Bioefficacy of β-carotene dissolved in oil studied in children in Indonesia[1–3]

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ABSTRACT

Background: More information on the bioefficacy of carotenoids in foods ingested by humans is needed.

Objective: We aimed to measure the time required for isotopic enrichment of β-carotene and retinol in serum to reach a plateau, the extent of conversion of β-carotene dissolved in oil with use of β-carotene and retinol specifically labeled with 10 ^{13}C atoms, and the intraindividual variation in response.

Design: Indonesian children aged 8–11 y (n = 35) consumed 2 capsules/d, 7 d/wk, for ≤10 wk. Each capsule contained 80 μg [12,13,14,15,20,12′,13′,14′,15′,20′-$^{13}C_{10}$]β-carotene and 80 μg [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate. Three blood samples were drawn per child over a period of ≤10 wk. HPLC coupled with atmospheric pressure chemical ionization liquid chromatography–mass spectrometry was used to measure the isotopic enrichment in serum of retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of β-carotene with [$^{13}C_{10}$]β-carotene. The β-carotene in the capsules used had a *cis-trans* ratio of 3:1.

Results: Plateau isotopic enrichment was reached by day 21. The amount of β-carotene in oil required to form 1 μg retinol was 2.4 μg (95% CI: 2.1, 2.7). The amount of *all-trans*-β-carotene required to form 1 μg retinol may be lower.

Conclusions: The efficiency of conversion of this β-carotene in oil was 27% better than that estimated previously (1.0 μg retinol from 3.3 μg β-carotene with an unknown *cis-trans* ratio). The method described can be extended to measure the bioefficacy of carotenoids in foods with high precision, requiring fewer subjects than other methods. *Am J Clin Nutr* 2001;73:949-58.

KEY WORDS Bioavailability, β-carotene, conversion, retinol, vitamin A, children, Indonesia, stable isotope, carbon isotopes, ^{13}C, extrinsic labeling, LC-MS, liquid chromatography–mass spectrometry

INTRODUCTION

Vitamin A deficiency is a serious problem in many developing countries (1). Provitamin A carotenoids, such as β-carotene, are the major source of vitamin A in the diet of a large proportion of the world's population. Unfortunately, the bioavailability of carotenoids in fruit and vegetables and the conversion of carotenoids to retinol are lower (2, 3) than assumed previously (4).

Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (5). We define bioconversion as the fraction of a bioavailable nutrient (here, absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol). We define bioefficacy as the efficiency with which ingested nutrients (here, dietary provitamin A carotenoids) are absorbed and converted to the active form of the nutrient (retinol). Because 1 μmol β-carotene theoretically could form 2 μmol retinol, 100% bioefficacy would mean that 1 μmol dietary β-carotene (0.537 μg) is 100% absorbed and converted 100% to retinol, yielding 2 μmol retinol (0.572 μg). Thus, the amount of β-carotene required to form 1 μg retinol would be 0.537/0.572 = 0.94 μg.

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Various factors, ordered in the acronym SLAMENGHI, influence the bioefficacy of carotenoids. The factors are *S*) the species of carotenoid, *L*) molecular linkage, *A*) the amount of carotenoids consumed in a meal, *M*) the matrix in which the carotenoid is incorporated, *E*) effectors of absorption, *N*) the nutrient status of the host, *G*) genetic factors, *H*) host-related factors, and *I*) interactions (6, 7).

Until now, the bioefficacy of carotenoids was estimated in humans with use of oral-fecal balance techniques, measurement of plasma or chylomicron responses after single or multiple doses of carotenoids, and tracer methods. In the 1960s, 2 tracer studies measured the recovery of [^{14}C]β-carotene in lymph (8, 9). To date, only single doses of specifically extrinsically labeled [$^2H_8$]β-carotene (10–12), intrinsically labeled β-carotene biosynthesized in green algae grown with ^{13}C as the sole carbon source (13), and intrinsically labeled β-carotene from spinach grown on 30% ^2H_2O (14) have been administered for studying β-carotene metabolism in a limited number of human subjects (n = 1–5).

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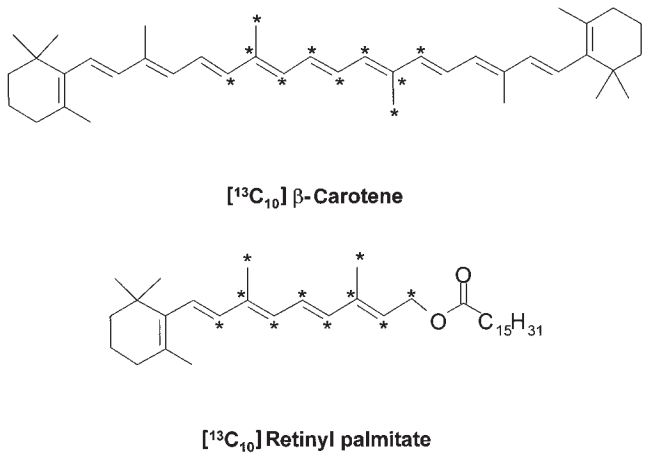

FIGURE 1. Molecular structure of synthesized [12,13,14,15,20,12′,13′,14′,15′,20′-$^{13}C_{10}$]β-carotene and [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate. The asterisks indicate the positions of the ^{13}C labels.

We developed a method that can quantify the bioefficacy of carotenoids with use of multiple low doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms. This enables the isotopic enrichment of both retinol and β-carotene in serum to reach a plateau. From the degree of labeling of retinol with 5 and 10 ^{13}C atoms and the doses of [$^{13}C_{10}$]-labeled β-carotene and retinol administered, the bioefficacy of β-carotene can be calculated. The aim of this study in children in Indonesia was to measure the extent of conversion of β-carotene dissolved in oil. We also measured the time required for the isotopic enrichment of β-carotene and retinol in serum to reach a plateau and the intraindividual variation in response.

SUBJECTS AND METHODS

Subjects

The study was conducted from December 1997 to May 1998. Schoolchildren aged 8–11 y (grades 3 and 4) from a rural village in the Bogor District, West Java, Indonesia, were asked to participate in the screening for this study. The purpose and procedures of the screening and the intervention were explained to the parents or guardians of these children. Almost all parents allowed their children to participate and signed an informed consent form. During the screening, a physician examined 140 children and each child's age, sex, weight, and height were recorded. Venous blood samples (4 mL) were drawn from apparently healthy, nonfasted children to measure hemoglobin concentrations and to prepare serum in which the concentrations of retinol and total carotenoids were measured. In addition, children were asked to provide a single stool sample that was examined for protozoan cysts and worm eggs. Children were also asked to record their food intake in a diary for 7 consecutive days and were interviewed once or twice during this week to check the quality of the data in the diaries (24-h recall).

Thirty-six children were then invited to participate in the intervention on the basis of their serum retinol concentrations: <0.70 μmol/L (low) or >1.05 μmol/L (high). Together with their parents, these children were informed about the intervention in more detail and their parents signed a second informed consent form. After stratification for weight, height, sex, and serum retinol concentration, the 36 children were randomly allocated to 3 groups of equal size that were studied for 4 wk (group 1), 7 wk (group 2), or 10 wk (group 3). This enabled us to assess the time required for the isotopic enrichment of retinol and β-carotene in serum to reach a plateau. To assess the effect of vitamin A status on bioefficacy, children with either low or high serum retinol concentrations were distributed throughout the 3 groups. It was not possible to carry out power calculations because this is a new method. Thus, we decided on a sample size of 6 children with low and 6 children with high serum retinol concentrations per group. From this number of children, it should be possible to measure the variation from which power calculations could be made for subsequent studies, even if there was limited dropout. The study was approved by the Medical Ethics Committee of the Ministry of Health, Indonesia; the Indonesian Institute of Science; and the Medical Ethical Committee of Wageningen University, Netherlands.

Study design

Each child received 2 capsules/d, 7 d/wk. Each capsule contained 80 μg [12,13,14,15,20,12′,13′,14′,15′,20′-$^{13}C_{10}$]β-carotene (analyzed value) and 80 μg [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate (44 retinol equivalents, or RE; analyzed value) (**Figure 1**). The oily mixture for the capsules was prepared by dissolving the labeled β-carotene and retinyl palmitate in highly unsaturated sunflower oil (>82% oleic acid and >10% linoleic acid; Hozol RBDW; Contined BV, Bennekom, Netherlands). *all-rac-α*-Tocopheryl acetate (Roche Nederland BV, Mijdrecht, Netherlands) was added to the oil as an antioxidant. These actions were carried out under subdued light. The capsules used in this study were made from bovine gelatin (Capsugel, Bornem, Belgium) and were filled with the oily mixture by multipipette. Each capsule contained 0.36 g oil and 150 μg vitamin E. The $^{13}C_{10}$-labeled retinol and β-carotene were synthesized at the Leiden Institute of Chemistry (15). These compounds were food grade, on the basis of criteria established by the US Pharmacopoeia (16), the Joint FAO/WHO Expert Committee on Food Additives (17), and the European Pharmacopoeia (18).

The capsules were consumed after a low-retinol, low-carotenoid meal Monday through Friday and after a chocolate wafer on the weekend. The foods provided contained some fat to promote the absorption of the retinol and β-carotene from the capsules. Five different menus were used, each of which contained a fixed combination of a lunch served at 1100 and an afternoon meal served at 1500. The 5 menus were randomly allocated over the days of each week. On weekdays, a dietitian supervised and recorded compliance with consumption of the capsules and attendance at meals. On the weekend and on holidays, a teacher supervised and recorded compliance with consumption of the capsules and whether each child received a wafer. Four village health volunteers prepared all meals during the intervention. For each ingredient, the recipes listed the amount to be purchased and the cleaned amount to be cooked. The amount of food to be distributed was listed for each dish. Children recorded their food consumption daily in a diary. For the foods provided, the children recorded the amount not eaten; for the foods not provided, they recorded which foods and drinks they had consumed and in which quantity. To check the quality of the data in the diaries, children were interviewed once per week about which foods and drinks they had consumed and in which quantity on the previous day (24-h recall).

Nonfasting blood samples (8 mL) were collected between 0930 and 1100 from an antecubital vein on days 0, 21, and 28 for

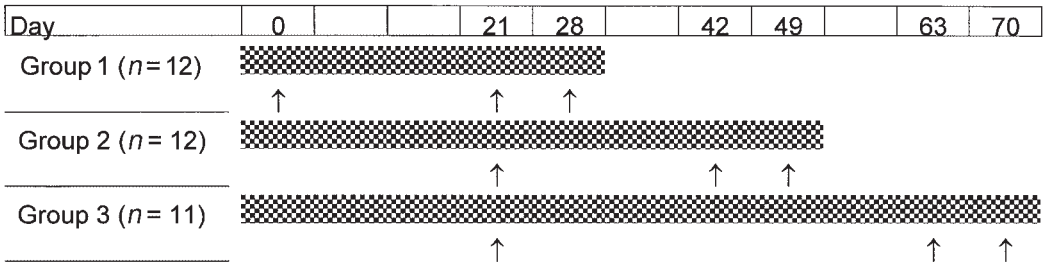

FIGURE 2. Study design of the intervention period. During the period shown by the shaded box, children consumed 2 capsules/d, 7 d/wk, and were provided a low-retinol, low-carotenoid diet 5 d/wk. Each capsule contained 80 μg [$^{13}C_{10}$]β-carotene and 80 μg [$^{13}C_{10}$]retinyl palmitate. Three blood samples were drawn per child as indicated by the arrows.

group 1; on days 21, 42, and 49 for group 2; and on days 21, 63, and 70 for group 3 (**Figure 2**). The children were examined by a physician on the day of blood collection and their weights and heights were measured. While the children wore a school uniform but no shoes, weight was measured to the nearest 0.1 kg with a digital electronic scale (770 alpha; Seca, Hamburg, Germany) and height was measured to the nearest 0.1 cm with a microtoise.

Methods

Estimation of energy and nutrient intakes

Concentrations of retinol and β-carotene in the capsules were analyzed by HPLC (19). To measure the nutrient contents of the meals, a duplicate sample of a menu was collected at random once per week. The random allocation was done in such a way that each menu was sampled once during the first 5 wk of the intervention and once during the last 5 wk. Duplicate samples of all dishes of both meals of a collection day were weighed before and after removal of the inedible portion (bones, peels, etc). The edible portions were then pooled, blended, and portioned into 3 plastic bags. All bags were stored at –20°C. Within 3 wk of collection, one bag of each duplicate sample was used to measure fat content by the Soxhlet method, protein content by the micro-Kjeldahl method, water content by evaporating the sample, and ash content by dry ashing the sample (20). Carbohydrate content was calculated by difference. Within 5 wk of collection, another bag of each duplicate sample was used to measure iron content (20). All analyses were carried out in duplicate at the laboratory of the Nutrition Research and Development Centre (NRDC) in Bogor. The last bag of each duplicate sample was stored for ≈2 mo before being packed on dry ice and transferred to the Division of Human Nutrition and Epidemiology in Wageningen. There the samples were stored for 2 mo at –80°C until analyzed for retinol and carotenoids by HPLC (19).

The energy content of the meals in kJ was calculated by multiplying the weight (in g) of fat, protein, and carbohydrates by 37, 17, and 17, respectively. Results of both duplicate samples per menu were averaged. Time and conditions of storage were adequate to obtain reliable results for the analyses carried out in food and serum (21–23). Energy and nutrient intakes during the screening and during the intervention were calculated by using the diaries, 24-h recalls, records of attendance at meals, and a computer program suite (KOMEET, version 2.0c, and VBS-EDIT, version 1.0; B-Ware Nutrition Software, Wageningen, Netherlands) with a nutrient database (Bg95K98) based on that developed by de Pee et al (24) to which the energy and nutrient contents of the foods provided (on the basis of the duplicate analyses) were added.

Analysis of blood and stool samples

Immediately after blood was collected, a portion of whole blood was removed to count white blood cells (25) and measure hemoglobin concentrations (26). These analyses were done on the day of blood collection at the NRDC. The remaining blood was placed on ice, protected from light, and centrifuged within a few hours (750 × *g* for 10 min at room temperature) at the NRDC. In serum samples collected during the screening, concentrations of retinol and total carotenoids were measured by HPLC at the NRDC (27). In serum samples collected during the intervention, concentrations of retinol and various carotenoids were measured by HPLC in Wageningen (28). For these latter analyses, serum was stored in a series of containers and frozen at –20°C for ≈2 mo before being packed on dry ice and transferred to Wageningen. All containers were then stored at –80°C. One container was stored for 2 mo until analyzed for both retinol and carotenoids by HPLC. Two other containers were stored for 4 mo before being packed on dry ice and transferred to the Department of Medicinal Chemistry and Pharmacognosy in Chicago. There the samples were stored for 2 mo at –80°C until analyzed for the degree of isotopic enrichment of retinol (29) and β-carotene (30) by HPLC coupled with atmospheric pressure chemical ionization liquid chromatography–mass spectrometry (APCI LC-MS).

For the LC-MS method, retinol and β-carotene were extracted from 0.2- and 1.0-mL serum samples, respectively, and analyzed by reversed-phase HPLC with a C_{30} column interfaced to a mass spectrometer equipped with positive ion APCI. To assess the ratio of labeled to unlabeled β-carotene, selected ion monitoring was carried out at mass-to-charge ratios (*m/z*) of 537 and 547. These ions corresponded to circulating β-carotene and orally administered [$^{13}C_{10}$]β-carotene, respectively. To assess the ratio of labeled to unlabeled retinol, selected ion monitoring was carried out at *m/z* 269, 274, and 279. These abundant fragment ions corresponded to the loss of water from the protonated molecule of circulating retinol, [$^{13}C_5$]retinol (metabolically formed from orally administered [$^{13}C_{10}$]β-carotene), and [$^{13}C_{10}$]retinol (formed by hydrolysis of orally administered [$^{13}C_{10}$]retinyl palmitate), respectively. Although the LC-MS method focused on the quantification of *all-trans*-β-carotene, the C_{30} HPLC method also resolves *cis* isomers from the *all-trans* peak. Therefore, this LC-MS method may be applied to the quantification of *cis* as well as *all-trans*-β-carotene.

The presence of protozoan cysts and worm eggs was diagnosed in stools by the Ridley method. The load of worm eggs was quantified by the Kato Katz method (31).

TABLE 1
Description of the β-carotene and retinol at plateau isotopic enrichment (CarRet PIE) mathematical model

Symbol	Description	Derivation	Example[1]
a	Dietary retinol intake (μg/d)		33.2
b	Retinol intake from capsules (μg/d)		87.4
r	Total retinol intake (μg/d)	$a + b$	120.6
d	Dietary β-carotene intake (μg/d)		125.0
e	β-Carotene intake from capsules (μg/d)		160.0
c	Total β-carotene intake (μg/d)	$d + e$	285.0
$E_{10,dR}$	Enrichment of dietary retinol with [$^{13}C_{10}$]retinol	b/r	0.725
$E_{10,dC}$	Enrichment of dietary β-carotene with [$^{13}C_{10}$]β-carotene	e/c	0.561
$E_{10,sR}$	Enrichment of serum retinol with [$^{13}C_{10}$]retinol	$M_{279,sR}/(M_{269,sR} + M_{274,sR} + M_{279,sR})^2$	0.080
$E_{5,sR}$	Enrichment of serum retinol with [$^{13}C_5$]retinol	$M_{274,sR}/(M_{269,sR} + M_{274,sR} + M_{279,sR})$	0.052
$E_{10,sC}$	Enrichment of serum β-carotene with [$^{13}C_{10}$]β-carotene	$M_{547,sC}/(M_{537,sC} + M_{547,sC})$	0.226
$P_{sR/dR}$	Proportion of serum retinol derived from dietary retinol	$E_{10,sR}/E_{10,dR}$	0.110
$P_{sR,dC}$	Proportion of serum retinol derived from dietary β-carotene	$E_{5,sR}/E_{10,dC}$	0.093
$P_{sC,dC}$	Proportion of serum β-carotene derived from dietary β-carotene	$E_{10,sC}/E_{10,dC}$	0.403
$F_{dR/dC}$	Vitamin A activity of β-carotene in oil compared with that of retinol in oil	$(P_{sR/dC}/P_{sR/dR}) \times (r/c)$	0.355
A_{dC}	Amount of β-carotene in oil required to form 1 μg retinol (μg)	$1/\bar{F}_{dR/dC}$[3]	—[4]
X_{dC}	Maximal bioefficacy of dietary β-carotene if absorption and conversion are both 100% (%)	100	100
Y_{dC}	Amount of β-carotene required to form 1 μg retinol if bioefficacy is 100% (μg)	0.94	0.94
B_{dC}	Bioefficacy of β-carotene in oil: the efficiency with which β-carotene in oil is asborbed and converted to retinol (%)	$(X_{dC} \times Y_{dC})/A_{dC}$	—[4]

[1] Sample calculation based on data from a 9-y-old boy on day 21.
[2] Where $M_{279,sR}$ is the signal measured by liquid chromatography–mass spectrometry at *m/z* 279.
[3] Where $\bar{F}_{dR/dC}$ is the mean of averaged values from each subject.
[4] Can be calculated only at the group level.

Calculation of the bioefficacy of β-carotene converted to retinol

A mathematical model was developed that uses the isotopic enrichment in serum of both β-carotene with [$^{13}C_{10}$]β-carotene and retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol at plateau isotopic enrichment. This model, which we refer to as CarRet PIE, was used to estimate carotenoid bioavailability and bioconversion and, thus, bioefficacy. This stable-isotope method is based on the isotopic enrichment of retinol and β-carotene in serum reaching a plateau during multiple dosing with [$^{13}C_{10}$]retinol and [$^{13}C_{10}$]β-carotene. The intake of retinol and β-carotene from other sources is kept constant and as low as possible. It is assumed that such retinol and β-carotene mixes completely with labeled retinol and labeled β-carotene. The bioefficacy of dietary provitamin A (in this study, β-carotene in oil) compared with that of dietary retinol was calculated as shown in the first 3 columns of **Table 1**. To assess the time required for isotopic enrichment of β-carotene and retinol in serum to reach a plateau, the regression coefficients of vitamin A activity of β-carotene in oil compared with that of retinol in oil were calculated for each group, pooling the data for the second and third time points in groups 2 and 3.

Statistical methods

Data are shown as means and 95% CIs or SDs (in the case of descriptive measures). Variables that were not normally distributed are expressed as medians and 25th to 75th percentiles. Kruskal-Wallis tests were carried out to compare energy and nutrient intakes among groups during the screening and during the intervention and to compare characteristics among groups. Wilcoxon's tests were carried out to compare energy and nutrient intakes between the screening and the intervention and to compare energy and nutrient intakes between the data obtained from the diaries and those obtained from the 24-h recalls. Friedman's tests were carried out to compare serum retinol and β-carotene concentrations on various blood collection days within each group (eg, for group 2 on days 21, 42, and 49). Kruskal-Wallis tests were carried out to compare the following variables among groups on day 21: serum retinol concentration, serum β-carotene concentration, isotopic enrichment of retinol with [$^{13}C_5$]retinol and with [$^{13}C_{10}$]retinol, isotopic enrichment of β-carotene with [$^{13}C_{10}$]β-carotene, and vitamin A activity of β-carotene in oil compared with that of retinol. Mann-Whitney *U* tests were carried out to compare the same variables within each group on each blood collection day between those with initial low and high serum retinol concentrations. Spearman correlation coefficients were calculated between serum retinol concentration at screening and vitamin A activity of β-carotene in oil compared with that of retinol on days 21, 28, 42, 49, 63, and 70. Friedman's tests were used to compare the *cis-trans* ratio in serum β-carotene on days 0, 21, and 28 within group 1. To assess the time required for the isotopic enrichment of β-carotene and retinol in serum to reach a plateau, a Kruskal-Wallis test was used to compare the regression coefficients of the vitamin A activity of β-carotene in oil compared with that of retinol among groups. A one-sample *t* test was used to test whether the regression coefficient differed from 0 when the 3 groups were pooled.

The intraindividual CV was assessed by one-way analysis of variance with use of the vitamin A activity of β-carotene in oil compared with that of retinol as the dependent variable and subject number as a factor. The root mean square (residual SD) is a measure of reproducibility. The intraindividual CV was derived by dividing this SD by the mean vitamin A activity of β-carotene in oil compared with that of retinol for all data.

All tests were two-sided and P values <0.05 were considered significant. The computer package SPSS (version 7.5.3; SPSS Inc, Chicago) was used for all statistical calculations.

TABLE 2
Characteristics of the population on day 21[1]

	Total (n = 16 girls, 19 boys)	Group 1 (n = 6 girls, 6 boys)	Group 2 (n = 6 girls, 6 boys)	Group 3 (n = 4 girls, 7 boys)
Age (y)	9.3 ± 0.8[2]	9.3 ± 0.9	9.6 ± 0.8	9.0 ± 0.8
Weight (kg)	23.5 ± 2.9	24.4 ± 3.1	23.2 ± 3.1	22.8 ± 2.5
Height (m)	1.25 ± 0.06	1.27 ± 0.06	1.23 ± 0.07	1.24 ± 0.06
Serum retinol (μmol/L)	0.86 ± 0.20	0.85 ± 0.22	0.92 ± 0.23	0.81 ± 0.13
Serum β-carotene (μmol/L)	0.14 ± 0.05	0.14 ± 0.07	0.14 ± 0.05	0.13 ± 0.04
Hemoglobin (g/L)	126.0 (121.0, 131.0)[3]	124.5 (118.5, 128.0)	126.5 (121.3, 132.5)	127.0 (121.0, 133.0)
Parasitic infestation (% with positive stool)[4]	62	67	64	55
Ascaris lumbricoides	38	42	45	27
Trichuris trichuria	59	67	55	55
No parasitic infestation (%)	38	33	36	45
Egg load in infested children (epg)[5]				
Ascaris lumbricoides	400 (50, 850)	150 (25, 475)	450 (75, 5075)	400 (0, 3000)
Trichuris trichuria	100 (50, 188)	125 (50, 225)	100 (38, 150)	150 (50, 250)

[1] There were no significant differences among groups.
[2] $\bar{x}$ ± SD.
[3] Median (25th, 75th percentile).
[4] Parasitic infestation, as diagnosed by the Kato Katz and Ridley method (31), and egg load, as quantified by the Kato Katz method (31), were determined at screening only; n = 34. Infestation with *Entamoeba hystolitica* and hookworm was not found in the feces of these children.
[5] Eggs per gram feces as diagnosed by the Kato Katz method (31) for those infested with *Ascaris lumbricoides* and *Trichuris trichuria*, respectively.

RESULTS

On the first day of the intervention, we made minor changes in the distribution of the children throughout the 3 groups because some children were absent. As a result, group 1 comprised 7 children with an initial serum retinol concentration <0.70 μmol/L (low) and 5 children with an initial serum retinol concentration >1.05 μmol/L (high), group 2 comprised 4 children with initial low and 8 children with initial high serum retinol concentrations, and group 3 comprised 6 children with initial low and 6 children with initial high serum retinol concentrations. Serum retinol concentrations of samples obtained during the intervention, which started 2.5 mo after the screening, showed that classification of children according to vitamin A status could not be maintained. Therefore, the effect of vitamin A status on the bioefficacy of β-carotene could not be assessed.

Data on the isotopic enrichment of retinol with [$^{13}C_{10}$]retinol were not available for 2 children from group 1 on day 28 and for 1 child from group 3 on day 21. For this latter child, no data were available on the isotopic enrichment of retinol with [$^{13}C_5$]retinol on day 21. Therefore, for these days, the amount of β-carotene required to form 1 μg retinol could not be calculated for these children. Data are presented for 35 of the 36 children enrolled because one child from group 3 (with an initial high serum retinol concentration) withdrew from the study.

The descriptive characteristics of the children on day 21 are given in **Table 2**; on this day, data were available for all children. No significant differences in any characteristic were found among the 3 groups.

The drawing of the third blood sample from 3 children (one from group 1 and 2 from group 2) was postponed for 1 wk because the children had been absent for 4 d during the week before the planned blood drawing day. During the additional week, these children continued on the experimental regimen. Blood was drawn only on days on which a child did not show signs of infection as judged by a physician. White blood cell counts in all blood samples taken were $<1 \times 10^{10}$/L, indicating lack of apparent infection on the day of blood collection (25).

Children in groups 1, 2, and 3 consumed 2 capsules/d for 27, 41, or 69 d, respectively. On day 22, which was a religious Muslim holiday, no capsules were provided. Records of attendance showed that 96% of the capsules were consumed (25th and 75th percentiles: 91%, 100%). Data from the 3 children for whom blood sampling was postponed for 1 wk were not included in these capsule compliance calculations, but were included in all other calculations. The median compliance of groups 1, 2, and 3 was 100%, 98%, and 92%, respectively. Correction of data for the lower compliance of group 3 did not change the conclusions of this study.

Energy and nutrient intakes

Energy and nutrients provided and consumed during the screening and the intervention are shown in **Table 3**. No food or capsules were provided during the screening period. Energy and nutrient intakes during the screening and the intervention did not differ significantly among the 3 groups studied, except for retinol intake during the intervention (20, 16, and 8 μg retinol/d for groups 1, 2, and 3, respectively). Because these differences were not biologically relevant, only intake data from the total group are shown. Energy, fat, protein, carbohydrate, iron, and retinol intakes were significantly higher during the intervention than during the screening ($P < 0.05$). The additional retinol was derived from the capsules (87 RE/d). During the intervention, retinol intake from foods not provided by us was low (15 RE/d). β-Carotene intake was significantly lower during the intervention than during the screening. β-Carotene intake from the food and capsules provided was 223 μg β-carotene/d, of which 160 μg/d was derived from the capsules and 63 μg/d from the foods. During the intervention, β-carotene intake from foods not provided by us was 155 μg/d.

Estimates of energy, fat, protein, carbohydrate, iron, and retinol intakes based on the diaries were higher than those based on the 24-h recalls. Except for retinol, these differences were significant (data not shown). The estimate of β-carotene intake based on the diaries was lower than that based on the 24-h-recalls, but this difference was not significant.

TABLE 3
Energy and nutrients provided and consumed daily during the screening and the intervention[1]

	Screening	Intervention			
			Consumed		
	Consumed[2]	Provided[3] in menus	From food provided and capsules[4]	From other foods[2]	Total[5]
Energy (MJ)[6]	5.1 (4.7, 5.6)[7]	5.80	4.1 (3.9, 4.2)	3.1 (2.8, 3.4)	7.2 (6.9, 7.5)
Fat (g)	26 (23, 30)	33 [21][8,9]	24 (23, 25)	17 (15, 19)	41 (39, 43)
Protein (g)	31 (28, 35)	28 [8][8,9]	19 (18, 20)	19 (17, 20)	38 (36, 40)
Carbohydrates (g)	237 (213, 261)	239 [70][8,10]	128 (125, 132)	140 (120, 160)	268 (247, 289)
Iron (mg)	11.3 (10.0, 12.6)	1.5[9]	7.6 (7.4, 7.9)	6.8 (6.0, 7.6)	14.4 (13.6, 15.3)
Retinol (μg)	38 (22, 53)	—[11]	87 (87, 87)	15 (11, 19)	102 (98, 106)
Total β-carotene (μg)	964 (612, 1317)	100[12]	223 (219, 227)	155 (116, 195)	378 (339, 417)

[1] n = 35.
[2] Energy and nutrient intakes were calculated by using the diaries and a computer program (*see* Methods).
[3] Mean energy and nutrient contents of 5 menus on the basis of analysis of duplicate portions.
[4] Energy and nutrient intakes were calculated by using the records of attendance at meals, the diaries (in which the children recorded the amount not eaten), and a computer program. Irrespective of compliance with capsule consumption, 87 μg retinol, 160 μg β-carotene, 0.7 g fat, and 26 kJ energy was added to the daily intake of these nutrients and energy.
[5] Sum of energy and nutrient intakes during the intervention from food, both provided and not provided, and capsules.
[6] Energy was calculated by multiplying the weight (g) of fat, protein, and carbohydrates by 38, 17, and 17 kJ, respectively.
[7] $\bar{x}$; 95% CI in parentheses.
[8] % of energy in brackets.
[9] Analyses were carried out at the Nutrition Research and Development Centre, Bogor, Indonesia.
[10] Calculated by difference.
[11] Below the limit of detection of ≈3.5 pg/g food.
[12] Analyses were carried out at Wageningen University. Total β-carotene is the sum of *all-trans*-β-carotene (74 μg) and *cis*-β-carotene (26 μg). Other carotenoids in foods provided were lutein (228 μg) and α-carotene (26 μg).

Bioefficacy of β-carotene converted to retinol

Shown in **Table 4** are the concentrations of retinol and β-carotene in serum during the intervention. There were no significant differences in concentrations of either retinol or β-carotene in serum within groups (eg, for group 2 on days 21, 42, and 49) or among groups (eg, on day 21 for groups 1, 2, and 3). Although labeled β-carotene can be synthesized as >95% in the *all-trans* configuration, the β-carotene in the capsules used in this study had a *cis-trans* ratio of 3:1. Because β-carotene intake from the capsules was low (160 μg/d), the *cis-trans* ratio of serum β-carotene was also low (ranging from 0.01 to 0.18) and did not change significantly during the intervention (day 0 compared with days 21 and 28 in group 1; data not shown).

Also shown in Table 4 is the isotopic enrichment in serum of retinol with both [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of β-carotene with [$^{13}C_{10}$]β-carotene. There were no significant differences in the isotopic enrichment of retinol and β-carotene or in the vitamin A activity of β-carotene in oil compared with that of retinol among groups on day 21. Serum concentrations of retinol and β-carotene, the degree of isotopic enrichment of retinol and β-carotene, and the vitamin A activity of β-carotene in oil compared with that of retinol also did not differ significantly within any group on any blood collection day between those with initial low and high serum retinol concentrations, except for the serum retinol concentration on day 49 in group 2 (data not shown). There was no significant correlation between the serum retinol concentration at screening and the vitamin A activity of β-carotene in oil compared with that of retinol on days 21, 28, 42, 49, 63, and 70 (correlation coefficients ranged from –0.142 to 0.289).

To calculate the vitamin A activity of β-carotene in oil compared with that of retinol, we used the formulas described in Table 1. Data from a 9-y-old boy on day 21 are shown in Table 1 as an example. Shown in **Figure 3** are values for the mean vitamin A activity of β-carotene in oil compared with that of retinol per group per day. The coefficient of the regression of the vitamin A activity of β-carotene in oil compared with that of retinol was not significantly different among groups. When the 3 groups were pooled, the regression coefficient was not significantly different from 0 (P = 0.137). Thus, in these children, isotopic enrichment of retinol and β-carotene in serum reached a plateau by day 21. On the basis of data from days 21 to 70, the mean vitamin A activity of β-carotene in oil compared with that of retinol was 0.4149 (95% CI: 0.3637, 0.4661). This corresponds to 2.4 μg (95% CI: 2.1, 2.7) β-carotene required to form 1 μg retinol in the body. If the data are recalculated assuming that *all-trans*-β-carotene and *cis*-β-carotene are absorbed to the same extent and that the vitamin A potency of *cis*-β-carotene is 50% of that of *all-trans*-β-carotene, the vitamin A activity of β-carotene in oil compared with that of retinol is higher: 0.6638 (95% CI: 0.5819, 0.7458), corresponding to 1.5 μg (95% CI: 1.3, 1.7) β-carotene required to form 1 μg retinol.

The intraindividual variation in response comprises both the biological variation within subjects and the variation in the chemical analyses. The latter was arbitrarily set at 0 because it is thought to be much lower than the biological variation within subjects. The SD of the vitamin A activity of β-carotene in oil compared with that of retinol was 0.0895, which, divided by the mean based on all data (0.4149), resulted in an intraindividual CV of 22%.

TABLE 4
Serum concentrations of retinol and β-carotene and degree of isotopic enrichment of retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of *all-trans*-β-carotene with [$^{13}C_{10}$]β-carotene[1]

	Day 0: Group 1	Day 21			Day 28: Group 1	Day 42: Group 2	Day 49: Group 2	Day 63: Group 3	Day 70: Group 3
		Group 1	Group 2	Group 3					
Retinol									
Concentration (μmol/L)	0.82 (0.67, 0.98)	0.83 (0.62, 1.08)	0.89 (0.79, 1.10)	0.83 (0.69, 0.92)	0.80 (0.64, 0.92)	0.84 (0.76, 0.98)	0.88 (0.76, 1.05)[2]	0.91 (0.70, 1.00)	0.78 (0.55, 0.91)
$^{13}C_5$ enrichment[3]	0	0.027 (0.021, 0.042)	0.033 (0.027, 0.045)	0.020[4] (0.017, 0.047)	0.031 (0.024, 0.045)	0.048 (0.032, 0.062)	0.051 (0.035, 0.075)	0.048 (0.037, 0.064)	0.048 (0.035, 0.059)
$^{13}C_{10}$ enrichment[5]	0	0.052 (0.027, 0.079)	0.052 (0.043, 0.076)	0.050[4] (0.015, 0.064)	0.059[4] (0.046, 0.082)	0.070 (0.054, 0.110)	0.071 (0.058, 0.079)	0.056 (0.032, 0.085)	0.060 (0.032, 0.073)
β-Carotene									
Concentration (μmol/L)	0.09 (0.07, 0.20)	0.14 (0.08, 0.16)	0.14 (0.11, 0.17)	0.13 (0.09, 0.17)	0.11 (0.09, 0.18)	0.18 (0.10, 0.23)	0.16 (0.11, 0.24)	0.15 (0.13, 0.21)	0.16 (0.10, 0.20)
$^{13}C_{10}$ enrichment[6]	0	0.26 (0.18, 0.28)	0.23 (0.19, 0.30)	0.24 (0.21, 0.24)	0.25 (0.17, 0.30)	0.23 (0.18, 0.29)	0.24 (0.18, 0.28)	0.27 (0.16, 0.31)	0.26 (0.17, 0.32)

[1] Median (25th, 75th percentile). Data were obtained from group 1 (n = 12) on days 0, 21, and 28; from group 2 (n = 12) on days 21, 42, and 49; and from group 3 (n = 11) on days 21, 63, and 70. Concentrations of retinol and β-carotene in serum were not significantly different among groups (eg, on day 21 for groups 1, 2, and 3) or within groups (eg, for group 2 on days 21, 42, and 49). There were also no significant differences in the isotopic enrichment of retinol and β-carotene among groups on day 21.

[2] The serum retinol concentration of those with an initial serum retinol concentration < 0.70 μmol/L [0.74 (0.58, 0.86)] was significantly lower than that of those with an initial serum retinol concentration >1.05 μmol/L [0.90 (0.86, 1.11)], P = 0.028.

[3] $M_{274,sR}/(M_{269,sR} + M_{274,sR} + M_{279,sR})$, where $M_{274,sR}$ is the signal measured by liquid chromatography–mass spectrometry at m/z 274 and sR is serum retinol.

[4] n = 10.

[5] $M_{279,sR}/(M_{269,sR} + M_{274,sR} + M_{279,sR})$.

[6] $M_{547,sC}/(M_{537,sC} + M_{547,sC})$, where sC is serum β-carotene.

Parasitic infestation and anthropometry

The prevalence and intensity of intestinal parasites were low in the children studied. Therefore, we expect that the effect of intestinal parasites on the findings of this study was negligible.

On days 0, 21, and 28 there were no significant differences in weight and height among the 3 groups. Within groups 1 and 2, height was constant and body weight increased by ≈1 kg during the intervention (28 and 49 d, respectively). Within group 3, height increased by ≈1 cm and body weight by ≈1 kg during the intervention (70 d). Because these changes were not expected to affect the outcome of the study, no adjustments in the statistical analyses were made for them.

DISCUSSION

In this study of Indonesian schoolchildren, 1.0 μg retinol was derived from 2.4 μg β-carotene dissolved in oil. The intraindividual CV was low (22%). Furthermore, the isotopic enrichment of retinol and β-carotene in serum reached a plateau by day 21.

The method used in this study has several advantages. First, the doses of labeled β-carotene and retinol used were very low and thus did not perturb the metabolism of unlabeled retinol and β-carotene. Second, the stable isotopes used entail no health risk and thus can be applied to study humans. Third, the multiple doses enabled the isotopic enrichment of both retinol and β-carotene in serum to reach a plateau. This plateau makes analysis of data and subsequent calculation and interpretation of results easier than in single-dose studies in which data are analyzed with use of complicated mathematical models. Single-dose studies can be useful for qualitatively studying the kinetics of the bioavailability and bioconversion of β-carotene. Fourth, administering specifically labeled [$^{13}C_{10}$]β-carotene plus [$^{13}C_{10}$]retinol enables the latter to be distinguished from circulating retinol (mainly [^{12}C]retinol) and from retinol formed in the body from the [$^{13}C_{10}$]β-carotene administered ([$^{13}C_5$]retinol). This is impossible after the administration of uniformly labeled retinol and β-carotene. Without administering retinol, although it would be possible to distinguish between circulating retinol and retinol formed in the body from labeled β-carotene, it would be impossible to establish retinol absorption and thus to quantify retinol and β-carotene bioavailability.

Tang et al (12) developed a stable-isotope method that uses single doses of [$^2H_8$]β-carotene and [$^2H_8$]retinyl acetate given on separate occasions up to 2 y apart. It would have been possible to administer [$^2H_8$]β-carotene and [$^2H_8$]retinyl acetate simultaneously with subsequent measurement of [$^2H_8$]retinol and [$^2H_4$]retinol, thus eliminating differences related to giving the labeled compounds at different times. However, the low isotopic purity of the ^2H-labeled compounds used in Tang et al's method would decrease the signal-to-noise ratio, especially when enrichment approaches baseline. Thus, the use of not only ^2H-labeled compounds but also single doses would possibly contribute to a lower intrinsic accuracy of Tang's method compared with our method.

A fifth advantage of our method is that the APCI LC-MS method developed for this study does not require derivatization or saponification of serum samples before analysis, whereas the use of gas chromatography–mass spectrometry does require these labor-intensive processes (32). Intrinsic labeling yields molecules with a variable number of ^{13}C or ^2H atoms. This unnecessarily complicates the use of MS for the detection and subsequent quantification of bioavailability. Finally, for this study, we synthesized extrinsically and specifically labeled retinol and β-carotene. These compounds have several advan-

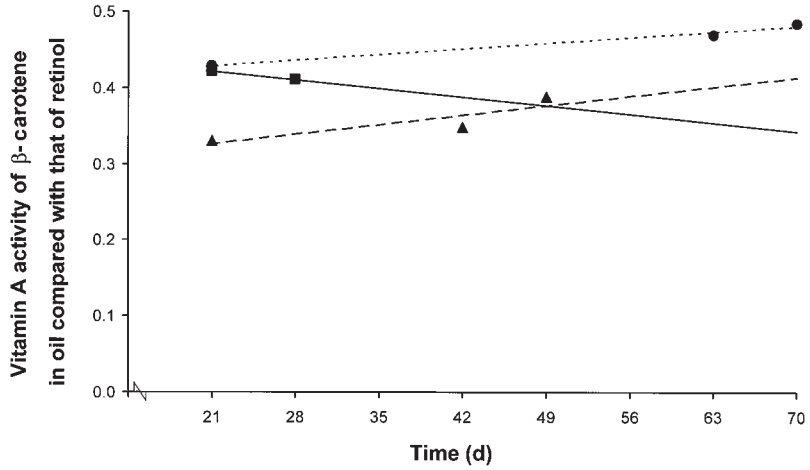

FIGURE 3. Vitamin A activity on a weight basis of β-carotene (with a *cis-trans* ratio of 3:1) dissolved in oil compared with that of retinol. Data are means of values obtained from subjects in each group on various days: for group 1 (■) on days 21 (n = 12) and 28 (n = 10); for group 2 (▲; n = 12) on days 21, 42, and 49; and for group 3 (●) on days 21 (n = 10), 63 (n = 11), and 70 (n = 11). For calculation of the regression coefficient, the mean values on days 42 and 49 in group 2 and on days 63 and 70 in group 3 were calculated. The regression coefficients for group 1 (solid line), group 2 (dashed line), and group 3 (dotted line) were 0.0049, 0.0016, and 0.0015, respectively.

tages, such as the high incorporation of ^{13}C atoms (99%) and the absence of scrambling (ie, leakage of ^{13}C atoms to positions in the molecule other than those in which they were initially incorporated). Scrambling occurs more often when deuterium is used for labeling, thus requiring measurement at different masses in the MS method. Summation of the signals leads to higher analytic variation. In addition, the labeled compounds can be synthesized as 95% in the *all-trans* configuration, which is the configuration in which they occur in nature.

The low intraindividual CV in the vitamin A activity of β-carotene in oil compared with that of retinol was derived in part from the experimental design. The high compliance with capsule consumption (96%) also contributed to the low variation observed. Unfortunately, as noted above, only 25% of the labeled β-carotene was present as *all-trans*-β-carotene. Although care was taken to prevent isomerization of synthesized *all-trans*-β-carotene, *cis*-isomerization of labeled β-carotene probably occurred during capsule preparation (33). Isomerization did not continue after capsule preparation because the ratio of *cis*-β-carotene to *all-trans*-β-carotene in the capsules was measured by HPLC after 9, 22, 29, and 50 d of storage at 4 and 30°C and remained stable. More knowledge on the metabolism of *cis*-β-carotene is required to decide whether the conversion factor given here may be even lower.

In this study, 2.4 μg β-carotene was required to form 1 μg retinol. If the efficiency with which dietary β-carotene is absorbed and converted to retinol in the body is 100%, then 0.94 μg β-carotene would have been required. Thus, in this study, bioefficacy was only 39%. At this stage of development of our model, it is not possible to quantify bioavailability and bioconversion separately. Note that in this study none of the values for the vitamin A activity of β-carotene in oil compared with that of retinol were ≥1.06, which corresponds to a bioefficacy of β-carotene of ≥100%. Vitamin E was added to the capsules as an antioxidant for the oil, but we do not know to what extent the vitamin E affected the bioefficacy of the β-carotene in oil.

The isotopic enrichment of retinol and β-carotene in serum had reached a plateau when first measured on day 21. Therefore, we now plan to examine whether the plateau of isotopic enrichment is reached earlier.

Estimates of intake of several nutrients derived from the 24-h recalls were lower than the estimates from the dietary records, as reported earlier (34, 35). The main purpose of the 24-h recall method was to make the children aware of their food intake. Neither of the methods used provides reliable measurements of food intake at the individual level, but both were appropriate for assessing whether differences in food intake existed among the 3 groups during the intervention and between the screening and the intervention.

Dietary intake of β-carotene was higher during the screening than during the intervention because we provided a low-retinol, low-carotenoid diet during the intervention, but there were no significant differences in serum β-carotene concentrations between days 0 and 21. This can largely be explained by the low bioavailability of β-carotene from food. For energy and all other nutrients, intakes were higher during the intervention than during the screening, indicating that in this age group dietary records may overestimate food intake.

This advanced stable-isotope technique can be extended to address the important question of the vitamin A potency of β-carotene in fruit and vegetables. Although this method has not yet been tested for its ability to measure the bioefficacy of β-carotene in fruit and vegetables, it is thought to be capable of doing so if the following design is used. Subjects would be studied for 2 consecutive periods of a maximum of 3 wk each. During both periods, the subjects would consume 2 or 3 capsules/d, 7 d/wk, and each capsule would contain small doses (maximum: 50 μg) of [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinyl palmitate. The subjects would be provided a low-retinol, low-carotenoid diet during both periods; during the second period, this diet would be supplemented with 2 or 3 portions of fruit or vegetables per day. Blood samples, preferably 2 fasting samples taken 2 d apart, would be drawn at baseline and at the end of each period. The degree of isotopic enrichment in serum retinol and β-carotene would be measured as in this study. Then, the change in isotopic enrichment during the second period (compared with the first

period) would be calculated. The extent of dilution in isotopic enrichment during the second period indicates the bioefficacy of β-carotene in fruit or vegetables.

Because of the precision of this method, fewer subjects would be required to measure the bioefficacy of dietary β-carotene than were required with the methods used previously. To illustrate this, we performed sample size calculations for a hypothetical study in which the vitamin A activity of β-carotene in oil is assessed in 2 groups, one with high and one with low serum retinol concentrations. To determine which difference in bioefficacy can be expected, we can use the findings of Villard and Bates (36) that vitamin A–deficient rats had roughly a 27% higher β-carotene 15,15′-dioxygenase activity than did control rats, indicating that vitamin A deficiency increases bioconversion. We can then calculate the minimum sample size required to detect a 27% difference between 2 groups in the vitamin A activity of β-carotene in oil compared with that of retinol, with a power of 0.90 and an α value of 0.05, with the following formula (37):

$$n = (u + v)^2 \times (SD_1^2 + SD_2^2)/(\mu_1 - \mu_2)^2 \qquad (1)$$

where n is the sample size of groups 1 and 2; u is the one-sided percentage point of the normal distribution corresponding to 100% − the power (eg, if power = 90%, 100% − power = 10% and $u = 1.28$); v is the proportion (%) of the normal distribution corresponding to the required two-sided significance level (eg, if $\alpha = 0.05$, $v = 1.96$); SD_1 and SD_2 are the SDs of the measurement, 0.0895; and μ_1 and μ_2 are the mean vitamin A activity of β-carotene in oil compared with that of retinol in groups 1 and 2, respectively.

Assuming that μ_1 (high serum retinol group) is 0.4149 and μ_2 is 27% higher (0.5269), the group sample size is 14 when based on data from 1 sample per period or 7 when based on data from 2 samples per period (where SD is $SD/\sqrt{2}$). Because a plateau of isotopic enrichment of serum retinol and β-carotene is reached by 21 d, this would result in 14 or 28 subjects participating for 21 d, resulting in 294 or 588 subject days, respectively. From experience in our laboratories, reducing sample size is the most cost-effective measure for reducing the costs of a dietary controlled trial, and can even counterbalance the use of compounds labeled with a stable isotope and LC-MS analyses, both of which are relatively expensive.

In conclusion, in this study of 35 children, 2.4 μg (95% CI: 2.1, 2.7) β-carotene (with a *cis-trans* ratio of 3:1) dissolved in oil was equivalent to 1 μg retinol in the body. This is 27% less than the 3.3 μg β-carotene (with an unknown *cis-trans* ratio) dissolved in oil proposed by a committee of the International Union of Pure and Applied Chemistry in 1959 (38), a value that was based on 2 studies in a limited number of subjects (4, 39). This value of 3.3 μg is quoted in the FAO/WHO guidelines of 1967 (40) and 1988 (41). Although our findings are in line with earlier data, this is the first time this method was used. Therefore, our findings need to be confirmed. The magnitude of the effect of several SLAMENGHI factors on carotenoid bioefficacy can be studied quantitatively with this stable-isotope method. This will enable a more accurate evaluation of food-based approaches to eliminating vitamin A deficiency.

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REFERENCES

1. Beaton GH, Martorell R, Aronson KJ, et al. Effectiveness of vitamin A supplementation in the control of young child morbidity and mortality in developing countries. ACC/SCN State-of-the-Art Series: nutrition policy discussion paper 13. Toronto: University of Toronto, 1993.
2. de Pee S, West CE, Muhilal, Karyadi D, Hautvast JGAJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. Lancet 1995;346:75–81.
3. de Pee S, West CE, Permaesih D, Martuti S, Muhilal, Hautvast JGAJ. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and β-carotene in schoolchildren in Indonesia. Am J Clin Nutr 1998;68:1058–67.
4. Hume EM, Krebs HA, eds. Vitamin A requirement of human adults. An experimental study of vitamin A deprivation in man. Medical Research Council (Vitamin A Sub-Committee of the Accessory Food Factors Committee). London: His Majesty's Stationary Office, 1949. (Medical Research Council special report series 264.)
5. Jackson MJ. The assessment of bioavailability of micronutrients: introduction. Eur J Clin Nutr 1997;51(suppl):S1–2.
6. de Pee S, West CE. Dietary carotenoids and their role in combating vitamin A deficiency: a review of the literature. Eur J Clin Nutr 1996;50(suppl):S38–53.
7. Castenmiller JJM, West CE. Bioavailability and bioconversion of carotenoids. Annu Rev Nutr 1998;18:19–38.
8. Goodman DS, Blomstrand R, Werner B, Huang HS, Shiratori T. The intestinal absorption and metabolism of vitamin A and β-carotene in man. J Clin Invest 1966;45:1615–23.
9. Blomstrand R, Werner B. Studies on the intestinal absorption of radioactive β-carotene and vitamin A in man. Conversion of β-carotene into vitamin A. Scand J Clin Lab Invest 1967;19:339–45.
10. Novotny JA, Dueker SR, Zech LA, Clifford AJ. Compartmental analysis of the dynamics of β-carotene metabolism in an adult volunteer. J Lipid Res 1995;36:1825–38.
11. Burri BJ, Park JY. Compartmental models of vitamin A. Adv Exp Med Biol 1998;445:225–37.
12. Tang G, Qin J, Dolnikowski GG, Russell RM. Vitamin A equivalence of β-carotene in a woman as determined by a stable isotope reference method. Eur J Nutr 2000;39:7–11.
13. Parker RS, Swanson JE, Marmor B, et al. Study of β-carotene metabolism in humans using ^{13}C-β-carotene and high-precision isotope ratio mass spectrometry. Ann N Y Acad Sci 1993;691:86–95.
14. Tang G, Grusak MA, Qin J, Dolnikowski GG, Russell RM. Vitamin A activity of spinach determined by isotope reference method. Report of the XIX International Vitamin A Consultative Group meeting. Washington, DC: ILSI, 1999 (abstr).
15. Lugtenburg J, Creemers AFL, Verhoeven MA, et al. Synthesis of ^{13}C labeled carotenoids and retinoids. Pure Appl Chem 1999;71:2245–51.
16. United States Pharmacopoeia. The pharmacopoeia of the United States of America. 12th ed. Rockville, MD: US Pharmacopoeial Convention, 1990.
17. Joint FAO/WHO Expert Committee on Food Additives. Compendium of food additive specifications. Combined specifications from the first to the thirty-seventh meeting 1956–1990. FAO Food Nutr Pap 1992;52:25–31.
18. European Pharmacopoeia. 2nd ed. Sainte-Ruffine-les-Metz, France: Maisonneuve, 1993.

19. Hulshof PJM, Chao X, van de Bovenkamp P, Muhilal, West CE. Application of a validated method for the determination of provitamin A carotenoids in Indonesian foods of different maturity and origin. J Agric Food Chem 1997;45:1174–9.
20. Nio OK. Daftar analysis bahan makanan. (List of analysis of food ingredients.) Jakarta, Indonesia: Universitas Indonesia, Fakultas Kedokteran, 1992 (in Indonesian).
21. Greenfield H, Southgate DAT. Food composition data. Production, management and use. London: Elsevier Science Publishers, 1992.
22. Craft NE, Brown ED, Smith JC Jr. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988;34:44–8. (Published erratum appears in Clin Chem 1988;34:1505.)
23. Comstock GW, Alberg AJ, Helzlsouer KJ. Reported effects of long-term freezer storage on concentrations of retinol, beta-carotene, and alpha-tocopherol in serum or plasma summarized. Clin Chem 1993; 39:1075–8.
24. de Pee S, West CE, van Staveren WA, Muhilal. Vitamin A intake of breastfeeding women in Indonesia: critical evaluation of a semi-quantitative food frequency questionnaire. In: de Pee S, ed. Food-based approaches for controlling vitamin A deficiency: studies in breastfeeding women in Indonesia. PhD thesis. Wageningen Agricultural University, Wageningen, Netherlands, 1996.
25. Pijlman G, Sanders GTB. Beknopte handleiding voor het klinisch-chemisch laboratorium-onderzoek. (Concise manual for clinical chemistry laboratory tests.) Lochem, Netherlands: Tijdstroom BV, 1976 (in Dutch).
26. Wintrobe MM. Nutritional anaemias: report of a WHO scientific group, Geneva, 13–17 March 1967. World Health Organ Tech Rep Ser 1968;405.
27. Thurnham DI, Smith E, Flora PS. Concurrent liquid-chromatographic assay of retinol, alpha-tocopherol, beta-carotene, alpha-carotene, lycopene, and beta-cryptoxanthin in plasma, with tocopherol acetate as internal standard. Clin Chem 1988;34:377–81.
28. Craft NE, Wise SA, Soares JH Jr. Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. J Chromatogr 1992;589:171–6.
29. van Breemen RB, Nikolic D, Xu X, et al. Development of a method for quantitation of retinol and retinyl palmitate in human serum using high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. J Chromatogr A 1998;794:245–51.
30. Wang Y, Xu X, Van Lieshout M, et al. A liquid chromatography-mass spectrometry method for the quantification of bioavailability and bioconversion of β-carotene to retinol in humans. Anal Chem 2000;72:4999–5003.
31. Polderman AM, Rijpstra AC, Blotkamp J, van Knapen F, Laarman J. Medische parasitologie: handleiding bij de laboratoriumdiagnostiek. (Medical parasitology: manual for laboratory diagnostic tests.) 2nd ed. Houten, Netherlands: Bohn Stafleu Van Loghum, 1993 (in Dutch).
32. Swanson JE, Wang YY, Goodman KJ, Parker RS. Experimental approaches to the study of β-carotene metabolism: potential of a ^{13}C tracer approach to modeling β-carotene kinetics in humans. Adv Food Nutr Res 1996;40:55–79.
33. Chandler LA, Schwartz SJ. HPLC separation of *cis-trans* carotene isomers in fresh and processed fruits and vegetables. J Food Sci 1987;52:669–72.
34. Cameron M, van Staveren WA. Manual on methodology for food consumption studies. Oxford, United Kingdom: Oxford University Press, 1988.
35. Burk M. Analysis of food consumption survey data for developing countries. FAO Food Nutr Pap 1980;16:101.
36. Villard L, Bates CJ. Carotene dioxygenase (EC 1.13.11.21) activity in rat intestine: effects of vitamin A deficiency and of pregnancy. Br J Nutr 1986;56:115–22.
37. Kirkwood BR. Essentials of medical statistics. Oxford, United Kingdom: Blackwell, 1988.
38. IUPAC. The vitamin A potency of beta-carotene. London: Butterworth Scientific Publications, 1959.
39. Wagner K-H. Die experimentelle Avitaminose A beim Menschen. (Experimentally low vitamin A status in humans.) Z Physiol Chem 1940;264:153–88 (in German). (Published erratum appears in Z Physiol Chem 1940;264:59.)
40. FAO/WHO. Requirements of vitamin A, thiamine, riboflavin and niacin. FAO Food Nutr Ser 1967;8:15–6, 23–5.
41. FAO/WHO. Requirements of vitamin A, iron, folate and vitamin B_{12}. FAO Food Nutr Ser 1988;23:17–8, 29–30.