

## Extraction of Carotenoids from Feces, Enabling the Bioavailability of $\beta$ -Carotene to Be Studied in Indonesian Children

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Previously, we have presented a method for quantifying  $\beta$ -carotene bioavailability based on analysis in serum, following administration of <sup>13</sup>C-labeled  $\beta$ -carotene. Because stool samples can be collected noninvasively, we have now extended the method to measure the bioavailability based on measurements in feces. An extraction method was developed to enable measurement of concentrations and degree of isotopic enrichment of retinol, retinyl palmitate and carotenoids in feces. Relative bioavailability of  $\beta$ -carotene from pumpkin ( $n = 6$ ) was found to be 1.8 times (interval, 0.6, 5.5) greater than that from spinach ( $n = 8$ ), based on data from feces compared with 1.7 times (interval, 0.9, 3.1) based on data from serum.

**KEYWORDS:** Bioavailability; carotenoids;  $\beta$ -carotene; retinol; vitamin A; humans; serum; feces; HPLC; LC-MS; stable isotopes; <sup>13</sup>C; liquid chromatography-mass spectrometry; children; Indonesia; oil; fruit; vegetables

### INTRODUCTION

Vitamin A deficiency is a problem in developing countries (1–4). Supplementation with vitamin A of children under 5 years of age in such countries reduces morbidity and mortality by 23%, probably because of improved immunocompetence (1). Improved vitamin A status can be achieved by reducing the demand for vitamin A or by increasing the effective supply of vitamin A or by combining both these approaches. Three factors determine the effective supply of vitamin A: the consumption of foods and pharmanutrients (i.e., dietary supplements), the content of vitamin A or its precursor in food or pharmanutrients consumed, and the bioefficacy of vitamin A or its precursor in the food or pharmanutrients. Unless the bioefficacy is sufficiently high, the effect of increasing the consumption of provitamin A-containing food and the provitamin A content of food consumed will be limited. Bioefficacy of provitamin A

carotenoids in plant foods is a particular problem, because plant foods are the major source of vitamin A in the diet of a large proportion of the world's population (5). Thus, the reduced estimate of bioefficacy of provitamin A (mainly  $\beta$ -carotene) from plant sources has greater meaning for their ability to achieve vitamin A sufficiency. The impact of the bioefficacy on the effective supply of vitamin A has received little attention until the 1990s, mainly because sensitive quantitative techniques were lacking (6).

Isotope techniques can provide accurate and precise estimates of bioavailability and bioefficacy. We developed a technique to quantify the bioavailability and bioefficacy of  $\beta$ -carotene in oil by using multiple low (<100  $\mu$ g) doses of  $\beta$ -carotene and retinol, each specifically labeled with 10 <sup>13</sup>C atoms. This technique has been tested in studies involving 35 (7) and 77 (8) Indonesian school children. In the latter study, the relative bioavailability and bioefficacy of  $\beta$ -carotene in spinach and pumpkin was also estimated. All results obtained from these studies were based on data from serum samples. Because stool samples can be collected noninvasively, the aim of the present study was to compare estimates of  $\beta$ -carotene bioavailability using data from serum with those based on data from feces. For this purpose, a sub-sample of children from our second study

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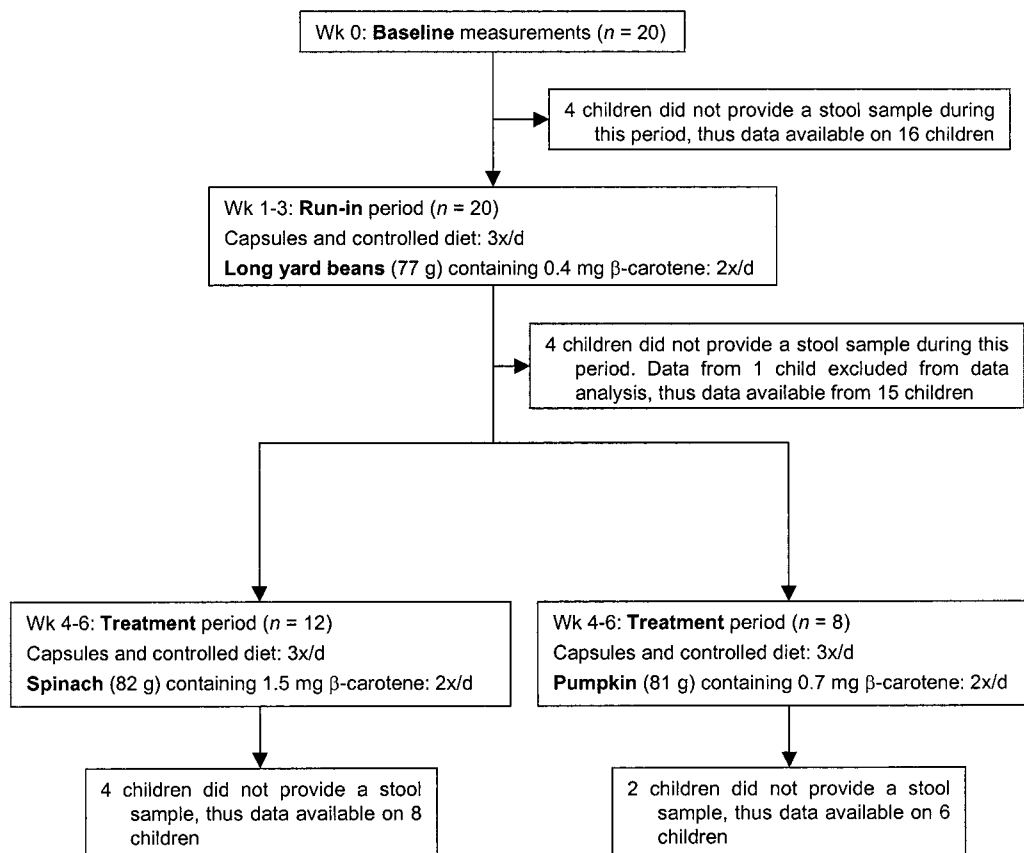
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**Figure 1.** Study design. Capsules contained 31  $\mu\text{g}$  of [ $^{13}\text{C}_{10}$ ] $\beta$ -carotene and 21  $\mu\text{g}$  of [ $^{13}\text{C}_{10}$ ]retinyl palmitate. The basal diet was low in retinol and carotenoids. At baseline and at the end of the run-in and treatment periods, blood and stools were collected. Wk = week.

(8) were asked to collect all their stools for 2 consecutive days, three times during the study. In feces, we measured the same variables as in serum, which required the development of an extraction technique to use in conjunction with existing HPLC (9) and liquid chromatography coupled with mass spectrometry methods (10, 11). We also extended the mathematical model to estimate the bioavailability of  $\beta$ -carotene. It is not possible to quantify the bioefficacy of  $\beta$ -carotene based on measurements in feces because the bioconversion of  $\beta$ -carotene is a metabolic process that occurs in the body.

## MATERIALS AND METHODS

**Definitions.** In this paper, a number of terms are used that need to be defined. Bioavailability is defined as the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (12) (i.e., the amount absorbed). Bioconversion is the fraction of a bioavailable nutrient (here, absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol). Bioefficacy is the fraction of an ingested nutrient (here, dietary provitamin A carotenoids) that is absorbed and converted to the active form of the nutrient (retinol) (7).

**Subjects.** The study was conducted from March to May 1999 in a rural village in Bogor District, West Java. Screening procedures and selection criteria of the study have been reported in detail elsewhere (8). There were no additional eligibility criteria for inclusion in the sub-sample of children being asked to collect their stools during the study. During the baseline measurements, 20 of the 80 apparently healthy school children (9 boys and 11 girls), aged 7–12 y, participating in the study were randomly selected to collect their stools during the study (Figure 1). The parents or guardians of these children were informed about the purpose and procedures of the stools collection and gave their written informed consent. At the end of the run-in period of 3 weeks, children were randomly allocated to receive either spinach ( $n = 12$ ) or pumpkin ( $n = 8$ ) during the subsequent treatment period of

3 weeks. 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 the Division of Human Nutrition of Wageningen University, The Netherlands.

**Study Type and Intervention.** The study was a randomized controlled dietary trial with two treatment arms—spinach and pumpkin—and a parallel design (Figure 1). During the study, comprising a 3-week run-in period followed by a 3-week treatment period, each child received capsules (3/d for 7d/wk) containing 31  $\mu\text{g}$  of [12,13,14,15,20,12',13',14',15',20'- $^{13}\text{C}_{10}$ ] $\beta$ -carotene (analyzed value) and 21  $\mu\text{g}$  of [8,9,10,11,12,13,14,15,19,20- $^{13}\text{C}_{10}$ ]retinyl palmitate (calculated based on analysis, equivalent to 12  $\mu\text{g}$  of retinol). Each capsule also contained 0.35 g of highly unsaturated sunflower oil and 0.12 mg of vitamin E as an antioxidant for the oil. Preparation of the labeled materials (13) and capsules is described elsewhere (8). Children consumed the capsules with a low-retinol, low-carotenoid meal, 3 times/d. During the run-in period; the lunch and afternoon meals were supplemented with a portion of 77 g of yard-long beans (*Vigna unguiculata*), containing 0.4 mg of  $\beta$ -carotene/portion (analyzed value). During the treatment period, children received daily either two portions of 82 g of spinach ( $n = 12$ ; *Amaranthus tricolor*) or 81 g of pumpkin ( $n = 8$ , *Cucurbita moschata*), containing 1.5 mg and 0.7 mg of  $\beta$ -carotene/portion, respectively (analyzed values). Preparation of the meals is described elsewhere (8). During the study, members of the research team supervised and recorded compliance of consumption of the capsules and meals. Main outcome measures of the study were the dilution of the degree of isotopic enrichment of retinol and  $\beta$ -carotene in serum and in feces during the treatment period (compared with the run-in period). By use of either data from serum or from feces, the relative bioavailability of  $\beta$ -carotene in spinach and pumpkin was estimated. Samples of serum and feces collected at the end of the run-in period were used for measurement of the bioavailability of  $\beta$ -carotene in oil.

**Measurements.** During baseline measurements (week 0) and at the end of the run-in period (week 3) and treatment period (week 6), a physician examined the children, and each child's weight, height, mid-

upper-arm-circumference (MUAC) were measured. During the one-week baseline measurements, children were asked to collect their stools over a 48 h-period (noon Day 2 until noon day 4). In the morning of day 4 of the baseline period, blood samples were drawn. Three days before the end of the run-in and of the treatment periods, children were asked to collect all their stools for 48 h (noon day 19 until noon day 21 and noon day 40 until noon day 42). At the end of each period, on Days 21 and 42, blood samples were drawn from the children (one sample/child/period). None of the children showed signs of infection, as judged by a physician, on days of blood collection. Thus, all blood samples could be collected on the days as intended.

Blood samples were used to obtain data as described previously (8): leukocytes counts, hemoglobin concentration, hematocrit values, concentration of retinol and various carotenoids, and degree of isotopic enrichment of retinol and  $\beta$ -carotene. Stool samples were used to obtain data on the following: prevalence and intensity of parasitic infestation (as described in 8); concentration of retinol, retinyl esters, and various carotenoids; and degree of isotopic enrichment of retinol and  $\beta$ -carotene.

Estimates of the energy and nutrient intakes were based on a combination of sources: diaries in which children daily recorded, both qualitatively and quantitatively, consumption of foods and drinks not provided by us; records of attendance and of consumption of the 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 (bg95k99t; 7) based on that developed by de Pee and colleagues (14) to which the energy and nutrient contents of the foods provided (on the basis of duplicate analyses of meals and supplements) were added. Collection of the duplicate samples and analysis of the energy and nutrient intakes in these samples has been described elsewhere (8). In this paper, we will only report retinol and carotenoid intakes from food provided.

**Collection of Feces Samples.** For collecting stool samples, each period, children received a plastic bucket and 10 plastic bags labeled with their ID number. They received instructions for the collection, storage (i.e., at a cool place in the dark) and transport of their stools. Children transported the samples to school in black plastic bags on the day of collection or the next morning. At school, the samples were stored in a cool box with chilling elements. Daily, this box was transported from school to the laboratory where the samples were stored at 4 °C. At the end of each 48-h period, samples from each child were pooled and homogenized. The weight of each pooled and homogenized sample was recorded. If necessary (for sample handling), a known amount of water was added to the samples before homogenization. Each homogenized sample was portioned over  $\leq 3$  plastic boxes, each containing up to 150 g of feces. All boxes were stored at  $-20$  °C for  $\approx 2$  mo. These procedures were done at the Nutrition Research and Development Centre in Bogor before the samples were packed on dry ice and transferred to Wageningen. There, the samples were stored for  $\approx 10$  mo at  $-20$  °C until thawed, pooled, homogenized and portioned over three plastic tubes containing up to 20 g. All tubes were stored at  $-20$  °C for  $\approx 4$  months before analysis.

**Extraction of Human Feces.** For the extraction of human feces, we extended a method for measuring the concentrations of provitamin A carotenoids in foods (9). Beginning 16 h prior to analysis, samples were allowed to thaw in the dark at 4 °C. An aliquot (2 g) was extracted in duplicate with 4 g of  $\text{Na}_2\text{SO}_4$ , 0.5 g of  $\text{CaCO}_3$ , 30 mL of tetrahydrofuran (THF) containing 0.01% butylated hydroxytoluene (BHT), and 1 mL of an internal standard in THF/methanol (3:1 v/v) containing a known amount of retinyl acetate (ca. 1  $\mu\text{g}$ ) in a 100-mL measuring cylinder, using a rod mixer (Polytron PT 20 OD, Kriens/Luzern, Switzerland) at moderate speed (speed 4) three times for 1 min. The solution was allowed to stand for 3–5 min, after which the extract was filtered through a filter paper (Whatman paper no 54, diameter 11 cm) in a glass funnel. The residue was re-extracted twice with 30 mL of THF. The volume of the combined filtrates was reduced to near dryness at 35 °C under nitrogen in a rotary evaporator (Büchi, Flawil, Switzerland). Using a glass pipet, 25 mL of THF/methanol (3:1 v/v) containing 0.01% BHT was added to the concentrated filtrate. After homogenizing, this sample was portioned over one crimpcap vial (1.5 mL; 403682, Phase Separation LTD, Deeside, UK) closed with a crimpcap (412115, Phase Separation) and three vials (1.8 mL; A4954–

010, Thermo Separation Products, San Jose, CA) closed with a screwcap. At the end of each day of sample preparation, the 1.5-mL vials without screwcaps were placed into the tray (protected from light, kept at 4 °C) of the HPLC system for duplicate injections of 25  $\mu\text{L}$ . The 1.8-mL vials were stored in containers at  $-80$  °C for  $\leq 2$  weeks before being packed on dry ice and transferred to the Department of Medicinal Chemistry and Pharmacognosy in Chicago. There, the extracts were stored at  $-80$  °C for  $\leq 2$  weeks before measurement of the degree of isotopic enrichment of  $\beta$ -carotene (11) and retinol (10;11).

**Storage of Feces Extracts.** The effect of storage of fecal extracts on the concentrations of  $\beta$ -carotene and lutein (the most prevalent carotenoids found in feces in this study) was assessed. For this purpose, two samples were extracted in duplicate following the above protocol. The extract was portioned ( $6 \times$  ca. 4 mL) over tubes (8-mL heavy duty centrifuge tubes with screw cap, Kimble Glass Inc, Vineland, NJ), and stored under nitrogen at  $-80$  °C for periods up to 5 weeks. On the day of extraction and once per week during the next 5 weeks, one tube from each sample was opened and 1.5 mL was transferred to a crimpcap vial closed with a crimpcap, from which 25  $\mu\text{L}$  was injected in duplicate into the HPLC system.

The average coefficient of variation (CV) over the 5-week period for each sample was 5% (range, 4–6%) for  $\beta$ -carotene and 5% (range, 2–8%) for lutein. For each sample, Spearman correlation coefficients were calculated between “week” and “ $\beta$ -carotene or lutein” concentration to assess whether there was any change in concentration. Only for one sample was the correlation between  $\beta$ -carotene and weeks significant. Because the mean CV was  $< 15\%$  and because there was no drift with time in concentration, we concluded that extraction of feces in Wageningen and shipment of the extracts to Chicago within 2 weeks, where they would be injected in the LC-MS system within 2 weeks, would not cause significant losses of carotenoids. In fact, small losses would not be a problem, because measurement is based on the ratio of labeled to unlabeled compounds, provided that such losses affect labeled and unlabeled compounds to a similar extent.

**Quality Control.** Samples from each child were analyzed within one run to minimize analytical variation. In each run, a control sample was extracted in duplicate and injected into the HPLC system for monitoring the stability of the analytical procedure over time. The control sample for the HPLC analysis was homogenized baby food from one lot obtained from Nutricia BV (Zoetermeer, The Netherlands) comprising 42% carrots (*Daucus carota*), 30% peas (*Pisum sativum*), 21% low fat milk, and 1% parsley (*Petroselinum crispum*) as described earlier (9). For each sample run, a new jar of baby food was opened. The average carotenoid concentrations of duplicate control samples were required to be within 2 SD of the mean concentrations measured when extracted and analyzed by HPLC, as described by Hulshof and colleagues (9), over the past 5 y. If the values obtained were not within 2 SD of the mean, the sample run was repeated. With each sample run, two blank samples were included. No retinol and carotenoids were detected in these blank samples.

**HPLC Analysis.** The HPLC system and the source and preparation of internal and external standards have been described elsewhere (9). A reversed phase column was used, and the mobile phase comprised a mixture of methanol, THF, and water containing 0.1% triethylamine, which was pumped isocratically at a flow of 0.7 mL/min. For the first 15 s, the solvent ratios were 88:2:10 (v/v/v), followed by 30 s in which the solvent concentrations changed to 92.5:7.5:0, which was maintained for the remainder of the 25 min runtime. There was a 5 min equilibration period between each run. Samples were injected in duplicate: all samples were injected once when elution of retinol and retinyl palmitate was monitored at 325 nm; after which, all samples were injected for a second time when elution of various carotenoids was monitored at 450 nm. Carotenoids, retinol, and retinyl palmitate were identified by comparing their retention times with those of standards. In 2 out of 45 samples, there was a peak eluting with a retention time the same as that of retinol. However, spectral analysis of these peaks showed that they had a different absorption spectrum than the retinol standards had. Further analysis of these peaks by mass spectrometry showed conclusively that these peaks were not retinol. Quantification was done by internal standard method, and calibration was performed in bracketing mode by a three-level calibration line. A solvent blank was included

in each run. Detector responses were linear over the concentration range: Pearson's correlation coefficients were  $>0.99$  for all standards. Total *cis*- $\beta$ -carotene was quantified from the standard line for all-trans- $\beta$ -carotene, assuming the same detector response for both carotenoids (9). The quantitation limit was set at 10 times the minimal detectable level, which in turn was defined as the amounts of lutein,  $\alpha$ -carotene, and  $\beta$ -carotene resulting in peak-heights 3 times the baseline noise. Data from duplicate samples were averaged and expressed as nmol/g feces.

**APCI LC-MS Analysis.** For the LC-MS analysis, the feces extracts were analyzed by reversed-phase HPLC with a  $C_{30}$  column interfaced to a mass spectrometer equipped with positive ion atmospheric pressure chemical ionization (APCI). To assess the ratio of labeled to unlabeled  $\beta$ -carotene, selected ion monitoring was carried out at mass-to-charge ratios ( $m/z$ ) of 537 and 547. These ions corresponded to endogenous  $\beta$ -carotene (thus including  $\beta$ -carotene derived from unlabeled dietary  $\beta$ -carotene) and orally administered [ $^{13}C_{10}$ ] $\beta$ -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 endogenous retinol (thus including retinol derived from unlabeled dietary retinol), [ $^{13}C_5$ ]retinol (metabolically formed from orally administered [ $^{13}C_{10}$ ] $\beta$ -carotene), and [ $^{13}C_{10}$ ]retinol (formed by hydrolysis of orally administered [ $^{13}C_{10}$ ]retinyl palmitate), respectively. Quality control measures and technical details of the LC-MS methods and equipment did not deviate from the protocol described in the original papers describing these methods (10, 11).

**Reagents and Standards.** HPLC-grade solvents methanol and THF (Labsan, Stillorgan Industrial Park Co., Dublin, Ireland) were used without further purification. BHT and triethylamine were obtained from Sigma Chemical Co. (St Louis, MO) while  $Na_2SO_4$  and  $CaCO_3$  were from Merck (Darmstadt, Germany).

**Calculation of the Bioavailability of  $\beta$ -Carotene.** From the degree of isotopic enrichment of  $\beta$ -carotene with [ $^{13}C_{10}$ ] $\beta$ -carotene and retinol with [ $^{13}C_5$ ]retinol and [ $^{13}C_{10}$ ]retinol in serum and feces at plateau isotopic enrichment during the run-in period, the bioavailability of  $\beta$ -carotene in oil was calculated using the CarRet PIE mathematical model as described earlier (7) and in Table 1, respectively. The plateau can be reached during prolonged consumption of multiple doses of [ $^{13}C_{10}$ ]retinol and [ $^{13}C_{10}$ ] $\beta$ -carotene. Note that the formula for determining the bioavailability of  $\beta$ -carotene in oil using data from feces is based on an oral-fecal balance.

During the run-in period, the intake of retinol and  $\beta$ -carotene from sources other than the capsules was kept constant and low. During the treatment period, the intake of retinol and  $\beta$ -carotene from the capsules was similar to the intake during the run-in period. The intake of retinol from other food sources was similar to the intake during the run-in period. The only difference between the two periods was the treatment (i.e., spinach or pumpkin, rich in  $\beta$ -carotene). It is assumed that  $\beta$ -carotene and retinol released from the food matrix and available for absorption mixes completely with labeled  $\beta$ -carotene and retinol. Changes in the degree of isotopic enrichment of retinol and  $\beta$ -carotene in serum and feces (versus the run-in period) reflect the bioavailability of  $\beta$ -carotene in spinach and pumpkin.

Despite the consumption of large amounts of  $\beta$ -carotene in spinach and pumpkin in the treatment period, the degree of isotopic enrichment of retinol in serum did not change dramatically during the treatment period. Although, in the spinach group, there was a statistically significant change in the isotopic enrichment of retinol with [ $^{13}C_{10}$ ]retinol, the mathematical model could not be used to obtain absolute data on the bioavailability and bioefficacy of  $\beta$ -carotene in spinach and pumpkin. However, the relative bioavailability was estimated as described elsewhere (8), and for clarity, are repeated in Table 1. We extended the CarRet PIE mathematical model to estimate the relative bioavailability of  $\beta$ -carotene in spinach and pumpkin using data from feces (Table 1).

**Statistical Evaluation.** Because the sample size was relatively small ( $<20$ ), normality of data was not tested, and data are expressed as medians and 25th to 75th percentiles, except for baseline characteristics, which are expressed as mean  $\pm$  SD. As the run-in period serves as a control for the treatment period, data collected during the run-in period

are shown separately for each treatment group. Results obtained using data from the run-in and treatment periods were compared between the spinach and pumpkin groups for variables referred to in Table 1. For all comparisons, Mann-Whitney  $U$  tests were used. Within each treatment group, Wilcoxon tests were carried out to compare the variables referred to in Table 1 between the run-in and treatment periods. All tests were two-sided, and  $P$  values  $<0.05$  were considered significant. The computer package SPSS (version 10.0.5; SPSS Inc, Chicago) was used for all statistical calculations.

## RESULTS

**Baseline Characteristics and Experimental Regimen.** Data are presented for 17 (9 boys, 8 girls) of the 20 children enrolled because two girls in the pumpkin group and one in the spinach group provided only one stool sample at baseline. Mean age of the children was  $9.1 \pm 1.2$  y (SD). At baseline, children weighed  $24.0 \pm 3.8$  kg, were  $1.25 \pm 0.06$  m high, and had a mid-upper-arm-circumference of  $18.2 \pm 1.6$  cm. At baseline, the mean serum concentration of retinol was  $0.78 \pm 0.26$   $\mu$ mol/L and of  $\beta$ -carotene  $0.14 \pm 0.08$   $\mu$ mol/L. In feces, the baseline concentration of  $\beta$ -carotene was  $0.011 \pm 0.010$   $\mu$ mol/g, while no retinol was detected in feces. At baseline, the hemoglobin concentration was  $101.6 \pm 7.3$  g/L and the hematocrit  $0.32 \pm 0.03$  L/L. In stools of 65% of the children, intestinal parasites were prevalent at baseline (no data available on one child). Anthropometric data, hemoglobin concentrations, hematocrit values, and prevalence and intensity of intestinal parasites during the study are reported elsewhere (8). In both periods and in both groups, the compliance to the capsule consumption was  $>92\%$ , which means that the children followed the experimental regimen very conscientiously.

During the baseline and run-in periods, the average weight of feces samples collected over the 48-h per period was  $168 \pm 94$  and  $130 \pm 62$ , respectively. During the treatment period, the average weight of feces samples collected over the 48-h for the spinach and pumpkin groups was  $174 \pm 71$  and  $122 \pm 60$ , respectively. Although children were instructed to provide all their stools over a 48-h period, some children did not provide a single stool sample in some, one, or all periods. In retrospect, children mentioned that their feces collection had often been incomplete. However, we were unable to estimate the proportion of feces collected.

During both periods of the study, children obtained  $>70\%$  of their daily energy intake from the food provided. Therefore, and because food intake from the foods provided can be estimated more accurately and precisely (8), we will only use data on nutrient intakes from food (menus, supplements, and capsules) provided to describe the nutrient intakes during this study. Daily retinol and provitamin A intakes are shown in Table 1. Because during the treatment period  $>82\%$  of provitamin A intakes was  $\beta$ -carotene, we refer to the bioavailability as the bioavailability of  $\beta$ -carotene. During the run-in period, intake of lutein was approximately  $700$   $\mu$ g/d and intake of zeaxanthin and lycopene was  $<75$   $\mu$ g/d. During the run-in period, retinol and  $\beta$ -carotene intake from capsules was statistically significantly higher in the pumpkin than in the spinach group, but we expect this difference to be of little biological relevance. During the treatment period, daily intakes of  $\beta$ -carotene ( $5.55$  vs  $2.76$   $\mu$ mol/d) and  $\beta$ -cryptoxanthin ( $0.07$  vs  $0.02$   $\mu$ mol/d) was significantly higher in the spinach than in the pumpkin group, while intake of  $\alpha$ -carotene ( $0.25$  vs  $1.19$   $\mu$ mol/d) was lower. In both groups, intake of provitamin A carotenoids (calculated as the sum of the intake of  $\beta$ -carotene and half the intake of other provitamin A carotenoids) differed significantly between the two periods, because different recipes were used

**Table 1.** Description of the  $\beta$ -Carotene and Retinol at Plateau Isotopic Enrichment (CarRet PIE) Mathematical Model (7) Using Data from Serum and Feces

symbol	description	derivation	run-in period		treatment period		
			spinach group ( <i>n</i> = 9)	pumpkin group ( <i>n</i> = 6)	spinach group ( <i>n</i> = 8)	pumpkin group ( <i>n</i> = 6)	
<i>a</i>	retinol intake from diet ( $\mu\text{mol/d}$ ) <sup>a</sup>		0	0	0	0	
<i>b</i>	retinol intake from capsules ( $\mu\text{mol/d}$ )		0.122	0.126	0.122	0.124	
<i>d</i>	$\beta$ -carotene intake from diet ( $\mu\text{mol/d}$ ) <sup>a</sup>		(0.120, 0.124) <sup>b</sup> z	(0.124, 0.126) z, y	(0.105, 0.124)	(0.118, 0.124) y	
<i>e</i>	$\beta$ -carotene intake from capsules ( $\mu\text{mol/d}$ )		1.52	1.69	5.71	3.36	
<i>C</i> <sub>SR</sub>	total retinol concentration in serum ( $\mu\text{mol/l}$ ) <sup>c</sup>		(1.42, 1.65) y	(1.50, 1.81) y	(4.86, 5.81) z, y	(3.23, 3.39) z, y	
<i>E</i> <sub>10,SR</sub>	enrichment of retinol in serum with [ <sup>13</sup> C <sub>10</sub> ]retinol		0.168	0.173	0.168	0.170	
<i>E</i> <sub>5,SR</sub>	enrichment of retinol in serum with [ <sup>13</sup> C <sub>5</sub> ]retinol		(0.165, 0.170) z	(0.172, 0.175) z, y	(0.144, 0.170)	(0.162, 0.175) y	
<i>C</i> <sub>SC</sub>	total $\beta$ -carotene concentration in serum ( $\mu\text{mol/l}$ ) <sup>c</sup>		0.67	0.64	0.72	0.80	
<i>C</i> <sub>FC</sub>	total $\beta$ -carotene concentration in feces (nmol/g) <sup>c</sup>		(0.56, 1.03)	(0.52, 0.78) y	(0.69, 1.09)	(0.72, 0.85) y	
<i>E</i> <sub>10,SC</sub>	enrichment of $\beta$ -carotene in serum with [ <sup>13</sup> C <sub>10</sub> ] $\beta$ -carotene	$\frac{M_{279,SR}}{(M_{269,SR} + M_{274,SR} + M_{279,SR})^d}$	0.0462	0.0697	0.0520	0.0617	
<i>E</i> <sub>10,FC</sub>	enrichment of $\beta$ -carotene in feces with [ <sup>13</sup> C <sub>10</sub> ] $\beta$ -carotene	$\frac{M_{274,SR}}{(M_{269,SR} + M_{274,SR} + M_{279,SR})}$	(0.0319, 0.0589) z, y	(0.0541, 0.0826)z	(0.0184, 0.0658) y	(0.0529, 0.0702)	
<i>E</i> <sub>5,SC</sub>	enrichment of $\beta$ -carotene in serum with [ <sup>13</sup> C <sub>5</sub> ] $\beta$ -carotene	$\frac{M_{547,SC}}{(M_{537,SC} + M_{547,SC})}$	0.0475	0.0634	0.0519	0.0571	
<i>E</i> <sub>5,FC</sub>	enrichment of $\beta$ -carotene in feces with [ <sup>13</sup> C <sub>5</sub> ] $\beta$ -carotene	$\frac{M_{547,FC}}{(M_{537,FC} + M_{547,FC})}$	(0.0296, 0.0663)	(0.0432, 0.0839)	(0.0142, 0.0759)	(0.0420, 0.0715)	
<i>BV</i> <sub>dCo</sub>	bioavailability (%) of $\beta$ -carotene in oil <sup>e</sup> using data from serum <sup>f</sup>		0.16	0.13	0.31	0.51	
	using data from feces <sup>g</sup>		(0.12, 0.22) y	(0.11, 0.20) y	(0.22, 0.37) z, y	(0.43, 0.58) z, y	
<i>BV</i> <sub>dCs<sub>p</sub></sub>	relative bioavailability of $\beta$ -carotene in spinach and pumpkin using data from serum		4	5	21	10	
			(2, 12) y	(1, 12) y	(1, 34)y	(2, 24) y	
			0.1425	0.1643	0.0869	0.0486	
			(0.0940, 0.2343) y	(0.1129, 0.2280) y	(0.0612, 0.01354) z, y	(0.0228, 0.0744) z, y	
			0.0691	0.0639	0.0118	0.0361	
			(0.0495, 0.1668) y	(0.0535, 0.0941)	(0.0071, 0.0268) z, y	(0.0265, 0.0675) z	
			$\frac{[(E_{10,SC1} \times C_{SC1}) + (StC \times E_{5,SR1} \times C_{SR1})] \times b_1}{(E_{10,SR1} \times C_{SR1}) \times e_1} \times 100$	114 (67, 136)	75 (52, 85)		
			$\frac{[(2 \times e_1) - (E_{10,FC1} \times C_{FC1} \times W)]}{(2 \times e_1)} \times 100$				
			$\left\{ \frac{[(E_{10,SC2} \times C_{SC2}) + (StC \times E_{5,SR2} \times C_{SR2})] \times d_2}{e_2} \right\}_{sp}$			1.7 (0.9, 3.1) <sup>h</sup>	
			$\left\{ \frac{[(E_{10,SC2} \times C_{SC2}) + (StC \times E_{5,SR2} \times C_{SR2})] \times d_2}{e_2} \right\}_{pu}$				

Table 1. (Continued)

symbol	description	derivation	run-in period		treatment period	
			spinach group (n = 9)	pumpkin group (n = 6)	spinach group (n = 8)	pumpkin group (n = 6)
	using data from feces <sup>a</sup>	$\left[ \frac{(E_{10,C2} \times d_2)}{e_2} \right]_{pv} \left[ \frac{(E_{10,C2} \times d_2)}{e_2} \right]_{sp}$				1.8 (0.6, 5.5) <sup>h</sup>

<sup>a</sup> Intake of nutrient is intake from food provided. Retinol in food provided was below the limit of detection (thus,  $\leq 3$  ng/d).  $\beta$ -Carotene intake is the sum of the intake of other provitamin A carotenoids from food provided. During the run-in period in both groups,  $\alpha$ -carotene intake was approximately 0.17  $\mu$ mol/d and  $\beta$ -cryptoxanthin intake was negligible ( $<0.01$   $\mu$ mol/d). During the treatment period,  $\alpha$ -carotene was higher (1.2  $\mu$ mol/d) in the pumpkin than in the spinach (0.25  $\mu$ mol/d) group, while in both groups,  $\beta$ -cryptoxanthin intake was negligible ( $<0.07$   $\mu$ mol/d). <sup>b</sup> Median with 25th and 75th percentiles. Mann-Whitney U tests were carried out to compare values between groups within each period. Wilcoxon tests were carried out to compare values within each group between the run-in and treatment periods. <sup>c</sup> In feces collected over 48 h, concentrations of retinol were below the limit of detection (thus, excretion of retinol was  $\leq 2$  ng/d). <sup>d</sup> Where  $M_{279,SR}$  is the signal measured by liquid chromatography-mass spectrometry at  $m/z$  279. Values at baseline were subtracted from values during the run-in and treatment periods. If baseline samples were absent, the mean enrichment at baseline was subtracted from the values during the run-in and treatment periods. <sup>e</sup> Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (12). <sup>f</sup> Figures 1 and 2 in the subtitles refer to the period from which data were used (1 = run-in and 2 = treatment period). SIC refers to the stoichiometry of the cleavage of  $\beta$ -carotene to retinol in the body. We assume the most efficient stoichiometry (i.e., 1  $\mu$ mol  $\beta$ -carotene yields 2  $\mu$ mol retinol), thus obtaining minimum estimates for the bioavailability. With this stoichiometry,  $SIC = 0.5$ . <sup>g</sup> Where  $W_i$  is the weight of feces collected. Because complete collection of feces is a prerequisite for adequate use of this formula and feces collection was incomplete, bioavailability of  $\beta$ -carotene in oil was not calculated using median values of the numerator and denominator. Interval of spread of ratio calculated using the 25th and 75th percentiles of the median values of the numerator and denominator (16). A ratio  $>1$  indicates that the bioavailability of  $\beta$ -carotene in spinach is less than that of  $\beta$ -carotene in pumpkin. <sup>h</sup> In these formulas, the absorption of retinol is not taken into account as is done in the formulas for serum. This cannot be done, because no retinol was detected in feces. <sup>i</sup> Values within one row within one period differ significantly ( $P < 0.05$ ). <sup>j</sup> Values within one group differ significantly ( $P < 0.05$ ).

for the different vegetable supplements (total daily intake of provitamin A carotenoids shown in **Table 1**).

**Concentrations and Degree of Isotopic Enrichment of Retinol and Carotenoids in Serum and Feces.** Concentrations of retinol in serum and feces are shown in **Table 1**. Using HPLC and LC-MS, with limits of detection of 3.5 ng/kg feces and  $\leq 1$  pmol injected on-column, respectively, no retinol was detected in feces samples collected during this study, possibly because intake of retinol from food provided was negligible. Concentrations of total retinol in serum did not differ between the spinach and pumpkin groups in each period, nor between both periods within the spinach group. In the pumpkin group, serum retinol concentrations were significantly higher during the treatment period than during the run-in period.

Concentrations of carotenoids in serum and in feces did not differ between groups during the run-in period (**Table 1**, data for carotenoids other than  $\beta$ -carotene not shown). During the treatment period, concentrations of  $\beta$ -carotene, lutein,  $\alpha$ -carotene, and zeaxanthin in serum were higher in the pumpkin than in the spinach group, while concentrations of lycopene in feces were higher in the spinach than in the pumpkin group. Except for zeaxanthin, these differences reflect differences in intake of carotenoids from food provided (see previous section). In the spinach group, concentrations of  $\beta$ -carotene and lutein in feces and in serum differed significantly between the run-in and treatment periods. In the pumpkin group, concentrations of  $\beta$ -carotene, lutein,  $\alpha$ -carotene, and zeaxanthin in serum and those of  $\beta$ -carotene, lycopene, and lutein in feces differed significantly between the run-in and treatment periods.

During the run-in period, the degree of isotopic enrichment of retinol in serum with [ $^{13}\text{C}_{10}$ ]retinol differed significantly between the spinach and pumpkin groups (**Table 1**). In the spinach group, the degree of isotopic enrichment of retinol in serum with [ $^{13}\text{C}_{10}$ ]retinol increased significantly over the course of the treatment period, while in the same group, the isotopic enrichment of  $\beta$ -carotene in feces decreased significantly over the course of the treatment period. In both groups, the degree of isotopic enrichment of  $\beta$ -carotene in serum decreased significantly over the course of the treatment period. During the treatment period, the degree of isotopic enrichment of  $\beta$ -carotene in serum and feces differed significantly between the spinach and pumpkin groups. The degree of isotopic enrichment of  $\beta$ -carotene in serum was lower in the pumpkin than in the spinach group, while in feces, the enrichment was higher in the pumpkin than in the spinach group.

**Comparison of Bioavailability of  $\beta$ -Carotene Using Data from Feces with Data from Serum.** The bioavailability of  $\beta$ -carotene in oil compared with that of retinol in oil obtained using data from serum was 82% (25th–75th percentile, 62, 125) (not different between groups; **Table 1**). These calculations were based on data from 15 children instead of 16, because data from one of the children who participated in this feces collection procedure was omitted (15) from calculations of the bioavailability of  $\beta$ -carotene in oil if calculations were based on the total study population ( $n = 77$ ; 8). Data on the bioavailability of  $\beta$ -carotene in oil obtained using data from feces could not be calculated, because the calculations reflected a balance study, for which it is important that feces collection is complete and losses have not occurred during handling and storage of the feces.

Using data from serum, the bioavailability of  $\beta$ -carotene in pumpkin was 1.7 (interval, 0.9, 3.1) times that of  $\beta$ -carotene from spinach. The ratio was calculated using median values of the numerator and denominator. Interval of spread of the ratio

was calculated using the 25th and 75th percentiles of the median values of the numerator and denominator (16). Using data from feces, the bioavailability of  $\beta$ -carotene in pumpkin was 1.8 (interval, 0.6, 5.5) times that of  $\beta$ -carotene in spinach. A ratio of  $>1$  indicates that the bioavailability of  $\beta$ -carotene in pumpkin is more than that of  $\beta$ -carotene in spinach. No statistical tests were performed to compare these ratios, because of the low quantitative precision of such ratios.

## DISCUSSION

In this study in 20 Indonesian school children, bioavailability of  $\beta$ -carotene from pumpkin was 1.8 or 1.7 times greater than that from spinach, using data from feces and serum, respectively. Thus, results from serum and feces are comparable. Human feces samples could be extracted using the above protocol to liberate retinol, retinyl esters, and carotenoids from feces prior to injection into the HPLC or LC-MS systems.

From the interval of spread around the estimates based on feces (0.6, 5.5) and serum (0.9, 3.1), it can be seen that the precision of data from feces is probably lower. Although fecal mass is not a variable in the calculations of the bioavailability, we expect incomplete feces collection might have made feces samples less representative, and therefore we speculate that the lower precision of the estimates may be caused by incomplete collection of feces. The bioavailability of  $\beta$ -carotene in oil could not be estimated using data from feces, because collection of feces was incomplete. In future studies, especially when studying children of this age group, the proportion of feces collected could be assessed by feeding radio-opaque markers prior to and during feces collection and by assessing their recovery in feces by X-ray examination. There are a number of limitations on basing results on analyses in feces. First, because bioconversion is a process that occurs within the body, bioefficacy cannot be quantified directly using data from feces. Second, the amount of feces required for analysis is greater than that for serum samples, thus increasing the cost of sample storage and analysis. In addition, sample preparation of feces is much more labor-intensive and more manual steps are involved in it than for sample preparation of serum.

Nevertheless, results from this study show that this stable isotope technique can be used for the quantification of the relative bioavailability of  $\beta$ -carotene in food using data from feces. In addition, in conjunction with the use of stable isotopically labeled compounds, feces can be collected to study the fate of carotenoids in the gastro-intestinal tract. This is a rather unexplored area of research, because the degree of carotenoid degradation in the gastric environment or by bacteria (17) and the extent of endogenous secretion of carotenoids are largely unknown. Such data are necessary to interpret data on the bioavailability of carotenoids obtained using oral fecal balance techniques. In fact, this study shows once more how much caution should be taken when interpreting data from nonisotopic techniques. The intake of dietary  $\beta$ -carotene was 1.7 times higher in the spinach than in the pumpkin group. The concentration of carotene in feces in the spinach group (21 nmol/g) was 2.1 times higher than that in the pumpkin group (10 nmol/g). This would lead to the conclusion that  $\beta$ -carotene from both vegetables is bioavailable to a similar extent. The higher serum  $\beta$ -carotene and retinol concentrations in the pumpkin than in the spinach group indicate that  $\beta$ -carotene from both vegetables is bioavailable to a biologically relevant yet different extent. A conclusion that is in accordance with the results obtained using isotopic enrichment data from serum showing that  $\beta$ -carotene in pumpkin was 1.7 times more bioavailable than  $\beta$ -carotene in spinach.

For the study described in this paper, we adapted an HPLC method, based on a validated method, to liberate retinol, retinyl esters, and carotenoids from human feces to assess the concentration of  $\beta$ -carotene and other carotenoids. The feces extracts can be stored at  $-80^{\circ}\text{C}$  without considerable loss of carotenoids for at least 5 weeks. Therefore, for assessing the concentration of retinol and carotenoids in feces using HPLC and the degree of isotopic enrichment of retinol and  $\beta$ -carotene in feces using LC-MS, sample preparation has to be performed only once. This is not only less labor intensive, it also decreases the variation between data from the two measurements. The extraction procedure for feces described in this paper does not require freeze-drying of the samples or saponification, as in methods described by other authors (18, 19). With saponification, all retinyl esters are hydrolyzed to retinol. Thus, in studies in which the retinyl ester content of the diet is high, this structural information will be lost.

In this study, only an internal retinol standard has been used. In our opinion, a standard added at the beginning of the analysis should only be used to appraise losses of compounds of interest (retinoids and carotenoids) during the entire workup procedures and should not be used to appraise losses during extraction. Given the differences in stability of the compounds of interest and the standards, it is questionable whether recovery percentages of the standards do indicate true losses of the compounds of interest. In addition, because the standards are not intimately linked with the feces matrix, they might be extracted more easily than are the endogenous compounds. Moreover, an ideal commercially available internal carotenoid standard has not yet been encountered (20). In further studies, completeness of extraction could be assessed by varying the sample amount while other extraction conditions, except the amount of  $\text{Na}_2\text{SO}_4$ , would be kept constant (9). Recovery of the added internal retinol standard (retinyl acetate) was  $>89\%$ , which mainly indicates that the analytical work has been conducted accurately. No retinol and retinyl esters were detected in feces samples collected during this study. This might be due to degradation during extraction and processing. However, in our opinion, a more plausible explanation might be that the intake of retinol and retinyl esters from food provided was negligible. At the start of this study, we assumed carotenoids to be more sensitive to light than to heat, as long as temperatures were below  $40^{\circ}\text{C}$ . Therefore, we only instructed children to store the samples at a cool place in the dark. However, in further studies, collection of data on the stability of retinoids and carotenoids in feces during all stages of the sample collection, storage, and preparation would be very useful. From data from this subsample, the main study (8), and our previous study (7), there does not seem to be an effect of sex on the bioefficacy and/or bioavailability of  $\beta$ -carotene. However, because none of these studies were specifically designed for that purpose, the stable isotope technique described in this and other papers (6–8) should now be used for studying the effect of sex on the bioefficacy and/or bioavailability of carotenoids.

For the study described in this paper, we also extended the CarRet PIE mathematical model to estimate the relative bioavailability of  $\beta$ -carotene in spinach and pumpkin. In an intervention study in 188 school children in Indonesia by de Pee and colleagues (2), there were 4 dietary groups: low-retinol, low-carotenoid (negative control); dark-green leafy vegetables and carrots; yellow and orange fruits; and a retinol-containing diet (positive control). The ratio of the bioavailability of  $\beta$ -carotene in orange fruits to that of  $\beta$ -carotene in dark green leafy vegetables was 5.9 (interval: 4.0, 7.9). This was calculated

from the changes in serum  $\beta$ -carotene concentrations (corrected for intake of  $\beta$ -carotene from food). In our study, the bioavailability of  $\beta$ -carotene in pumpkin (an orange fruit) was also higher (1.7–1.8) than that of  $\beta$ -carotene in spinach (a dark green leafy vegetable). This ratio is smaller than when based on data from changes in serum concentrations of  $\beta$ -carotene. The stable isotope technique provides more accurate estimates of the relative bioavailability of  $\beta$ -carotene in food both when using data from serum and when using data from feces than techniques which are based on changes in serum  $\beta$ -carotene concentrations. This can be explained by the latter techniques failing to take into account the bioconversion of  $\beta$ -carotene to retinol. The mathematical model using data from feces data provides data on absorption independent of the rate of bioconversion. As described earlier (6, 8), the design of studies using this isotope technique should be altered by providing moderate amounts of retinol in food thus enabling quantification of the absolute bioavailability and bioefficacy of  $\beta$ -carotene in fruit and vegetables. Data from such studies will enable proper evaluation of the effective supply of vitamin A of various approaches to eliminating vitamin A deficiency.

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