
Lutein	0.165 ± 0.019 (0.103-0.227)
Zeaxanthin	0.088 ± 0.015 (0.000-0.117)
β-Cryptoxanthin	0.106 ± 0.018 (0.059-0.178)
α-Carotene	0.125 ± 0.023 (0.052-0.245)
Lycopene	0.166 ± 0.032 (0.104-0.351)

¹ $\bar{x} \pm \text{SEM}$ with range in parentheses; n = 7.**TABLE 2**Area under the plasma concentration versus time curves (AUCs) for ¹³C-lutein for 0-528 h after subjects ingested 1 mg ¹³C-β-carotene and 3 mg ¹³C-lutein

Subject	AUC (μmol · h/L)
1	3238.86
2	5762.88
3	3979.2
4	2483.66
5	5438.15
6	2014.75
7	1987.06
$\bar{x} \pm \text{SEM}$	3557.794 ± 591.1824

TABLE 3

Plasma ^{13}C - β -carotene area under the concentration-versus-time curve values for 0-528 h after subjects ingested 1 mg ^{13}C - β -carotene alone or 1 mg ^{13}C - β -carotene and 3 mg ^{13}C -lutein

Subject	Doses		Within-subject difference
	β -Carotene	β -Carotene plus lutein	
	$\mu\text{mol} \cdot \text{h/L}$		
1	27.778	32.704	-4.926
2	66.302	52.801	13.502
3	22.583	61.642	-39.059
4	46.244	34.609	11.635
5	71.047	23.643	47.405
6	26.006	25.096	0.909
7	11.359	41.632	-30.274
$\bar{x} \pm \text{SEM}$	38.760 ± 8.664	38.875 ± 5.341	-0.115 ± 10.946

TABLE 4

Plasma ^{13}C -retinol area under the concentration-versus-time curve values for 0-96 h after subjects ingested 1 mg ^{13}C - β -carotene alone or 1 mg ^{13}C - β -carotene and 3 mg ^{13}C -lutein

Subject	Doses		Within-subject difference
	β -Carotene	β -Carotene plus lutein	
	$\mu\text{mol} \cdot \text{h/L}$		
1	34.416	25.248	9.168
2	17.029	19.926	-2.897
3	-0.931	3.275	-4.206
4	10.467	5.0401	5.427
5	20.711	21.553	-0.841
6	15.126	21.728	-6.601
7	20.879	19.223	1.657
$\bar{x} \pm \text{SEM}$	16.814 ± 4.082	16.570 ± 3.290	0.243 ± 2.106

TABLE 5

Area under the plasma concentration-versus-time curve (AUC) values for ^{13}C - β -carotene and ^{13}C -retinol and ratio of AUCs for ^{13}C - β -carotene to AUCs for ^{13}C -retinol after subjects ingested 1 mg of ^{13}C - β -carotene with or without 3 mg of ^{13}C -lutein

Subject	^{13}C - β -Carotene dose			^{13}C - β -Carotene + ^{13}C -Lutein dose		
	^{13}C -Retinol (0-96 h AUC)	^{13}C - β -Carotene (0-528 h AUC)	^{13}C -Retinol : ^{13}C - β -carotene	^{13}C -Retinol (0-96 h AUC)	^{13}C - β -Carotene (0-528 h AUC)	^{13}C -Retinol : ^{13}C - β -carotene
	$\mu\text{mol} \cdot \text{h/L}$			$\mu\text{mol} \cdot \text{h/L}$		
1	34.416	27.778	1.239	25.248	32.704	0.772
2	17.029	66.302	0.257	19.926	52.801	0.377
3	-0.931	22.583	-0.041	3.275	61.642	0.053
4	10.467	46.244	0.226	5.041	34.609	0.146
5	20.711	71.047	0.292	21.553	23.643	0.912
6	15.126	26.006	0.582	21.728	25.096	0.866
7	20.879	11.358	1.838	19.223	41.632	0.462
$\bar{x} \pm \text{SEM}$	16.814 ± 4.081	38.760 ± 8.664	0.521 ± 0.254	16.570 ± 3.290	38.875 ± 5.341	0.289 ± 0.131

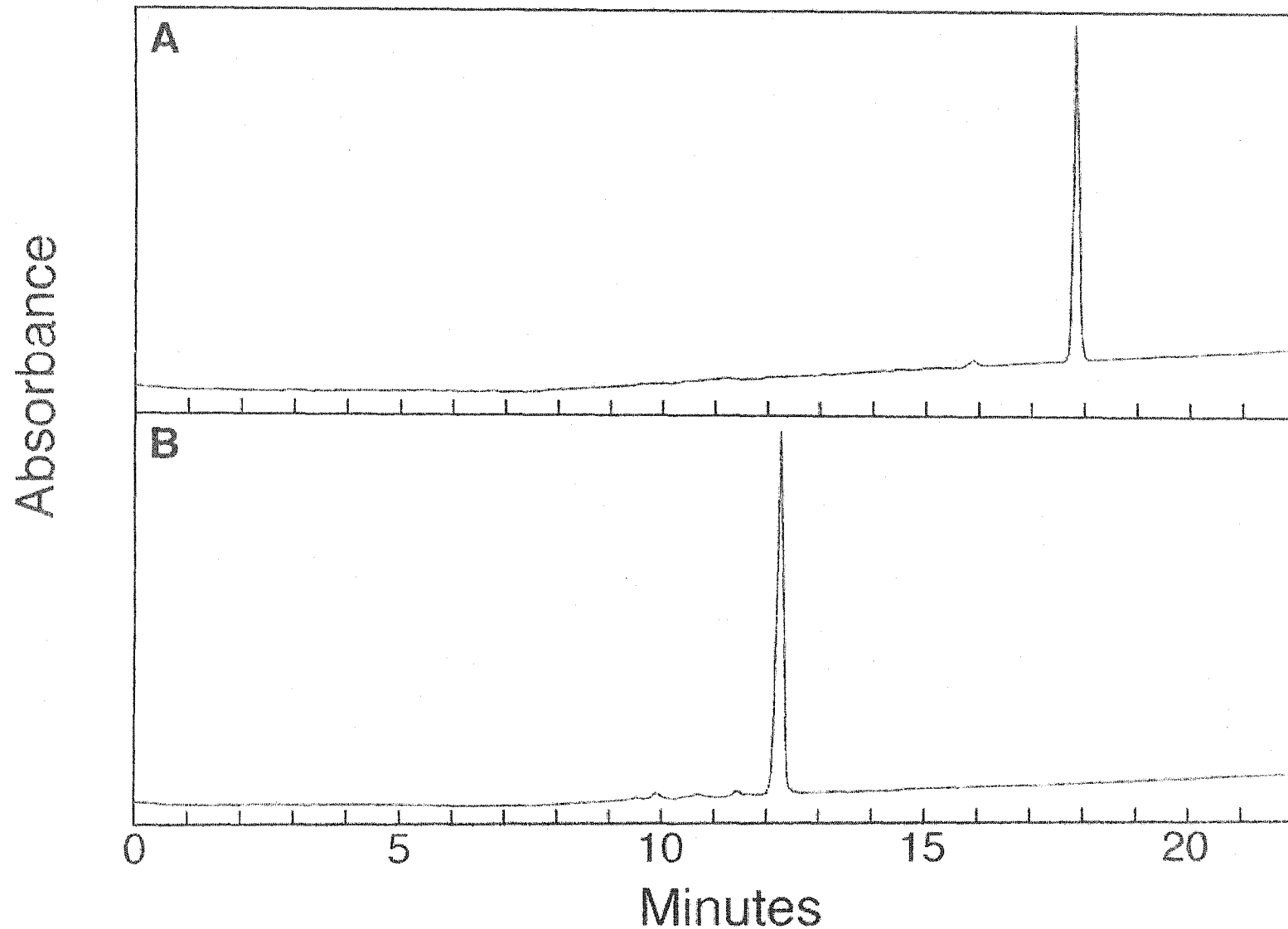


Figure 1. The uniformly-labeled ^{13}C - β -carotene (A) and ^{13}C -lutein (B) tracers were isolated in high purity from a biosynthetically-labeled algal biomass.

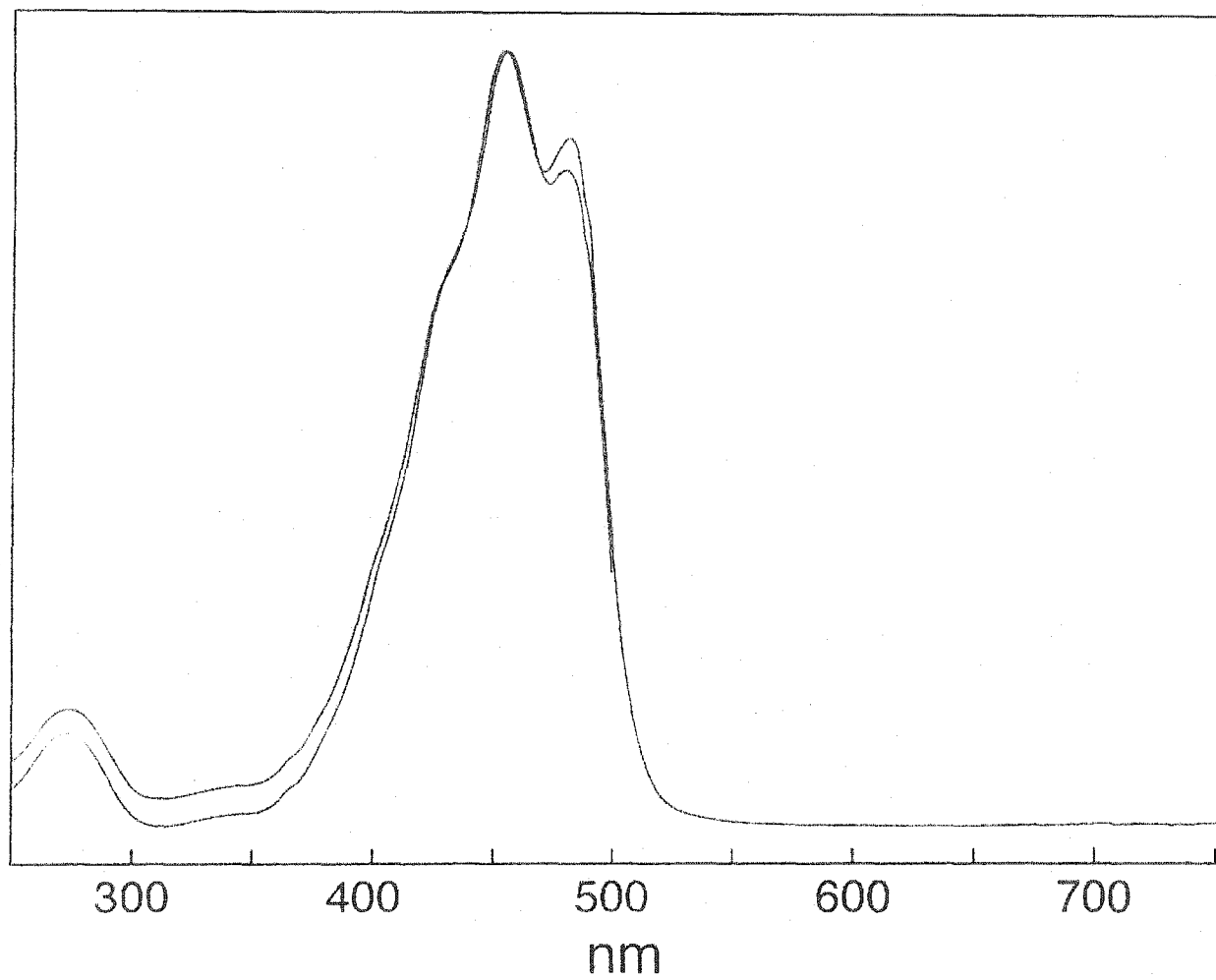


Figure 2. Near-identical ultraviolet/visible absorbance spectra of a commercial β -carotene standard (Sigma Scientific, St. Louis, MO) and the biosynthetically-labeled ^{13}C - β -carotene tracer.

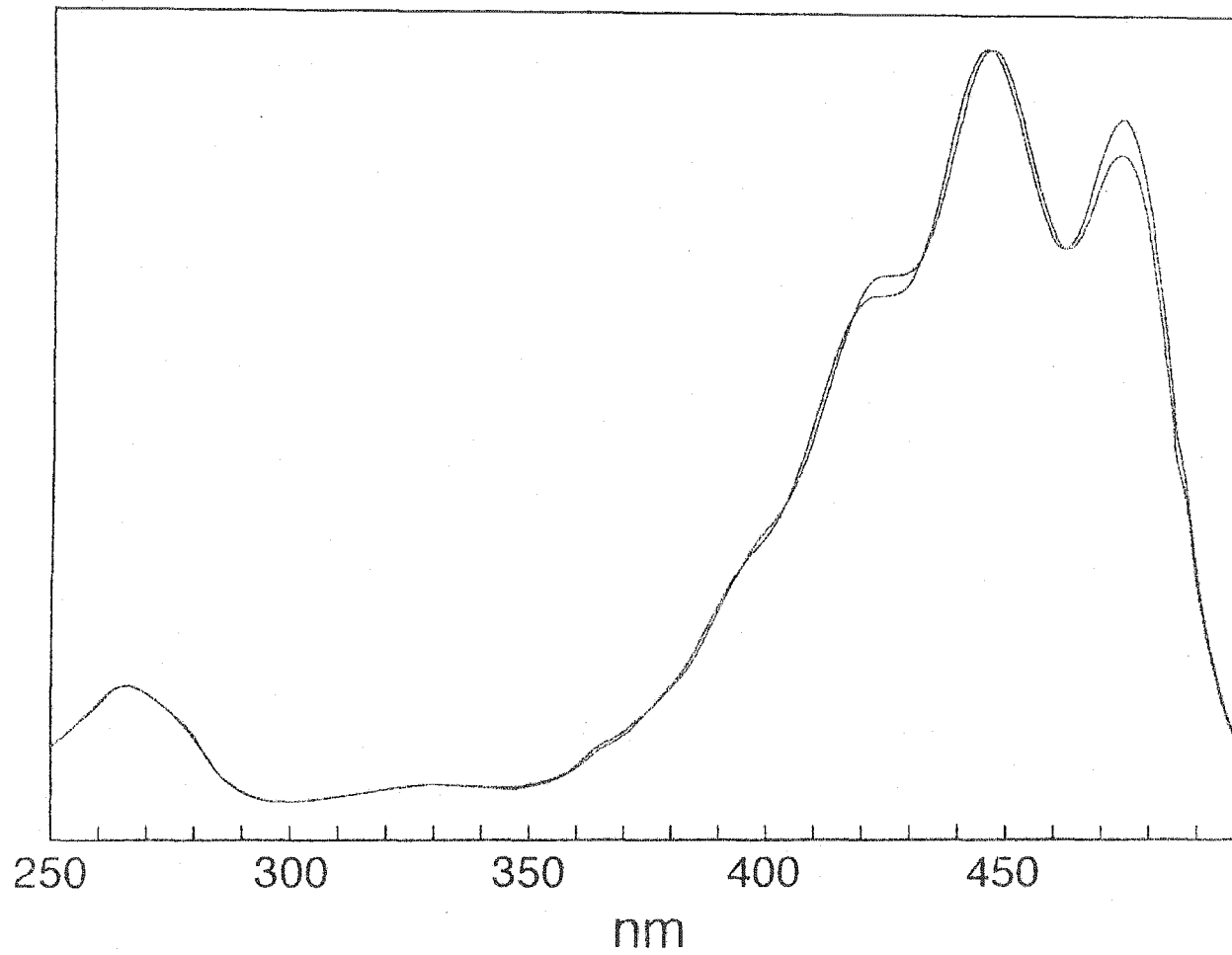


Figure 3. Near-identical ultraviolet/visible absorbance spectra of a commercial lutein standard (Kemin Industries, Des Moines, IA) and the biosynthetically-labeled ^{13}C -lutein tracer.

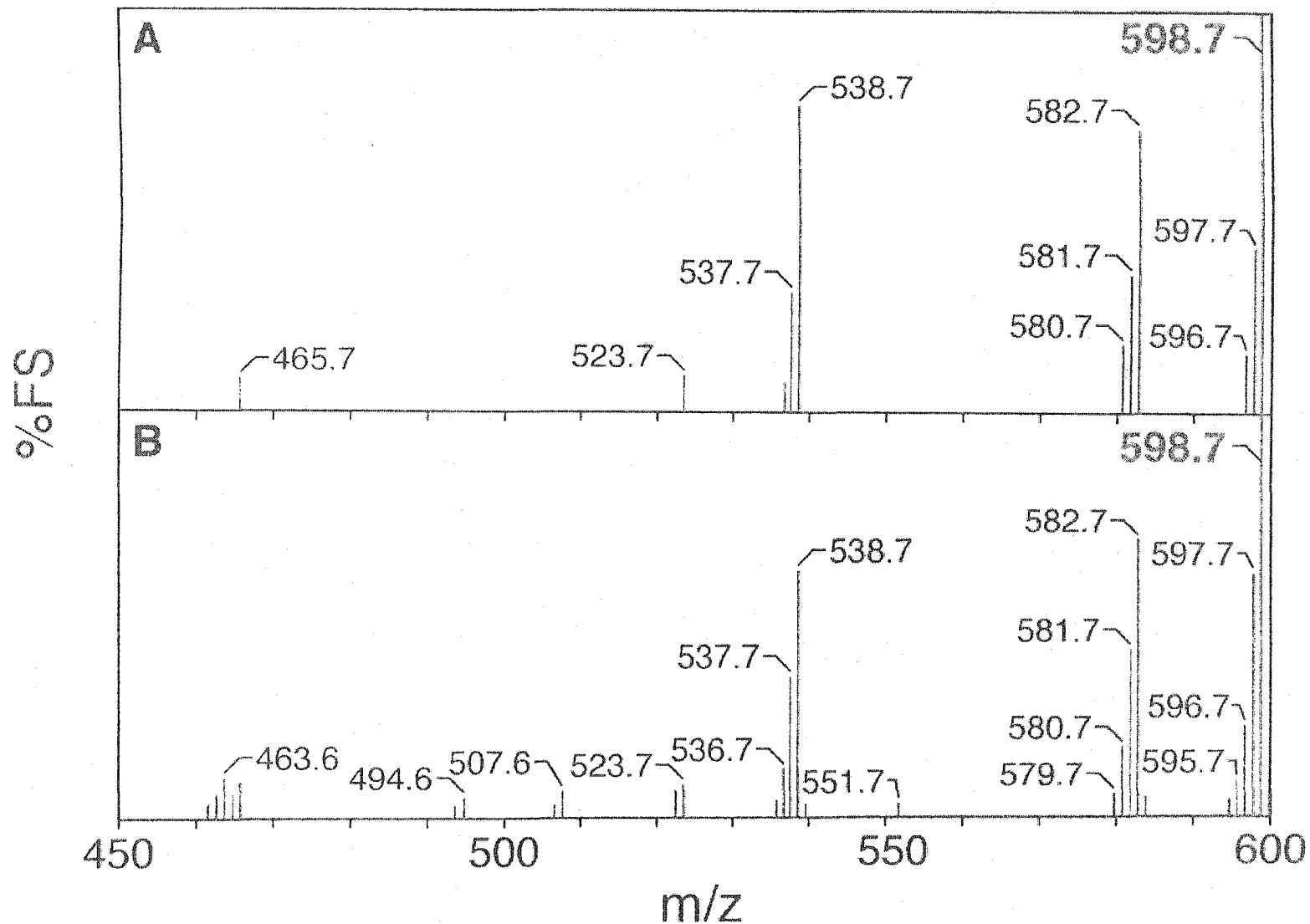


Figure 4. Electron ionization (70 eV) mass spectra of ^{13}C -perhydro- β -carotene, the thermally stable product of ^{13}C - β -carotene (A) and ^{13}C -lutein (B), showing the high ^{13}C -enrichment of the tracers.

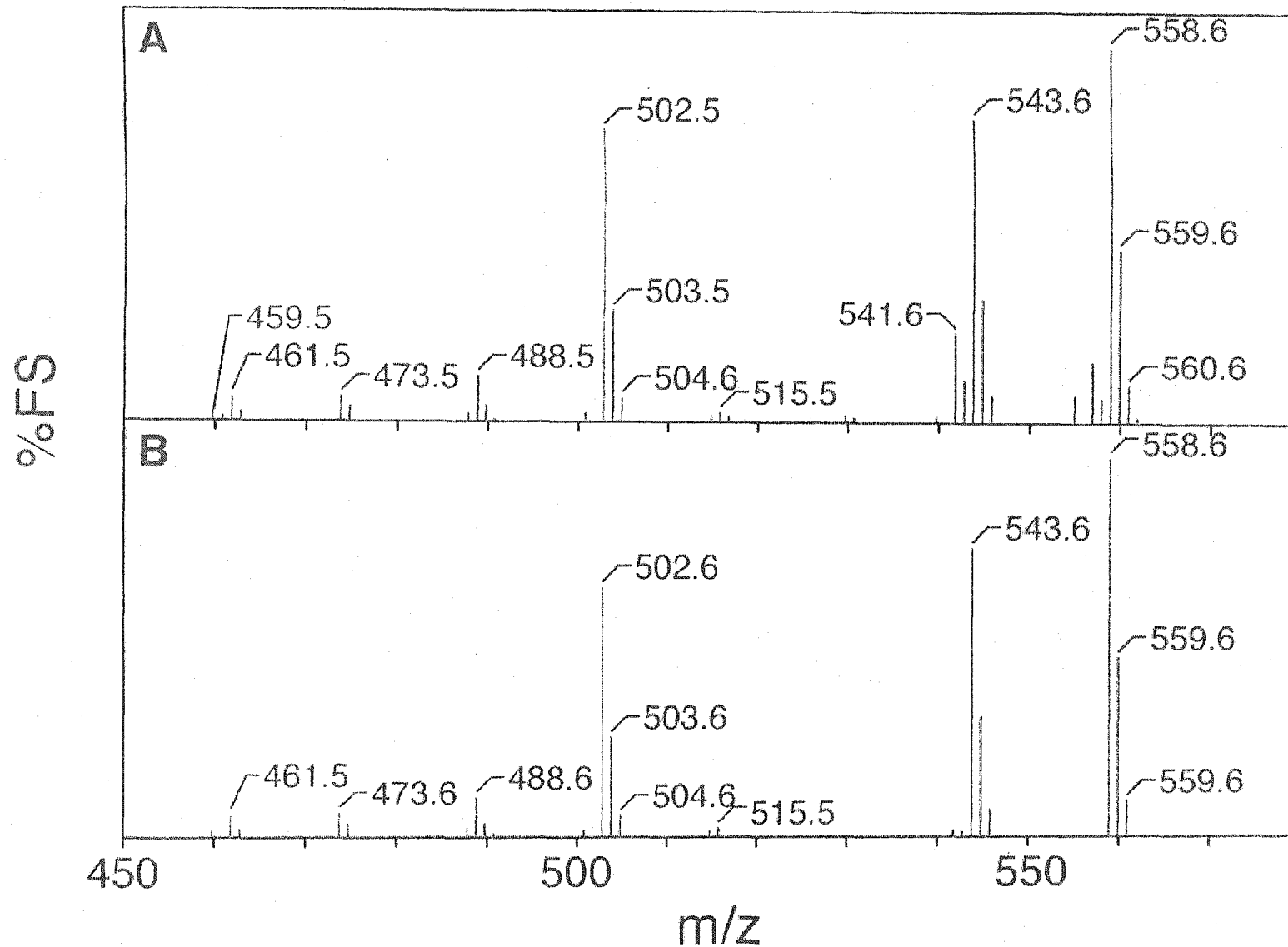


Figure 5. Electron ionization (70 eV) mass spectra showing similar fragmentation of the product of ^{13}C - β -carotene hydrogenation (A) and the major product of hydrogenolysis and hydrogenation of lutein (B).

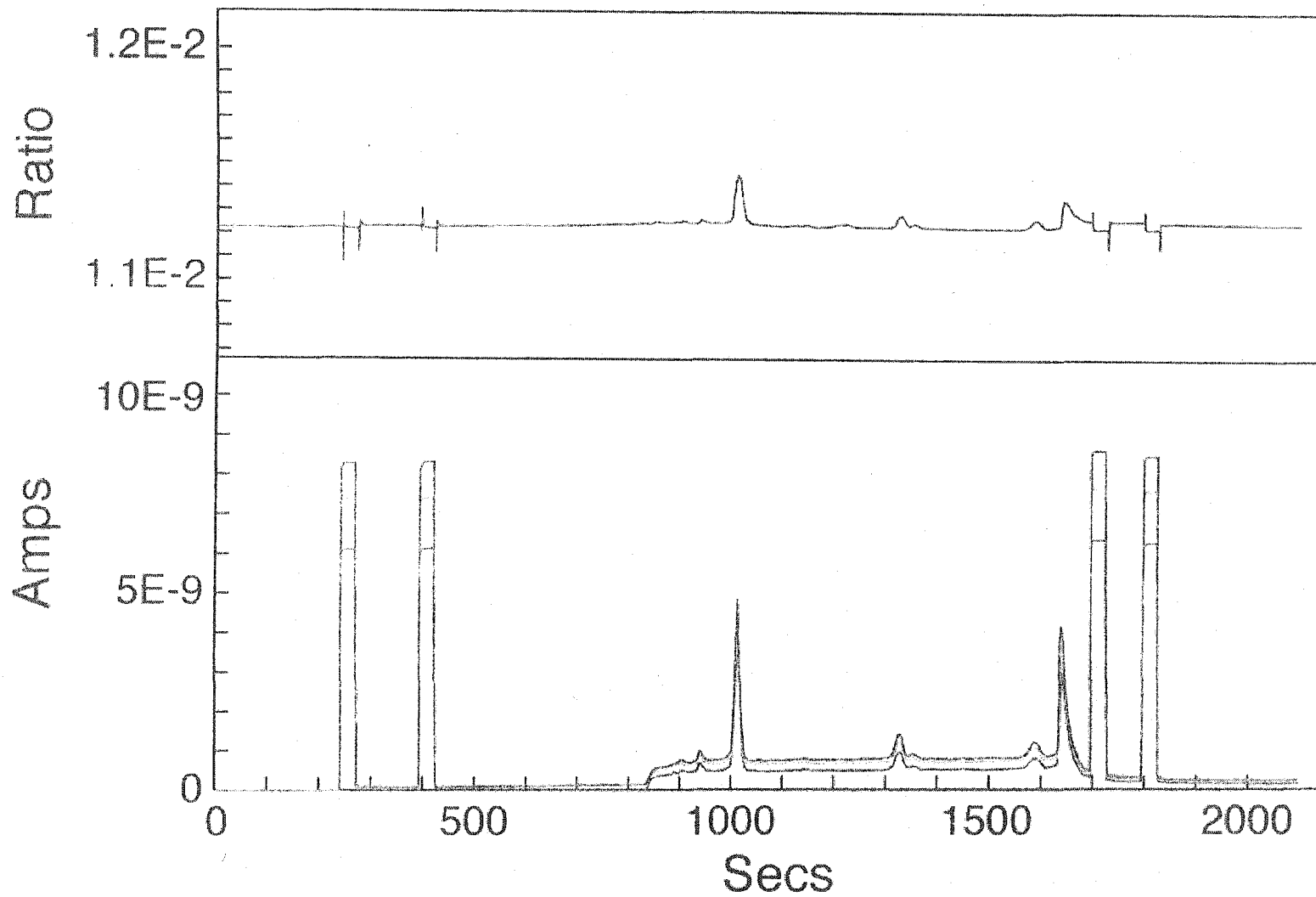


Figure 6. GC-C-IRMS chromatogram of the hydrogenated β -carotene fraction isolated from the plasma of a subject who ingested a combined dose of ^{13}C -lutein (3 mg) and ^{13}C - β -carotene (1 mg).

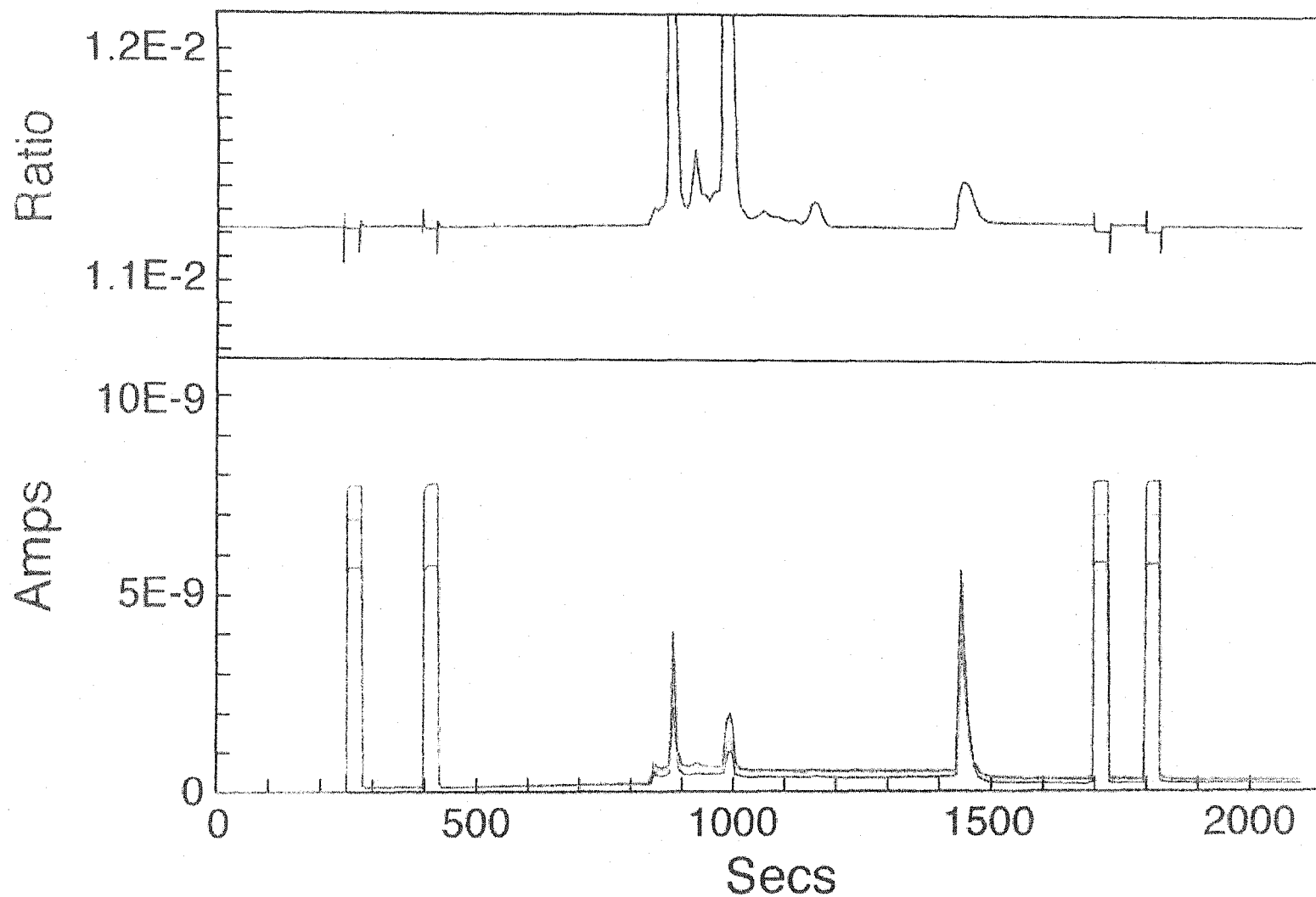


Figure 7. GC-C-IRMS chromatogram of the hydrogenated lutein fraction isolated from the plasma of a subject who ingested a combined dose of ^{13}C -lutein (3 mg) and ^{13}C - β -carotene (1 mg).

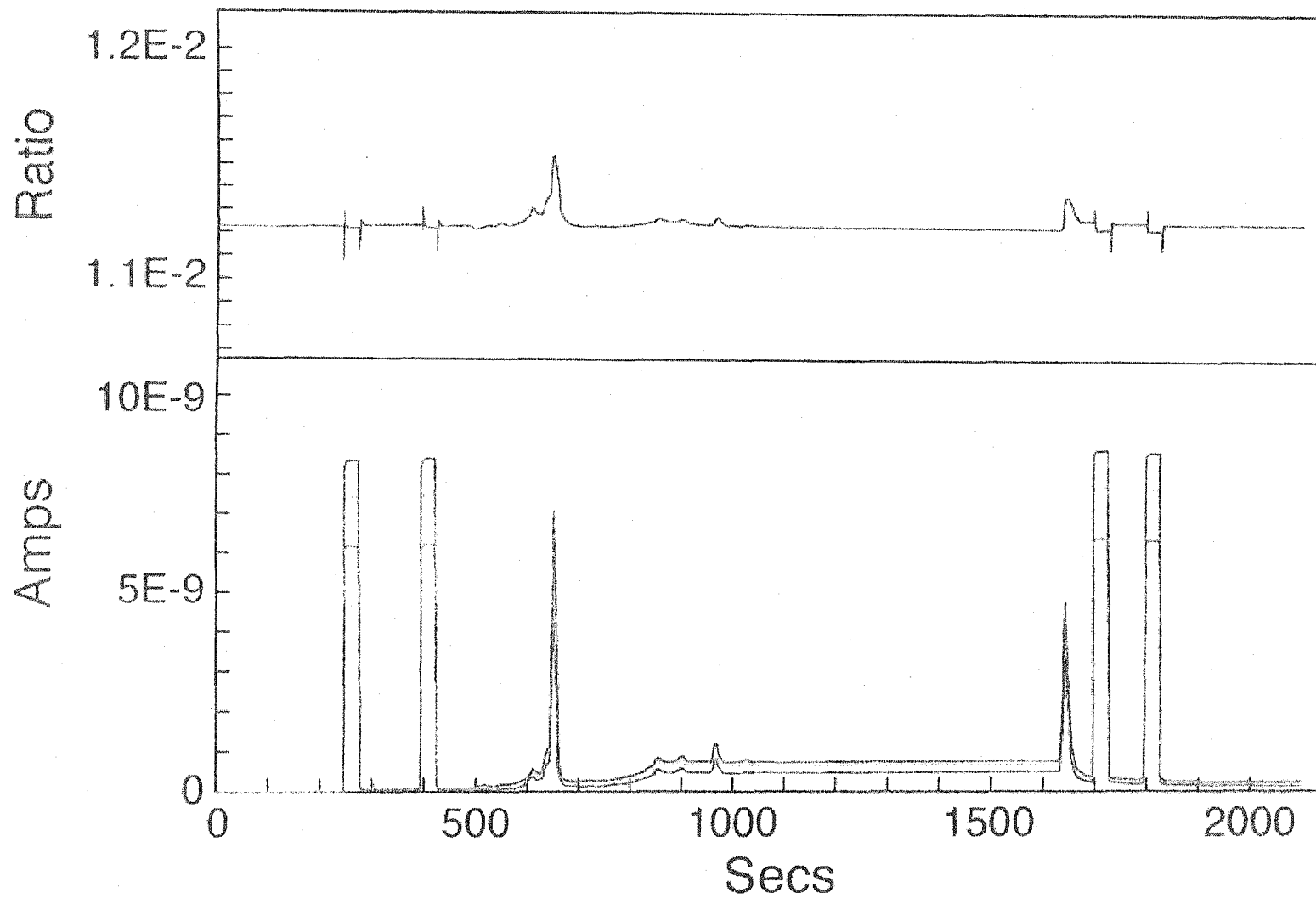


Figure 8. GC-C-IRMS chromatogram of the retinol fraction isolated from the plasma of a subject who ingested a combined dose of ^{13}C -lutein (3 mg) and ^{13}C - β -carotene (1 mg).

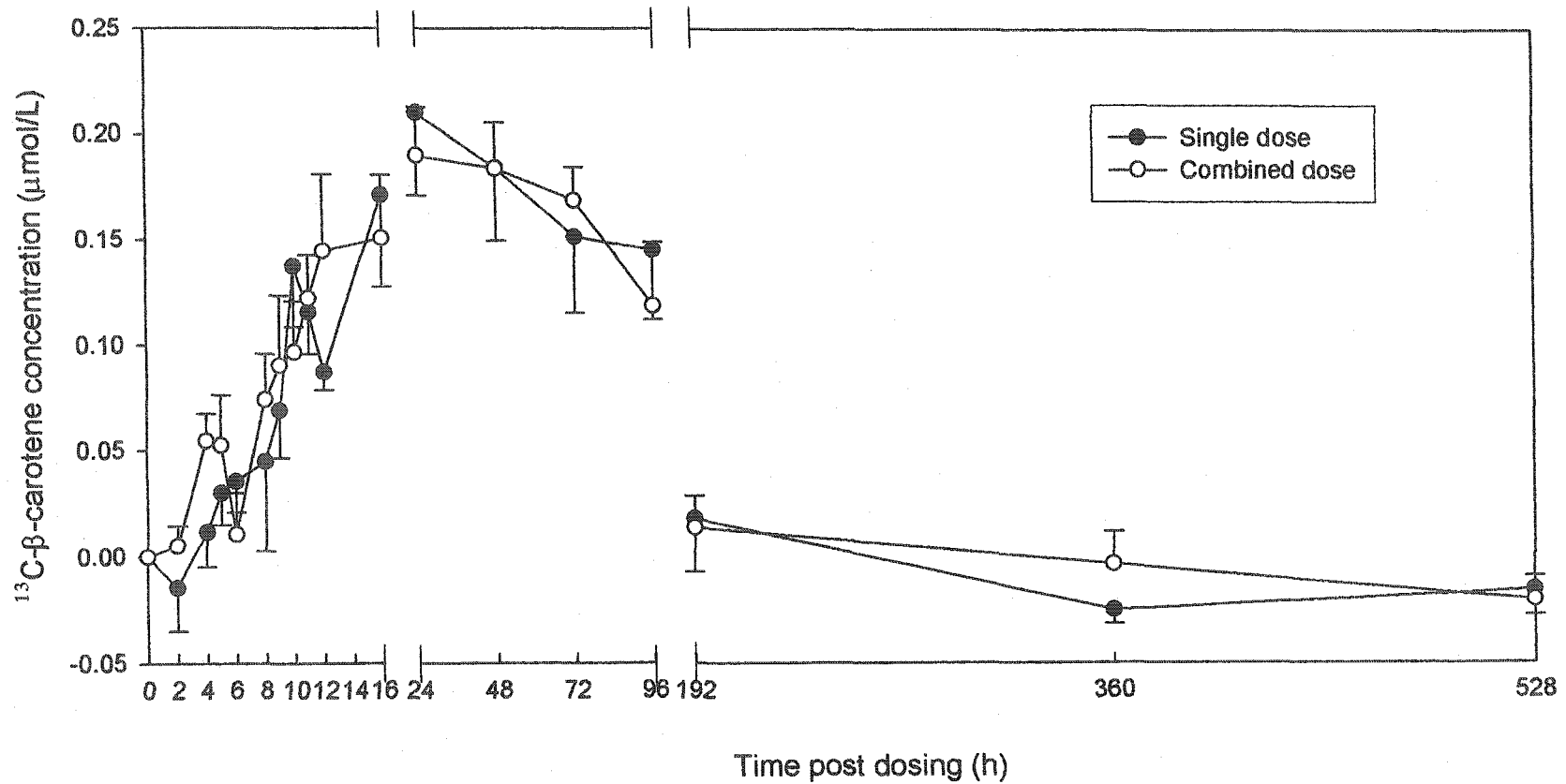


Figure 9. Appearance of ^{13}C - β -carotene in the plasma after subjects ingested 1 mg of ^{13}C - β -carotene with or without 3 mg of ^{13}C -lutein

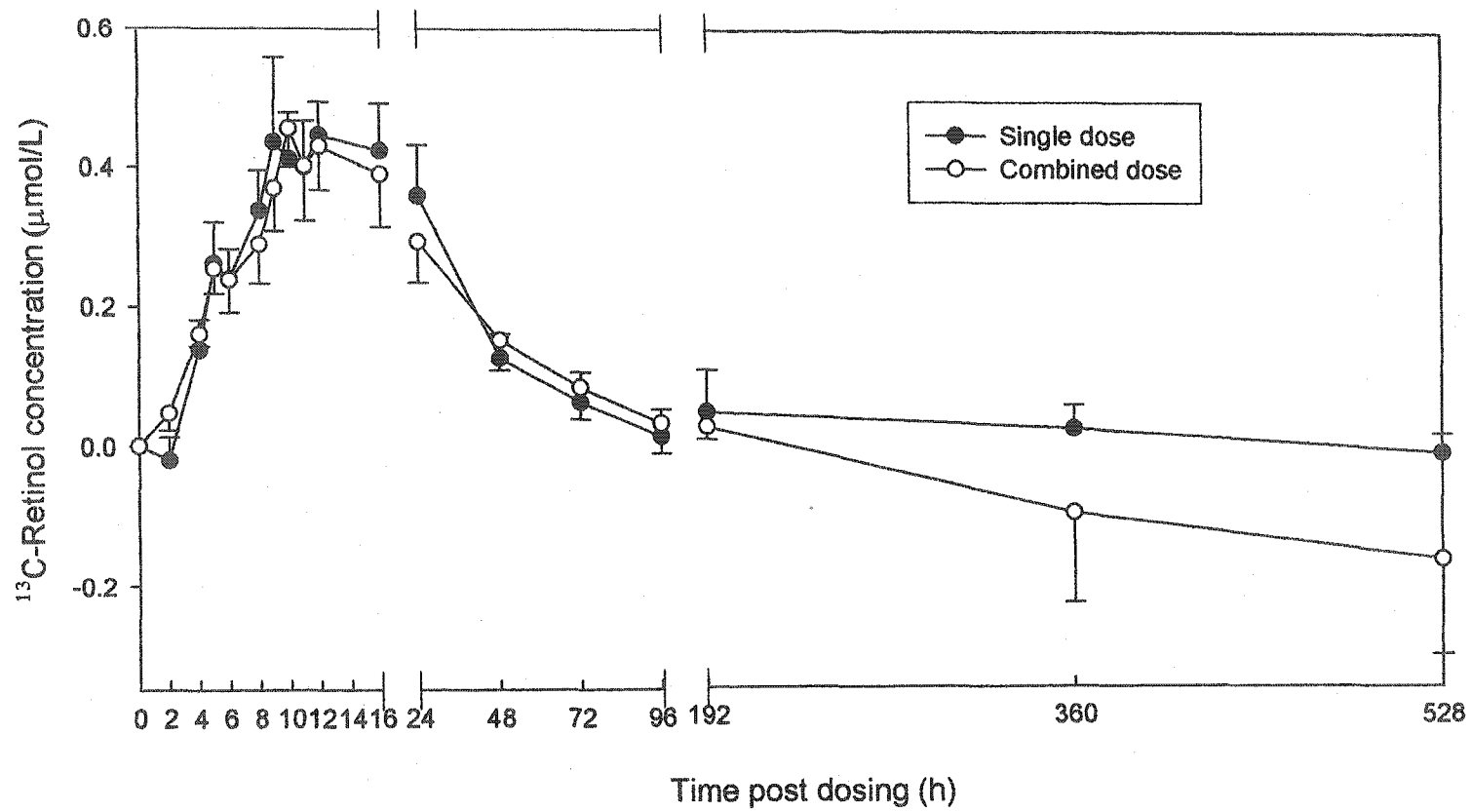


Figure 10. Appearance of ¹³C-retinol in the plasma after subjects ingested 1 mg of ¹³C-β-carotene with or without 3 mg of ¹³C-lutein

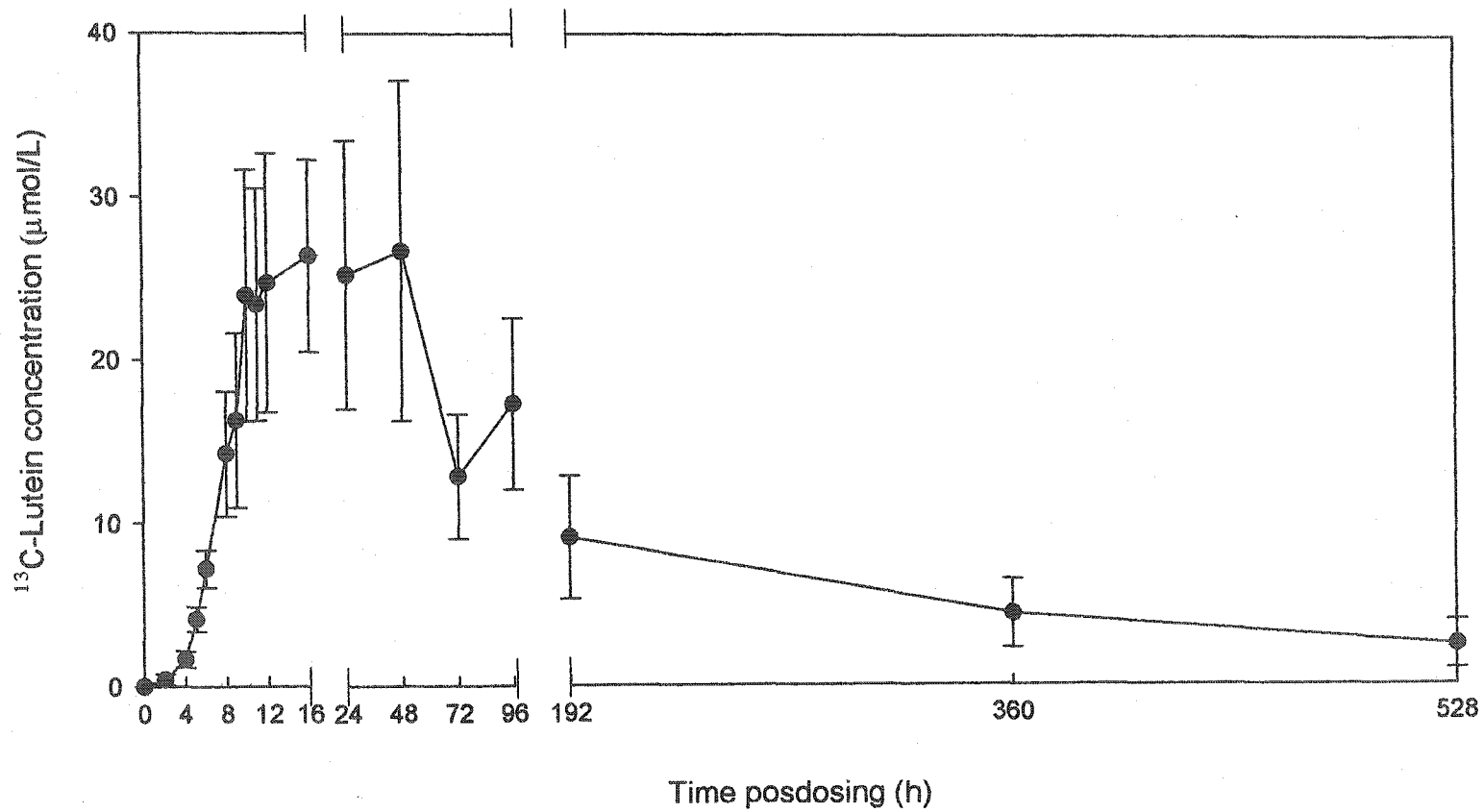


Figure 11. Appearance of ^{13}C -lutein in the plasma after subjects ingested 1 mg of ^{13}C - β -carotene and 3 mg of ^{13}C -lutein

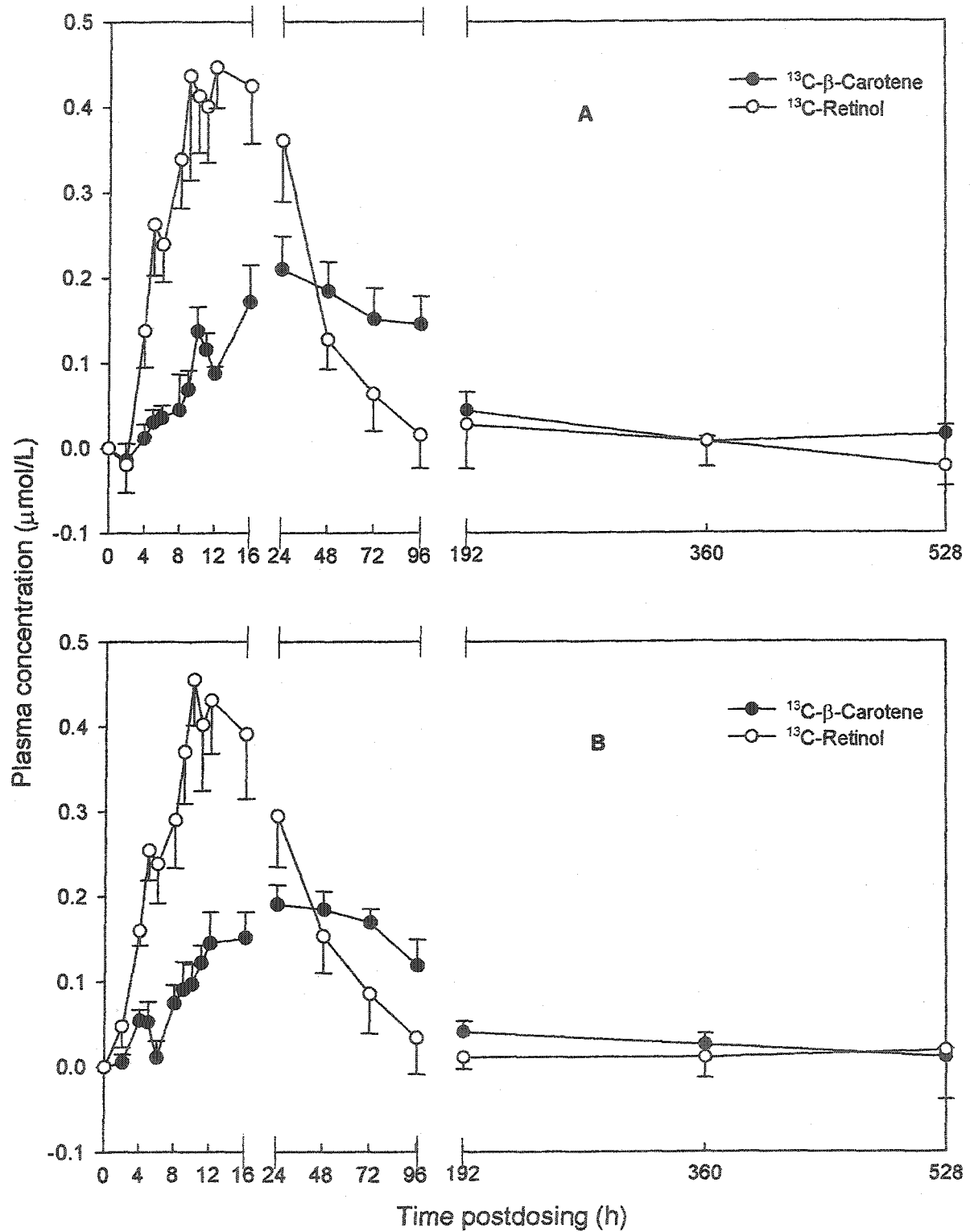


Figure 12. Comparison of the appearance of ^{13}C - β -carotene and ^{13}C -retinol in the plasma after subjects ingested (A) 1 mg of ^{13}C - β -carotene or (B) 1 mg of ^{13}C - β -carotene and 3 mg of ^{13}C -lutein (B)

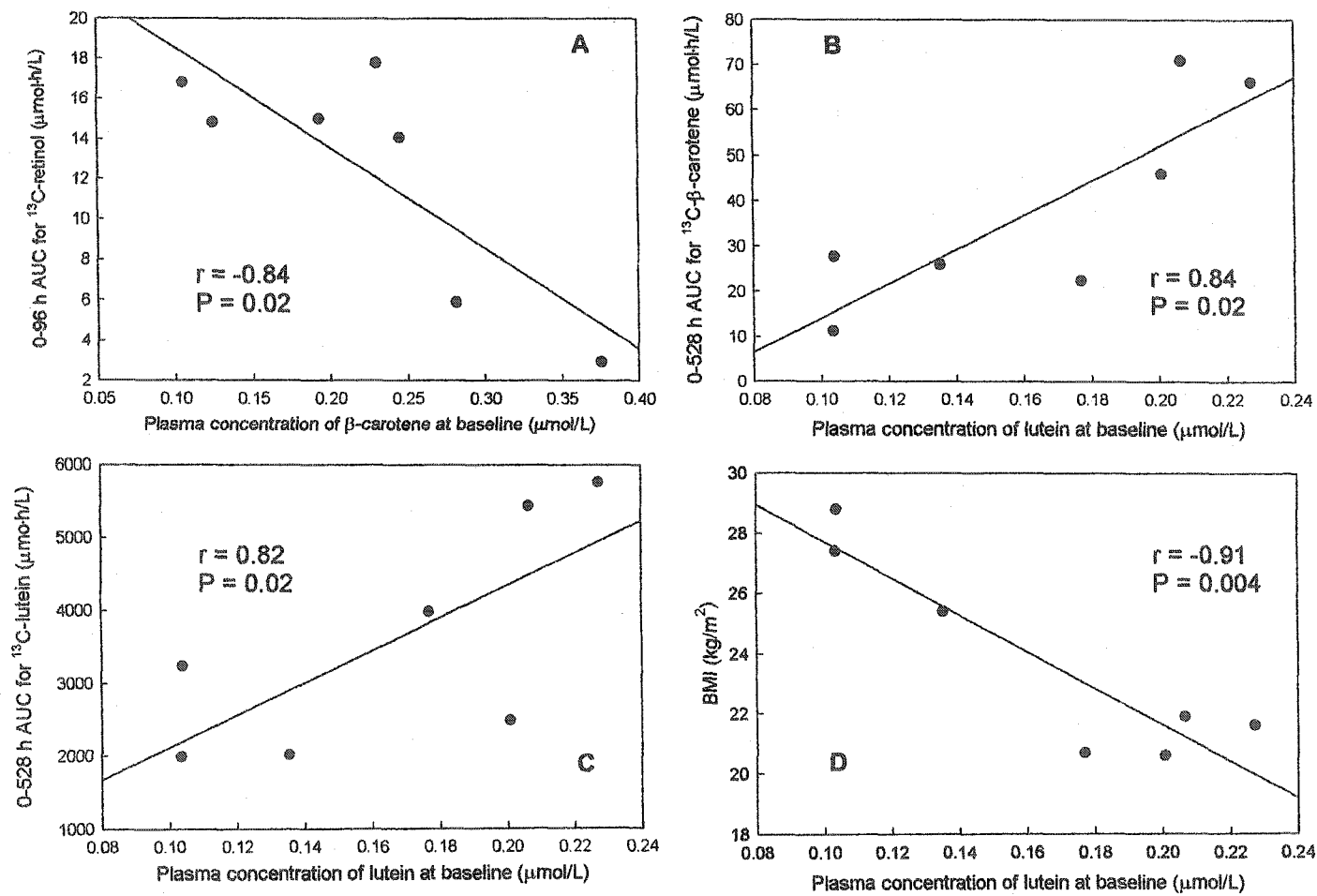


Figure 13. Correlation between plasma concentration of β -carotene at baseline and 0-96 h AUC for ^{13}C -retinol after the combined dose (A); correlation between plasma concentration of lutein at baseline and 0-528 h AUC for ^{13}C - β -carotene after the single dose (B); correlation of plasma lutein at baseline and 0-528 h AUC for ^{13}C -lutein (C); correlation of plasma concentration of lutein at baseline and BMI

GENERAL CONCLUSIONS

This study investigated in humans the effects of lutein on the bioavailability and bioconversion of β -carotene at physiological doses and at a lutein to β -carotene ratio typically found in dark-green leafy vegetables. The objectives were completed by using highly-enriched ^{13}C - β -carotene and ^{13}C -lutein tracers and highly-sensitive gas chromatography-combustion interfaced-isotope ratio mass spectrometry (GC-C-IRMS). In 7 young women, a 3-mg dose of lutein did not inhibit the bioavailability and bioconversion of 1 mg of concurrently ingested β -carotene. The responses to the β -carotene and lutein doses were highly variable among subjects. The results do not support the hypothesis that lutein is an inhibitory factor in the bioavailability and bioconversion of β -carotene at dose levels typically ingested from a single serving of dark green leafy vegetables. We had based our hypothesis on data from clinical studies, animal studies, and in vitro studies. Our results suggest caution must be taken when extrapolating animal and in vitro data to humans. Similarly, human studies that use pharmacological doses of carotenoids may not reflect the metabolism of physiological levels of carotenoids ingested in vegetables. It is important to develop methods that are not invasive so that they can be used in humans, and at the same time sensitive enough so that they can measure the responses to doses simulating dietary intakes. We and others have shown that highly enriched ^{13}C -labeled tracers and highly sensitive GC-C-IRMS are useful tools for achieving these research goals. Results from this study again raise the question as to the nature of the possible constitutive factor(s) responsible for the low bioavailability and bioconversion of β -carotene from dark green leafy vegetables. Does lutein interact with β -carotene differently when ingested in a food matrix as compared with the situation when both carotenoids are ingested in their pure forms? Could lutein have dual (both inhibitory and enhancing) or even multiple effects on bioavailability and bioconversion of β -carotene? Questions such as these still present challenges to the scientific community.

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