



Vitamin A equivalence of spirulina β -carotene in Chinese adults as assessed by using a stable-isotope reference method¹⁻⁴

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ABSTRACT

Background: Spirulina is a high-protein food supplement that contains carotenoids.

Objective: The objective of the study was to determine the vitamin A equivalence of spirulina β -carotene in humans.

Design: Spirulina was grown in a 23 atom% ²H₂O cultural solution. Spirulina β -carotene showed the greatest enrichment as [²H₁₀]trans β -carotene. Ten healthy Chinese men with a mean (\pm SD) serum retinol concentration of 1.7 ± 0.3 μ mol/L and a body mass index (in kg/m²) of 23 ± 3 consumed 5.8 μ mol [¹³C₁₀]retinyl acetate in oil as a reference dose with a breakfast containing 13 g fat. One week later, each subject consumed 7.9 μ mol trans β -carotene in spirulina with a breakfast containing 22 g fat. All subjects followed diets low in carotenoid and vitamin A. Forty blood samples were collected from each subject over a span of 56 d. Concentrations and enrichments of retinol and β -carotene in serum samples were determined by using HPLC and a mass spectrometer.

Results: Compared with the serum response to [¹³C₁₀]retinyl acetate dose, the mean conversion factor of spirulina β -carotene to retinol was 4.5 ± 1.6 (range: 2.3–6.9) by weight. It was estimated that 80% of the conversion occurred within the first 24 h after spirulina administration.

Conclusion: In a group of well-nourished, normal-weight Chinese men following low-vitamin A diets, 4.5 mg spirulina β -carotene consumed with 22 g fat has the same vitamin A activity as does 1 mg retinyl acetate. *Am J Clin Nutr* 2008;87:1730–7.

INTRODUCTION

Vitamin A is essential for life (1), and a severe deficiency of vitamin A causes blindness and mortality (2, 3). However, long-term excessive intake of preformed vitamin A can be harmful to humans by causing liver malfunction and birth defects (4). As observed in many studies, β -carotene is an effective (5) and safe (6, 7) source of vitamin A. High doses (180 mg/d) of β -carotene were given to humans for several months without any observed adverse effects other than changes in skin color (8). Increases in lung cancer risk were observed in long-term smokers who took a supplement of 20 mg β -carotene/d (9) and in asbestos workers who took a supplement of 30 mg β -carotene/d (10). However, a β -carotene supplement ≤ 25 mg/d was safe for nonsmokers (11). Dietary intake of β -carotene, usually ≤ 10 mg/d, can be a safe source of vitamin A (12–15). In many countries, plant foods rich in β -carotene are major sources of vitamin A. For example, in China, 59% of vitamin A comes from vegetables (16). However,

little has been known about the vitamin A activity of β -carotene in plant foods.

Vegetables intrinsically labeled with a stable isotope have been produced (17, 18), and a stable-isotope reference method was developed to calculate the vitamin A equivalence of β -carotene in vegetables (19). In the stable-isotope reference method (5), the subjects consumed a single dose or multiple doses of β -carotene and vitamin A labeled with stable isotopes. The labeled retinols from the doses were absorbed into the blood and diluted in the body pool of vitamin A. According to the molecular mass obtained by using a sensitive mass spectrometer, serum retinols derived from β -carotene, vitamin A, or endogenous unlabeled retinol were significantly different. To calculate the vitamin A equivalence of β -carotene, the amount of newly formed retinol from β -carotene was compared with that from vitamin A. One study using the stable-isotope reference method found that 20.9 mg spinach β -carotene or 14.8 mg carrot β -carotene could provide 1 mg retinol (20).

Spirulina is an alga that is rich in protein and carotenoids (mainly β -carotene) (21–23). One supplementation study in humans found that the incidence of a symptom of vitamin A deficiency, Bitot's spot, decreased from 80% to 10% after 1 g spirulina/d was provided for ≥ 150 d to 5000 preschool-age children in Madras, India (24). However, no data are available on a direct and quantitative assessment of the vitamin A value of spirulina β -carotene. In the present study, intrinsically deuterium-labeled

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spirulina was consumed by healthy Chinese men to evaluate the vitamin A equivalence of spirulina β -carotene by using a known amount of [$^{13}\text{C}_{10}$]retinyl acetate as a reference dose.

SUBJECTS AND METHODS

Subjects

Subjects were recruited from 3 villages on the outskirts of the city of Jining in the Shandong Province of China. The diets for this population were composed of cereal, vegetables, and a small amount of foods of animal origin. Study and recruitment information was advertised in the 3 villages, and men (aged 40–60 y, $n = 195$) voluntarily participated in the prescreening process.

Ten healthy men with normal but relatively low serum retinol concentrations were selected from the 195 volunteers and enrolled in the study. All subjects were nonsmokers or had stopped smoking >2 mo before the study, and they had not taken vitamins or other supplements in the previous month. No smoking, alcohol, supplements, or medicines were permitted during the preparation period and the study. All subjects were negative for *Helicobacter pylori* infection and hepatitis B surface antigen, had normal liver function, and had no metabolic disease or cardiovascular disease.

Written informed consent was obtained from all volunteers under the guidelines established by the Institutional Review Board (IRB) of Tufts-New England Medical Center, and by the Institute of Nutrition and Food Hygiene Ethical Review Committee of the Chinese Academy of Preventative Medicine.

Study design

The study procedure is shown in **Figure 1**. Two weeks before the study, the subjects were instructed to eat their usual diet at home but to avoid nutritional supplements and foods rich in β -carotene or vitamin A, such as spinach, carrots, green or red pepper, or liver products. Each subject kept a 3-d food record (2 weekdays and one weekend day) at the beginning of the study. After that 2-wk period, volunteers were housed for 10 d in a metabolic unit established in Jining. Meals low in carotenoids or vitamin A were served.

On day 1, after a fasting blood sample was drawn (0 h), and each subject was given 2 mg (5.8 μmol , corrected by 98% purity) vitamin A ([$^{13}\text{C}_{10}$]retinyl acetate) in an oil capsule (0.2 g corn oil)

with a carotene- and retinol-free liquid drink containing 10 g fat. The subjects consumed a vitamin A-free breakfast (3 g fat and 17 g protein with a total energy of 445 kcal) 1 h after dosing. Lunch and dinner (26% of energy from fat) were consumed 5 and 10 h after dosing, respectively. On day 8, each subject ate 5 g spirulina (powder in capsules) with the same liquid drink as on day 1. The subjects ate breakfast (12 g fat and 20 g protein with a total energy of 730 kcal) 1 h after dosing. The additional 9 g fat, over the amount of the breakfast on day 1, would help dissolve β -carotene, enhance intestinal uptake, and eventually benefit the conversion of β -carotene to retinol. Lunch and dinner (23% energy from fat) were served on the same time schedule as on day 1. In total, 71 and 70 g fat were consumed on days 1 and 8, respectively. For the first 10 d of the study, while the subjects were housed in the research unit, the diets they consumed contained 240 μg carotene/d and 154 μg vitamin A/d, calculated according to Chinese food composition tables (25).

Blood samples (10 mL) were collected from all subjects at 0 (fasting blood), 5, 6, 7, 8, 9, 11, and 13 h on days 1 and 8. Blood was drawn at 0, 5, and 11 h on days 2 and 9 (*see* Figure 1) and at 0 and 11 h on days 3 and 10. Fasting blood samples (10 mL) were obtained on days 4, 5, 6, 7, 11, 13, 15, 19, 23, 27, 32, 39, 46, and 56. Blood samples were covered by aluminum foil to protect them from light, kept in a 37 °C water bath for 30 min, and then centrifuged at 4 °C and 800 $\times g$ for 15 min. Serum was stored at -20 °C for ≤ 10 d and then packed with dry ice and transported to the Institute for Nutrition and Food Safety (Beijing, China), where it was stored at -70 °C. All frozen samples, on dry ice, were sent by air to the Carotenoids and Health Laboratory of the US Department of Agriculture (USDA)–Human Nutrition Research Center on Aging (HNRCA) at Tufts University (Boston, MA) for analysis.

The subjects were free-living from day 11 to day 56. Dietary instructions (a list of vegetables, fruit, and meats that could be eaten) were given to the subjects to avoid large intakes of vitamin A and β -carotene. Dietary record forms with directions were taken home and completed by all subjects for 3 d of each week.

Supplements

[$^{13}\text{C}_{10}$]retinyl acetate (8, 9, 10, 11, 12, 13, 14, 15, 19, 20- $^{13}\text{C}_{10}$ -retinyl acetate) with a chemical purity of 98% and an isotopic purity of >99% was purchased from Cambridge Isotope Laboratory (Andover, MA). Spirulina was grown hydroponically at the USDA-Agricultural Research Service Children's Nutrition Research Center in Houston, TX, by using 23 atom% deuterium oxide in the nutrient media. Algal cells (*Spirulina platensis*), obtained from the University of Texas Culture Collection of Algae (UTEX No. LB 2340; Austin, TX), were inoculated into 20-L glass carboys and were maintained in a greenhouse with the use of a mixture of sunlight and metal-halide lamps. Cells were grown in a sterilized artificial-seawater nutrient medium (26) enriched with deuterium oxide (23 atom%). Cell cultures were stirred at low speed to keep cells suspended. Individual cultures were grown for 10–12 d before being harvested, at which time cells were allowed to settle, were rinsed with distilled water, and were centrifuged at low speed to pellet the cells. Pelleted cells were freeze-dried and stored at -80 °C until they were shipped on dry ice to the USDA-HNRCA at Tufts University. The pelleted material was powdered, put in gelatin capsules, and

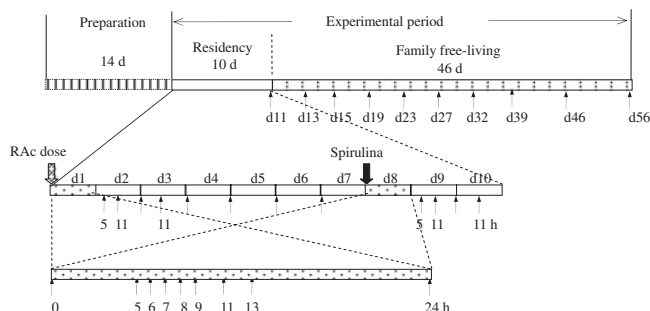


FIGURE 1. Experiment scheme of dosing and time for blood sample collection. Ten healthy Chinese men consumed 2 mg [$^{13}\text{C}_{10}$]retinyl acetate (RAC) as a reference dose and 5 g spirulina containing 4.3 mg [$^2\text{H}_{10}$] β -carotene on day 1 and day 8, respectively. \uparrow , Blood sample collection; d, day; h, hour of the day.



weighed. All supplements were subsequently kept at -80°C until they were given to volunteers or analyzed.

HPLC for retinol and carotenoid concentrations in baseline serum and in spirulina

HPLC (Waters 2695 separation module and 2996 photodiode array detector wavelength 250–600 nm; Waters Corporation, Milford, MA) and a Pronto SIL C₃₀ column (Bischoff Chromatography, Leonberg, Germany) were used to analyze retinol and carotenoid concentrations in baseline serum and spirulina. HPLC mobile phase included solvent A [methanol:*tert*-butyl methyl ether:water = 85:12:3 (by vol)] and solvent B (methanol:*tert*-butyl methyl ether:water = 8:90:2 by vol), both of which include 1.5% ammonium acetate in water (27).

To extract retinol and carotenoids from serum, 100 μL serum, 100 μL internal standards (retinyl acetate + echinenone), 500 μL saline (0.85%), and 4 mL of a ratio of chloroform to methanol [(C:M) 2:1, by vol] were added to a glass tube (16 \times 100-mm), mixed by using a vortex mixer (Labnet International Inc, Woodbridge, NJ) for 1 min, and then centrifuged at 4°C and $800 \times g$ for 10 min. The lower layer (chloroform layer) was collected into another glass tube (13 \times 100-mm) by using a glass pipette. Hexane (3 mL) was added to the aqueous layer, mixed by vortex, and centrifuged again. The hexane layer and the chloroform layer were combined and dried under N_2 on an N-EVAP nitrogen evaporator (Organomation Associates Inc, South Berlin, MA). The residue was dissolved in 100 μL of a mixture of ethanol and tetrahydrofuran at a ratio of 2:1 (by vol), mixed and sonicated for 20 s, and then transferred to a vial. The dissolved residual solution (20 μL) was injected into the HPLC. A linearly gradient HPLC pump-flow method began with 100% solvent A (A), changed to 45% A and 55% solvent B (B) in 21 min and held for 1 min, changed to 5% A and 95% B in 11 min and held for 4 min, and then changed to 100% A in 2 min and held for 10 min to equilibrate the system before injection of the next sample. The HPLC pump flow was set at 0.4 mL/min.

To analyze carotenoids in spirulina, 100 mg spirulina powder was weighed and put into a 50-mL glass vial, and then 3 mL methanol was added to the vial. Spirulina in the vial was homogenized by using a Polytron (PT1600E; Kinematica AG, Lucerne, Switzerland) at speed 10 for 30 s in an ice bath, and then it was washed with 5 mL methanol. The vial containing the mixture was stored at 4°C for 12 h. Then, the vial was mixed by vortex for 30 s and centrifuged at 4°C and $800 \times g$ for 5 min. The methanol layer was transferred to a 50-mL volumetric flask by using a pipette gun. Tetrahydrofuran was used to extract carotenoids 4 times (10 mL tetrahydrofuran for the first 3 times, and 5 mL for the 4th time); vortex mixing and centrifuging were involved each time. The tetrahydrofuran layers were collected into the methanol extract-containing volumetric flask, to which tetrahydrofuran was added to a volume of 50 mL and mixed well. The extract (1 mL) was evaporated under the N_2 flow of the N-EVAP (27). The residue was resuspended in 1 mL ethanol, and 20 μL was injected into the HPLC. Triplicate samples were analyzed.

A linearly gradient procedure started from 60% solvent A and 40% solvent B for 2 min, then changed to 17% A and 83% B in 7 min and held for 7 min, changed to 100% B in 6 min and held for 6 min, and changed back to 60% A and 40% B in 2 min. The HPLC pump flow was set at 0.4 mL/min. Retinol and carotenoid

concentrations were calculated according to the standard curves of *all-trans* retinol, α -carotene, *trans* β -carotene, 9-*cis* and 13-*cis* β -carotene (all: Sigma-Aldrich Inc, Saint Louis, MO) and lutein, zeaxanthin, and β -cryptoxanthin (all: Roche Vitamin Inc, Belvidere, NJ).

EMPOWER PRO software (version 2.0; Waters Corporation) was used to control the HPLC system and data analysis. Carotenoids and retinol were detected at 450 and 340 nm, respectively. The standard response curve of retinol was set at 340 nm under the mobile phase solvent composition of the HPLC system. The amount of retinol injected into the HPLC system was measured by using the spectrophotometer at 325 nm with the use of ethanol as solvent.

HPLC for collecting retinol fraction from serum

To collect retinol from serum, 200 μL serum was used; the same procedures as for baseline serum analysis were followed. The serum extract was dissolved in 75 μL of a ratio of ethanol to tetrahydrofuran [(E:T) 2:1 by vol], mixed and sonicated for 20 s, and then transferred to a vial. Sixty μL was injected into the HPLC.

A Waters HPLC (616 pump, 717 Plus Autosampler, and 996 photodiode array detector; Waters Corporation) with a Pecosphere C₁₈ column (PerkinElmer Life and Analytic Sciences, Shelton, CT) and an FC203 collector (Gilson Inc, Middleton, WI) were used to analyze the concentration and to collect retinol fraction from serum samples. The HPLC mobile phase included solvent A [methanol:tetrahydrofuran:water (M:T:W) = 50:20:30 by vol] and solvent B (M:T:W = 50:44:6 by vol) with 1.5% ammonium acetate in water. A linearly gradient solvent procedure started at 100% solvent A for 6 min, changed to 60% A and 40% B in 5 min, changed to 17% A and 83% B in 11 min, and changed to 100% B in 10 min. Finally, the gradient went back to 100% A in 2 min and held for 8 min to stabilize the system before the next sample was injected. The column temperature was 12°C , and the flow rate was 1 mL/min. Retinol was eluted at 9 min, so the eluant fluid from 8–10 min was collected into a 2-mL flask (Pyrex Labware; Corning Inc, Corning, NY).

Gas chromatography–electron capture negative chemical ionization–mass spectrometer for the enrichment of labeled retinol in serum

The retinol fraction from HPLC was dried under N_2 . We added 50 μL *N,O*-bis(trimethylsilyl) trifluoroacetamide (1%, BSTFA-TMCS; Pierce, Rockford, IL) to the residue, capped the flask with a stopper to avoid moisture, and it put into an oven at 100°C for 60 min. Then the flask was cooled to room temperature. The derivatized retinol (2–4 μL) was injected into a gas chromatography–electron capture negative chemical ionization–mass spectrometer (GC-ECNCI-MS, Agilent 6890 Series GC System and 5973 Network Mass Selective Detector; Agilent Technologies, Andover, MA). A Zebtron ZB-1ms Capillary GC column (Phenomenex Inc, Torrance, CA) was used to separate compounds. Helium and methane were used as the carrier gas and the reaction gas, respectively. The GC oven temperature was increased from 50°C to 220°C at a speed of $15^{\circ}\text{C}/\text{min}$, to 230°C at $5^{\circ}\text{C}/\text{min}$, and to 310°C at $20^{\circ}\text{C}/\text{min}$; it remained at 310°C for 5 min. This method produced a *trans* retinol peak at ≈ 14 min. The mass spectrometer was set to scan at a mass-to-charge ratio



(m/z) of 260 to 280. Retinol molecular mass was equal to 286, and lost one group of H_2O during the negative chemical ionization process, which made the major fragment of retinol at m/z 268 (19).

The enrichment of labeled retinol was calculated by using the following 2 equations:

$$\begin{aligned} & \text{Enrichment of } [^2H_5]\text{retinol from spirulina} \\ & = (\sum \text{ areas of } m/z \text{ 271–276} / \sum \text{ areas of } m/z \text{ 268–280}) \times 100 \quad (1) \end{aligned}$$

and

$$\begin{aligned} & \text{Enrichment of } [^{13}C_{10}]\text{retinol from } [^{13}C_{10}]\text{retinyl acetate} \\ & = (\sum \text{ areas of } m/z \text{ 276–280} / \sum \text{ areas of } m/z \text{ 268–280}) \times 100 \quad (2) \end{aligned}$$

Total body stores of vitamin A (in mmol retinol) were calculated as $0.00468 \times 10^{37(\text{isotope dose in mmol})} / [^{13}C]\text{retinol} : [^2H]\text{retinol}$ in serum 3 d after the isotope dose (28). $[^{13}C]\text{retinol} : [^2H]\text{retinol}$ instead of $[^2H]\text{retinol} : \text{unlabeled retinol}$ was used in the present study, assuming that retinol labeled by ^{13}C followed the same kinetics as did deuterated retinol.

The area under the curve (AUC) of labeled retinol was calculated by using INTEGRAL-CURVE of KALEIDAGRAPH software (version 3.5; Synergy Software, Reading, PA) using total serum response (nmol, as y axis) versus time (d, as x axis). The calculation is shown in the following equation:

$$\begin{aligned} & \text{Labeled retinol from spirulina } \beta\text{-carotene or} \\ & [^{13}C_{10}]\text{retinyl acetate} = \text{enrichment} \times \text{retinol concentration} \\ & \quad \times \text{body weight} \times 0.0435 \quad (3) \end{aligned}$$

where $\text{body wt} \times 0.0435 = \text{total-body serum volume}$.

Vitamin A equivalence was calculated by using the following equations:

$$\begin{aligned} & [^2H_5]\text{retinol from spirulina } [^2H_{10}]\beta\text{-carotene (nmol)} \\ & = (\text{AUC of } [^2H_5]\text{retinol} / \text{AUC of } [^{13}C_{10}]\text{retinol}) \times 5790 \quad (4) \end{aligned}$$

where 5790 is the reference dose (in nmol); and

$$\begin{aligned} & \text{Conversion factor of } \beta\text{-carotene to retinol (by wt)} \\ & = \beta\text{-carotene dose in spirulina (nmol)} \times (536.5 + 10) / \\ & [^2H_5]\text{retinol from the } \beta\text{-carotene dose (nmol)} \times (286 + 5) \quad (5) \end{aligned}$$

Postabsorptive conversion of β -carotene to retinol

$[^2H_5]\text{retinol}$ from spirulina $[^2H_{10}]\beta\text{-carotene}$ was assumed to follow the same serum kinetics as does $[^{13}C_{10}]\text{retinol}$ from the $[^{13}C_{10}]\text{retinyl acetate}$ dose, on the assumption that all $[^2H_5]\text{retinol}$ was formed in the intestine only. The time span of 24 h was chosen as the cutoff for the intestinal conversion and postabsorptive conversion. If no further conversion occurred, the ratio of $[^2H_5]\text{retinol}$ to $[^{13}C_{10}]\text{retinol}$ during the study should be as consistent as the ratio on day 1. The conversion calculations were obtained by using the following equations:

$$\begin{aligned} & \text{Calculated AUC of } [^2H_5]\text{retinol over } n \text{ d} \\ & = \text{AUC of } [^2H_5]\text{retinol over 1 d} / \text{AUC of } [^{13}C_{10}]\text{retinol} \\ & \quad \text{over 1 d} \times \text{AUC of } [^{13}C_{10}]\text{retinol over } n \text{ d} \quad (6) \end{aligned}$$

and

$$\begin{aligned} & \text{Percentage } [^2H_5]\text{retinol from postabsorptive conversion} \\ & = [(\text{AUC of } [^2H_5]\text{retinol over } n \text{ d} - \text{calculated AUC of} \\ & \quad [^2H_5]\text{retinol over } n \text{ d}) / \text{AUC of } [^2H_5]\text{retinol over } n \text{ d}] \\ & \quad \times 100 \quad (7) \end{aligned}$$

in both of which equations $n \text{ d} = 3, 7, 15, 24, 31, 38, \text{ or } 48 \text{ d}$.

Liquid chromatography–atmospheric pressure chemical ionization–mass spectrometer for β -carotene in spirulina and serum

To determine the percentage enrichment of labeled β -carotene, the β -carotene fraction was extracted and collected from the separation on the HPLC system (the same procedure as that used for collecting the retinol fraction, described above). After extraction of carotenoids from 1–2 mL serum by using hexane and a ratio of chloroform to ethanol (C:E) as described above, the carotenoid residue was resuspended in 200 μL chloroform and put on a Strata NH_2 -propyl open column (500 mg/3 mL; Phenomenex, Torrance, CA) conditioned with 4 mL hexane. Hexane (4 mL) was used as the eluent. The β -carotene in hexane was evaporated under N_2 . The residue was resuspended in 75 μL E:T (1:2 by vol), and 60 μL was injected into the HPLC with a C_{18} column. A linearly gradient solvent procedure started from 60% solvent A and 40% B, changed to 17% A and 83% B in 4 min and held for 4 min, changed to 100% B in 5 min and held for 25 min, then changed back to 60% A and 40% B in 2 min and held for 8 min to stabilize the system before injecting the next sample. β -Carotene was washed out at 17 min, and the eluant of 16–18 min was collected, dried down, resuspended in 60 μL ethanol, and injected into the liquid chromatograph–mass spectrometer [LC-MS (20, 29)].

The LC–atmospheric pressure chemical ionization–MS (LC-APCI-MS) system included an Agilent 1100 LC (Agilent Technologies) equipped with a C_{18} Prizm column (Keystone Scientific, Bellefonte, PA) and an Esquire mass spectrometer (Bruker Daltonic Inc, Billerica, MA). The LC mobile phase contained 75% methanol and 25% E:M:T (75:20:5 by vol). The mass spectrometer was set to scan at m/z 530–560 in 0.1-Da steps with a scan speed of 5.25 scans/s.

Statistical analysis

We used SPSS software (version 13.0; SPSS Institute, Chicago, IL) for statistical analyses. Bivariate correlation was used to find the correlations between variables. The paired t test was used to compare the concentrations of serum retinol and carotenoids before and after retinyl acetate and spirulina were ingested.

RESULTS

Subject characteristics and fasting serum concentrations of retinol and carotenoids at the beginning of the study are listed in **Table 1**. The mean (\pm SD) BMI and serum retinol concentration of the 10 subjects was 23.4 ± 3.1 and $1.71 \pm 0.33 \mu\text{mol/L}$, respectively. Mean serum concentrations of α -carotene, β -carotene, and β -cryptoxanthin were lower than the mean values in American men aged ≥ 20 y, but the mean serum concentration of lutein + zeaxanthin ($0.44 \mu\text{mol/L}$) was similar to the mean value in American men aged ≥ 20 y—ie, $0.40 \mu\text{mol/L}$ (30).



TABLE 1Subjects' fasting serum concentrations of retinol and carotenoids at the beginning of the study¹

Serum analyte	Value
	$\mu\text{mol/L}$
Retinol	1.71 \pm 0.33 (1.28–2.07)
Lutein	0.39 \pm 0.09 (0.25–0.49)
Zeaxanthin	0.05 \pm 0.01 (0.03–0.07)
β -Cryptoxanthin	0.05 \pm 0.04 (0.02–0.13)
α -Carotene	0.02 \pm 0.03 (0.00–0.09)
<i>trans</i> β -Carotene	0.15 \pm 0.12 (0.04–0.40)

¹ All values are $\bar{x} \pm \text{SD}$; range in parentheses. $n = 10$ subjects aged 48.1 \pm 5.7 y (range: 41.0–57.0 y) and with BMIs (in kg/m^2) of 23.4 \pm 3.1 (17.9–27.6).

The provitamin A carotenoid content in labeled spirulina consisted mainly of *all-trans* and *9-cis* β -carotene and included low concentrations of *13-cis* β -carotene and β -cryptoxanthin. Assuming that *9-cis* β -carotene, *13-cis* β -carotene, and β -cryptoxanthin have half the vitamin A activity of *trans* β -carotene, the total of calculated β -carotene equivalents in the labeled spirulina administered to the subjects was 7.9 μmol (4.3 mg) (Table 2).

According to the LC-MS results (Figure 2), the actual enrichment of β -carotene in spirulina was distributed at m/z 536–558. The m/z region 536–539 was assigned as the predominant natural abundance isotopomer of β -carotene, whereas the m/z region 540–558 was the β -carotene labeled by different degrees of deuteration with the highest enrichment at m/z 547 ($[\text{}^2\text{H}_{10}]$ *trans* β -carotene). Ninety-nine percent of spirulina β -carotene was enriched with deuterium.

Before consumption of deuterated spirulina, the extracted ion chromatograms (EIC) of serum samples showed only the endogenous, unlabeled β -carotene at m/z 536–539. After the spirulina β -carotene dose, β -carotene at m/z 536–539 and 540–558 both presented on EIC. The area of m/z 543–555 accounted for 94.5% of total area of m/z 540–558. That means the result of enrichment of $[\text{}^2\text{H}_5]$ retinol from spirulina $[\text{}^2\text{H}_{10}]$ β -carotene calculated by an m/z region of 271–276 was 94.5% of the true value. Thus, the true enrichment of labeled retinol was adjusted by dividing the result by 94.5%.

The mean (\pm SD) highest enrichment of $[\text{}^2\text{H}_5]$ retinol in the circulation was 3.7 \pm 1.3% (range: 2.4–6.6%), and the mean highest enrichment of $[\text{}^{13}\text{C}_{10}]$ retinol in the circulation was 7.8 \pm 1.4% (range: 5.2–9.8%). The greatest concentrations of $[\text{}^{13}\text{C}_{10}]$ retinol and $[\text{}^2\text{H}_5]$ retinol both appeared at 11–24 h after the

TABLE 2Carotenoid content in 5.0 g labeled spirulina consumed by the male subjects¹

Carotenoids	Value
	μmol
<i>trans</i> β -Carotene	6.68 \pm 0.50
<i>9-cis</i> β -Carotene	1.20 \pm 0.04
<i>13-cis</i> β -Carotene	0.43 \pm 0.02
Zeaxanthin	6.46 \pm 0.37
β -Cryptoxanthin	0.81 \pm 0.03
β -Carotene dose ²	7.90 \pm 0.52

¹ All values are $\bar{x} \pm \text{SD}$. $n = 3$.

² β -Carotene dose = (*trans* β -carotene + (*9-cis* β -carotene + *13-cis* β -carotene + β -cryptoxanthin)/2).

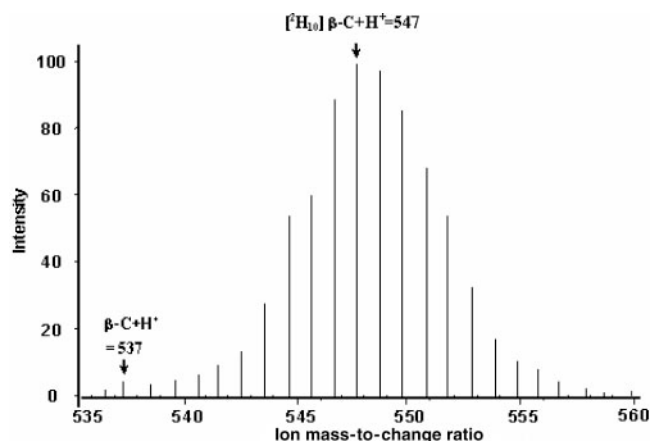


FIGURE 2. Enrichment profile of β -carotene (β -C) in deuterated spirulina determined by using a liquid chromatography–atmospheric pressure chemical ionization–mass spectrometer.

corresponding doses. Representative serum response curves are shown in Figure 3.

AUCs of the serum-labeled retinol response showed that 1 μmol retinyl acetate provided 287.7 nmol retinol and that 1 μmol spirulina β -carotene provided 134.6 nmol retinol over 48 d after the corresponding doses. In addition, 1 μmol spirulina

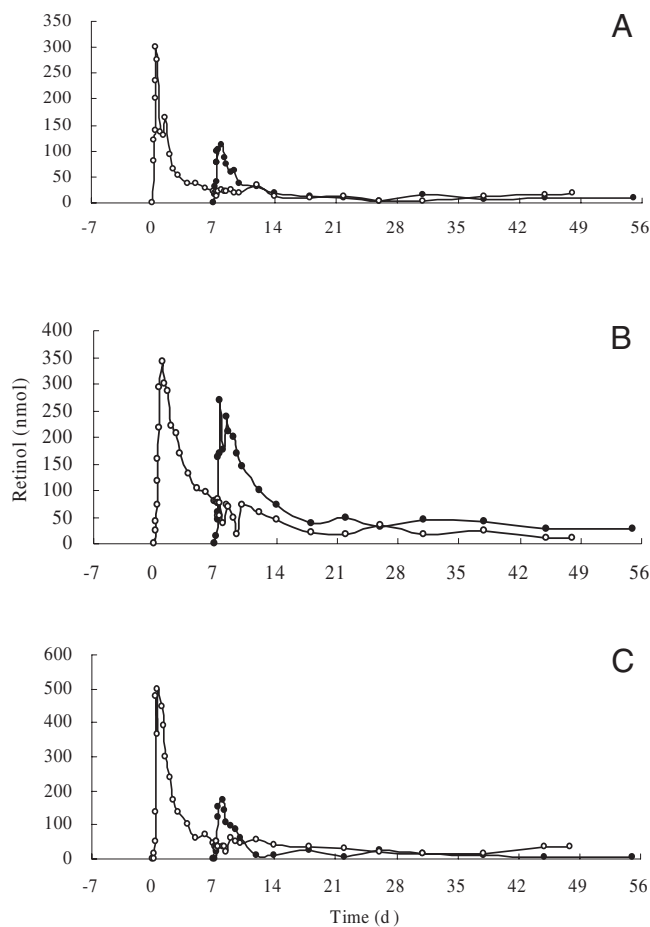


FIGURE 3. Serum response curves of $[\text{}^{13}\text{C}_{10}]$ retinol from retinyl acetate (\circ) and $[\text{}^2\text{H}_5]$ retinol from spirulina β -carotene (\bullet) in 3 subjects. A, subject 1; B, subject 6; C, subject 7.



TABLE 3

Area-under-the-curve (AUC) responses of serum retinol to spirulina β -carotene and retinyl acetate over 48 d after corresponding doses and AUC responses of serum β -carotene to spirulina β -carotene over 24 d after taking spirulina¹

Subject no.	AUC of [² H ₅]retinol ²	AUC of [¹³ C ₁₀]retinol ³	AUC of [² H ₁₀] β -carotene ⁴
1	89	178	57
2	109	300	73
3	69	184	77
4	74	231	151
5	133	200	36
6	316	391	254
7	111	405	377
8	163	227	27
9	100	349	178
10	182	412	67
$\bar{x} \pm$ SD	134.6 \pm 73.2	287.7 \pm 94.8	129.7 \pm 112.5
Range	69.1–315.9	178.0–411.6	26.8–377.2

¹ AUCs of labeled retinol were calculated by using INTEGRAL-CURVE of KALEIDAGRAPH software (version 3.5; Synergy Software, Reading, PA) using total serum response (nmol, as y axis) versus time (d, as x axis).

² Calculated as nmol \cdot d/ μ mol β -carotene dose.

³ Calculated as nmol \cdot d/ μ mol retinyl acetate.

⁴ Calculated as nmol \cdot d/ μ mol β -carotene dose.

β -carotene provided 129.7 nmol intact β -carotene over 24 d after spirulina administration, according to the AUC of the serum-labeled β -carotene response (Table 3). A significant correlation existed between the AUC of β -carotene and the baseline serum β -carotene concentration ($r = 0.89$, $P = 0.01$).

On the basis of the serum responses to the reference dose, 0.48 nmol or 0.26 mg retinol was formed from 1 nmol or 1 mg spirulina β -carotene. Thus, 4.5 mg spirulina β -carotene had the same vitamin A activity as did 1 mg retinol (Table 4). No correlation was found between subjects' total body stores of vitamin A (Table 4) and the conversion factors. A positive correlation was found between the subjects' total body stores of vitamin A and their serum baseline retinol concentrations ($r = 0.65$, $P = 0.04$).

The calculated AUCs of [²H₅]retinol over 1, 3, 7, 15, 24, 31, 38 and 48 d represented the AUC of retinol if the conversion of β -carotene to retinol can happen only in the intestine. When

additional [²H₅]retinol is formed after the intestinal absorption of spirulina β -carotene, it is called postabsorptive conversion of β -carotene. The additional retinol was estimated to be 10.4%, 18.1%, and 20.1% of the total converted retinol over 3, 15, and 48 d, respectively, after the spirulina β -carotene dose (Table 5).

In addition, mean circulating retinol concentrations increased from 1.71 \pm 0.33 to 1.85 \pm 0.15 μ mol/L ($P < 0.01$) 24 h after administration of 2 mg retinyl acetate. Mean circulating zeaxanthin, *trans* β -carotene, and β -cryptoxanthin increased from 0.05 \pm 0.01 to 0.07 \pm 0.02 μ mol/L, from 0.11 \pm 0.07 to 0.14 \pm 0.07 μ mol/L, and from 0.04 \pm 0.02 to 0.06 \pm 0.02 μ mol/L, respectively ($P < 0.01$ for all), 24 h after the administration of 5 g spirulina containing 3.7 mg zeaxanthin, 3.7 mg *trans* β -carotene, and 0.5 mg β -cryptoxanthin.

DISCUSSION

In the stable-isotope reference method, the labeled β -carotene can be administered as a single dose or in multiple doses. Although multiple doses were more related to usual dietary practice, the single-dose method can provide an accurate metabolic curve for isotope tracers because there are more blood samples and less influence from diets than in the multiple-dose method (29). In the current study, a single dose of spirulina β -carotene was given to the subjects 7 d after a single dose of labeled retinyl acetate to avoid the potential absorption competition. Diets low in vitamin A and β -carotene (≈ 174 μ g retinol activity equivalents/d, 19% of the US recommended dietary allowance or 37% of the intake recommended in China) were followed by the subjects to minimize the influence of subsequent dietary vitamin A or β -carotene on the labeled retinol metabolic curve.

We found that the mean conversion factor of spirulina β -carotene to vitamin A was 4.5 by weight, which was in the range of mean conversion factors (1.3–9.1 by weight) of commercial β -carotene (dose ≤ 20 mg) for normal converters (5, 29, 31). Several factors could contribute to the high vitamin A equivalence of spirulina β -carotene. As observed in previous studies, fat provided with the β -carotene dose was a major influencing factor for conversion efficiency. In the single-dose method, β -carotene was usually dissolved in more oil (6 mg in 6 g oil) than was retinyl acetate (3 mg in 170 mg oil) to improve the absorption and conversion (20, 29, 31). Edwards found that an increased retinol concentration in plasma triacylglycerol-rich lipoprotein

TABLE 4

Calculated vitamin A equivalence and conversion factor of spirulina β -carotene to vitamin A over 48 d after consuming spirulina

Subject no.	Total body stores of vitamin A		Retinol formed		Conversion factor
	mmol	nmol/nmol dose	mg/mg dose	g	
1	0.68	0.50	0.27	2.00 ¹	3.75
2	0.27	0.36	0.19	2.74	5.15
3	0.46	0.38	0.20	2.67	5.01
4	0.37	0.32	0.17	3.13	5.87
5	0.54	0.66	0.35	1.50	2.83
6	0.17	0.81	0.43	1.24	2.32
7	0.28	0.27	0.15	3.65	6.86
8	0.22	0.72	0.38	1.40	2.62
9	0.39	0.29	0.15	3.48	6.54
10	0.27	0.44	0.26	2.27	4.26
$\bar{x} \pm$ SD	0.37 \pm 0.16	0.48 \pm 0.19	0.26 \pm 0.10	2.41 \pm 0.87	4.52 \pm 1.63
Range	0.17–0.68	0.27–0.81	0.15–0.43	1.24–3.65	2.32–6.86

¹ Values in this column are molar.



TABLE 5

Serum retinol responses over 1, 3, 7, 15, 24, 31, 38, and 48 d after supplements and estimated postabsorptive bioconversion¹

Time after dose	AUC of [¹³ C ₁₀]retinol	AUC of [² H ₅]retinol	Calculated AUC of [² H ₅]retinol	[² H ₅]retinol from postabsorptive conversion
	<i>nmol · d</i>	<i>nmol · d</i>	<i>nmol · d</i>	%
Day				
1	201 ± 62	104 ± 28	—	—
3	548 ± 171	323 ± 102	296 ± 122	10.4 ± 15.7
7	830 ± 265	555 ± 205	458 ± 218	19.2 ± 19.4
15	1152 ± 337	765 ± 291	634 ± 284	18.1 ± 17.2
24	1358 ± 412	936 ± 374	739 ± 318	21.6 ± 13.3
31	1501 ± 466	1037 ± 445	814 ± 347	22.0 ± 11.3
38	1625 ± 510	1112 ± 509	876 ± 368	20.9 ± 11.6
48	1782 ± 553	1188 ± 585	957 ± 391	21.0 ± 12.0

¹ AUC, area under the curve. All values are $\bar{x} \pm SD$, $n = 9$. The data for subject no. 4 with negative postabsorptive bioconversion (-35.3% at 48 d after the dose) were not included in the table. The AUCs of labeled retinol were calculated by using INTEGRAL-CURVE of KALEIDAGRAPH software (version 3.5; Synergy Software, Reading, PA) with total serum response (nmol, as y axis) versus time (d, as x axis).

could be detected when carrot or spinach was consumed with 20 g fat instead of 1 g fat, but the fat amount did not affect the AUC of serum [²H₄]retinol from [²H₄]retinyl acetate (32). In our study, the labeled spirulina and the vitamin A doses were both administered with a liquid drink containing 10 g fat. An additional 9 g fat in the breakfast meal was provided 1 h after the spirulina dose to benefit the conversion. However, 71 and 70 g fat were consumed by the subjects on day 1 and day 8, respectively. Therefore, we adjusted the proportion of fat in the 3 meals, but the total fat and energy for the day did not change. We felt that this was an acceptable dietary practice when taking a fat-soluble supplement. The low vitamin A diets during the study may contribute to the high vitamin A equivalence. No control groups (no spirulina or vitamin A dose) were included to determine whether the vitamin A nutriture would change during the study period. Therefore, our results on the conversion factors were obtained under optimal conditions.

In addition, this efficient conversion of spirulina β -carotene may be due to the simple cell structure of spirulina, which is composed of protein and peptidoglycans that are easily digested (21). It has been reported that the absorption of iron in spirulina was significantly lower than that of the iron in ferrous sulfate and whole egg but significantly greater than that of the iron in whole wheat (33). In addition, the high natural free fatty acid (1–10%) in spirulina (21) may help the absorption of β -carotene (34).

It is well known that β -carotene could either be cleaved into retinol in the intestine by carotenoid monooxygenase I and II (35, 36) or be absorbed intact into the circulation (37). β -Carotene that is absorbed intact into the circulation is stored in human tissue or cleaved into retinol thereafter (37, 38). The part of the conversion that occurred after absorption of the β -carotene was called postabsorptive conversion of β -carotene. However, it was nearly impossible to directly differentiate between the retinol from preabsorptive conversion and that from postabsorptive conversion, because the same retinol was formed. A mathematical method using 24 h as a cutoff for preabsorption and postabsorptive conversion was developed to calculate the postabsorptive conversion (29). Haskell et al (39), using the deuterated retinol-dilution technique, found that plasma-labeled retinol peaked within the first 24 h. Dueker et al (40) reported that labeled retinol appeared at 5.5 h, and there was a linear rise for 28 h before the decline began. The metabolic curve of labeled retinol in the

present study showed that the greatest concentration of labeled retinol in serum from the vitamin A dose or from the spirulina β -carotene dose appeared at 11–24 h. Because the intestine is the most important organ for the conversion of β -carotene to retinol, and because all of the metabolic curves reached their peaks in 24 h, it is reasonable to use 24 h as the cutoff for intestinal and postabsorptive conversion. According to the calculations, only 1 in 10 subjects in the present study did not show postabsorptive conversion (Table 5).

Since the late 1970s, the nutritive value and health benefits of spirulina have been reported, and those reports triggered the commercial production of spirulina. Millions of people in the world have used it as a health food supplement, taking 3–20 g/d. Rarely have there been any reports of allergies or sensitivities (41). The present study, using the stable-isotope reference method, found that, when consumed with 22 g fat, 4.5 mg spirulina β -carotene provided 1 mg vitamin A for 10 well-nourished, normal-weight Chinese men. Therefore, 4.1 g spirulina powder could meet the US recommended dietary allowance of 900 μ g retinol activity equivalents for men.

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The authors' responsibilities were as follows—JW: conducted the human study, collected and analyzed samples and data, and wrote the manuscript; SY, JW, and ZW: supervised the human study and revised the manuscript; WL and YF, nurses: collected blood samples; GT and RR: designed the study and revised the manuscript; GT and JQ supervised the liquid chromatographic, gas chromatographic, and mass spectrometric analyses; PS: proposed the project and revised the manuscript; and MG-C: produced the labeled spirulina, analyzed the carotenoid content in spirulina, and transported the spirulina and liquid diets from United States to China. PS is affiliated with the University Hospital (Zurich, Switzerland) and Nestlé Foundation (Lausanne, Switzerland). None of the other authors had a personal or financial conflict of interest, and no financial benefit was obtained from this research study.

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