Vitamin A equivalency of β-carotene in healthy adults: limitation of the extrinsic dual-isotope dilution technique to measure matrix effect

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Data on the vitamin A equivalency of β-carotene in food are inconsistent. We quantified the vitamin A equivalency (μg) of β-carotene in two diets using the dual-isotope dilution technique and the oral–faecal balance technique. A diet-controlled, cross-over intervention study was conducted in twenty-four healthy adults. Each subject followed two diets for 3 weeks each: a diet containing vegetables low in β-carotene with supplemental β-carotene in salad dressing oil ('oil diet') and a diet containing vegetables and fruits high in β-carotene ('mixed diet'). During all 6 weeks, each subject daily consumed a mean of 55 (SD 0·5) μg $[^{13}C_{10}]$β-carotene and 55 (SD 0·5) μg $[^{13}C_{10}]$retinyl palmitate in oil capsules. The vitamin A equivalency of β-carotene was calculated as the dose-corrected ratio of $[^{13}C_5]$retinol to $[^{13}C_{10}]$retinol in serum and from apparent absorption by oral–faecal balance. Isotopic data quantified a vitamin A equivalency of $[^{13}C_{10}]$β-carotene in oil of 3·4 μg (95 % CI 2·8, 3·9), thus the bio-efficacy of the β-carotene in oil was 28 % in the presence of both diets. However, data from oral–faecal balance estimated vitamin A equivalency as 6:1 μg (95 % CI 4, 7) for β-carotene in the 'oil diet'. β-Carotene in the 'oil diet' had 2·9-fold higher vitamin A equivalency than β-carotene in the 'mixed diet'. In conclusion, this extrinsic labelling technique cannot measure effects of mixed vegetables and fruits matrices, but can measure precisely the vitamin A equivalency of the β-carotene in oil capsules.

β-Carotene: Vitamin A equivalency: Stable isotopes: Food matrix

The bio-efficacy (%) of β-carotene as a source of retinol is defined as the proportion of β-carotene ingested, which is absorbed and converted into vitamin A (retinol) in the body[1]. The vitamin A equivalency (μg) of β-carotene as a source of retinol is defined as the amount of β-carotene ingested, which is absorbed and converted into vitamin A (retinol) in the body[1]. According to the current guidelines 6 μg (FAO/WHO) or 12 μg (US Institute of Medicine) of β-carotene in a mixed diet have the same vitamin A equivalency as 1 μg retinol[2–4]. For supplemental β-carotene in oil the current guidelines are that 3·3 μg (FAO/WHO) or 2 μg (US Institute of Medicine) of β-carotene have the same vitamin A equivalency as 1 μg retinol[2–4].

Data concerning the bio-efficacy of β-carotene from various dietary sources are inconsistent and more data are needed. Data are required both for developing countries as well as for developed countries for calculating the 'true' nutrient value of food items from food composition tables. A number of factors influence the bio-efficacy of carotenoids, e.g. vitamin A status, health status, the food matrix in which the carotenoid is incorporated, processing of vegetables and fruits, and the presence of dietary fat and fibres[5–10].

Until now, various studies using serum or plasma response after consumption of diets rich in β-carotene from several vegetables and fruits have been carried out[10–13]. However, this technique has limited precision, thus requiring large numbers of volunteers to be studied for long periods of time. Accurate data of the bioconversion of dietary β-carotene to retinol without the absorption step are not available. It is accepted that after absorption of β-carotene, whether from oil or food, the

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metabolism of the molecule is similar. Thus the critical step in the conversion of β-carotene into vitamin A is the absorption of the molecule into the enterocyte[2–4]. It is generally assumed that 2 μg β-carotene in the enterocyte is equivalent to 1 μg retinol in the body, thus the estimated bioconversion is about 50 % for β-carotene and about 25 % for other provitamin A carotenoids[2–4]. In order to quantify how much β-carotene enters the enterocyte, stable isotope techniques have been developed since the 1990s[14,15]. However, these techniques and the studies in which they have been applied, have reported conflicting results[16–21].

In the present investigation, an extrinsic dual-isotope-labelling technique was used, which is based on attaining a plateau (reached by day 21) of isotopic enrichment of β-carotene and retinol in serum during prolonged daily intake of capsules containing low doses of β-carotene and retinol, each specifically labelled with ten ^{13}C atoms[1]. In addition to the administered $[^{13}C_{10}]$retinol, $[^{13}C_5]$retinol was measured in serum resulting from the central cleavage of $[^{13}C_{10}]$β-carotene. In addition to the dual-isotope dilution technique, an oral–faecal balance technique was used to estimate the apparent absorption of β-carotene over 72 h. The aim of the present diet-controlled intervention study was to quantify the vitamin A equivalency of β-carotene in healthy adults consuming two types of controlled western diets: the 'oil diet', which contained mainly supplemental β-carotene in oil as the source of β-carotene and the 'mixed diet', which contained mainly β-carotene from vegetables and fruits.

Subjects and methods

Recruitment of subjects

Healthy non-smoking adults aged 18–50 in the surroundings of Wageningen in the Netherlands were recruited for participation in the screening for the study by posters and advertisements in local newspapers. Written and verbal information was provided and informed consent forms were signed. The screening's examination included a health and lifestyle questionnaire, a FFQ[22], weight measurement (precise to 0·1 kg)

and height measurement (precise to 0·5 cm), and haematological analyses of blood, liver enzymes, creatinine, alkaline phosphatase and cholesterol. Exclusion criteria were as follows: haematological abnormalities, history of chronic diseases, including cancer, renal insufficiency, liver disease, diagnosed gastrointestinal disorders, surgery of gastrointestinal tract, use of (oral) drugs suspected of interfering with fat-soluble vitamin absorption, pregnancy, BMI <18 or >25 kg/m², smoking, abnormal dietary pattern, excessive alcohol consumption (>40 g/d), and consumption of carotenoids/vitamin/mineral supplements 6 weeks before and during the study. Subjects with low serum β-carotene (<0·28 μmol/l) and/or low serum retinol (<1·07 μmol/l) concentrations were also excluded from participation. Twenty-eight volunteers participated in the screening, and twenty-four subjects were selected to form two groups, which were matched for sex, age, BMI and habitual energy intake. The subject characteristics at baseline are shown in Table 1. The study was conducted at the Division of Human Nutrition, Wageningen University, the Netherlands. The research protocol was approved by the Medical-ethical Committee on Research Involving Human Subjects, Region Arnhem-Nijmegen, the Netherlands.

Study design

The study was designed as a cross-over intervention with two controlled diets in twenty-four healthy subjects. Each subject followed two diets for 3 weeks each; one diet containing vegetables low in β-carotene with supplemental β-carotene in salad dressing oil ('oil diet') and the other diet containing vegetables high in β-carotene ('mixed diet'). The subjects consumed capsules each day for 6 weeks during both diets. The capsules contained a mean of 55 μg/d $[^{13}C_{10}]$β-carotene and 55 μg/d $[^{13}C_{10}]$retinyl palmitate in oil (relative to their daily energy intake). On days 0, 1, 21, 22, 42 and 43 fasting blood samples (13 ml each) were obtained, and then kept in the dark at 4°C for 30 min before being centrifuged at 3000 rpm for 10 min at 4°C to separate cells from serum. Serum was stored at −80°C until analysis. Fasting was

Table 1. Characteristics of the subjects at baseline*

(Mean values and standard deviations)

	Group 1 (*n* 24)		Group 2 (*n* 24)		Laboratory references	
	Mean	SD	Mean	SD	Male	Female
Sex (male/female)	5/7		5/7			
Age (years)	21·8	4·7	22·2	3·4		
BMI (kg/m²)	22·3	2·2	21·3	2·0		
Habitual energy intake (MJ)	11·0	3·0	11·2	2·8		
Hb (mmol/l)	8·7	0·7	8·7	0·7	8·5–11·0	7·5–9·5
Haematocrit (l/l)	0·42	0·03	0·42	0·04	0·41–0·51	0·36–0·46
Erythrocytes (cells × 10¹²/l)	4·7	0·4	4·6	0·3	4·2–5·6	3·7–5·0
Leucocytes (cells × 10⁹/l)	7·5	1·1	6·9	1·2	4·0–10·0	
Thrombocytes (cells × 10⁹/l)	272	57	264	40	150–400	
Creatinine (μmol/l)	78	11	75	12	50–125	
Alanine aminotransferase (IU/l)	23	11	21	6	5–45	
Alkaline phosphatase (U/l)	84	23	66	11	40–125	
Cholesterol (mmol/l)	4·3	0·5	4·3	0·9	<6·5	

* Groups 1 and 2 were matched for sex, age, BMI and habitual energy intake. There were no differences between groups (two-tailed *t* tests for independent samples).

defined as not consuming any food or energy-containing drinks for 12 h prior to the blood sampling. On days 19, 20 and 21 and also on days 40, 41 and 42, complete 72 h faeces were collected directly after defecation at home (according to instructions), stored on dry ice ($-79°C$) in plastic bags with labels, and then transported to the $-80°C$ freezer at the research centre. The concentrations of carotenoids and retinol in duplicate diets, serum and in faeces were measured by HPLC, and the isotopic enrichments of retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of β-carotene with [$^{13}C_{10}$]β-carotene were measured using LC–MS.

Diets and compliance

Menus were designed for twelve levels of energy intake ranging from 7 to 18 MJ/d. The subjects were allocated to an energy intake level close to their habitual energy intake, which was estimated from a FFQ[22]. The 'oil diet' and the 'mixed diet' were designed according to the guidelines of 'good nutrition' from the Dutch Nutrition Board in The Hague, the Netherlands. Both diets were typical western diets with respect to the contribution of carbohydrates, proteins and fats to the energy intake (57, 13 and 30, respectively; Table 2). The menu was changed daily on a 3-week cycle; 90 % of energy was provided; all food was weighed out for each subject. The remaining 10 % of energy had to be chosen from a list of low-fat food items, which did not contain carotenoids or retinol, which had to be recorded in a diary. The diaries were inspected at least twice weekly. Body weight was recorded twice weekly and energy intake was adjusted, when necessary, to limit changes in weight to less than 2 kg. During weekdays, subjects consumed all their capsules and hot meal at noon at the research centre under supervision. Foods for their other meals and snacks (bread; margarine; meat and cheese; honey, jam, or sprinkles; fruit; milk and/or yoghurt; cookies) were packed for consumption at home, as was food for the weekends. The hot meal contained potatoes/pasta/rice, cooked vegetables, salad with

salad dressing, a piece of meat and dessert. The salad dressing for the 'oil diet' was supplemented with synthetic β-carotene (all-*trans* β-carotene, 30 % suspension in vegetable oil; Hoffmann-La Roche, Switzerland). The margarine was prepared by special order (Unilever, the Netherlands) and was not supplemented with retinol or β-carotene as is normally the case with margarine in the Netherlands. The ratio of β-carotene provided by fruits to vegetables was 1:2·4 in the 'oil diet' and 1:8·2 in the 'mixed diet'. The fruits were orange, apple, grapes, banana and melon. The cooked vegetables in the 'oil diet' were French beans, beetroots, snow peas, white cabbage, cauliflower, ratatouille, red cabbage and Chinese cabbage. The cooked vegetables in the 'mixed diet' were carrots in combination with French beans, green beans, snow peas, green peas, endive, leek, savoy cabbage and broccoli. Each vegetable originated from one batch and was analysed before the study to ensure the β-carotene content of each daily diet was similar. Each subject kept a diary for monitoring compliance to the diet, compliance to the intake of the capsules, compliance to fasting instruction, compliance to faeces collection, illnesses, medication used and the daily choice of low-fat food items. The composition of both diets was calculated using the Dutch Food Composition Table[23]. In order to analyse the nutrient content, duplicate diets of the 11 MJ menu were collected every day and stored at $-20°C$ in non-transparent buckets. On a weekly basis, seven diets were pooled, mixed thoroughly with 2·5 ml 20 % butylhydroquinone/kg food, and stored at $-20°C$ until analysis. The individual energy and nutrient intakes during the study were calculated by using the food composition data of the selected food items and the analysed values of the duplicate diets and adjusted to the individual energy intake level.

Chemical analysis of duplicate diets, vegetables and salad dressings

Duplicate samples of the meals were analysed for fat, protein, dietary fibre, moisture, ash, retinol and carotenoids at the Division of Human Nutrition. The fat concentration was measured gravimetrically after Soxhlet extraction with petroleum ether–diethyl ether (1:1, v/v)[24]. The protein concentration was measured as total nitrogen by the Kjeldahl method, multiplied by 6·25[24]. Dietary fibre was measured according to the Prosky procedure[24]. The moisture level was determined after drying for 10 h in a vacuum oven at 80°C, and the ash content was determined using a dry ashing procedure in a muffle furnace for 10 h at 550°C[25]. Available carbohydrates were calculated by difference.

For the analysis of the dietary retinol and carotenoids, food samples were homogenized and extracted with tetrahydrofuran. After evaporation of the solvent, the residue was saponified overnight at room temperature in 5 % ethanolic KOH containing 0·2 % pyrogallol. After addition of dichloromethane, KOH was extracted four times using water. The dichloromethane layer was filtered by using a water filter (597 HY $\frac{1}{2}$; Schleicher & Schuell) and evaporated to dryness under nitrogen at 35°C. The residue was dissolved in methanol–tetrahydrofuran (1:1, v/v) and analysed by HPLC on a Vydac 201TP54 reversed-phase column (C_{18}; 5 μm; 300 Å; 4 × 250 mm) using gradient elution with a mixture of methanol, tetrahydrofuran, water and triethylamine, as described

Table 2. Composition of the two controlled diets during the 6-week crossover intervention study*

	'Oil diet'	'Mixed diet'
Energy (MJ)	11·2	11·1
Fat (g)	90	89
Protein (g)	84	87
Carbohydrates (g)	373	363
Alcohol (g)	5	5
Fibres (g)	36	36
Retinol (μg)	137	116
Total β-carotene in salad dressing oil (μg)	3280	<1
Total β-carotene in vegetables and fruits (μg)	1615	6846
trans-β-Carotene (μg)	4895	6846
cis-β-Carotene (μg)	<1	628
Proportion *cis*:*trans* in diet	<1:100	13:87
α-Carotene (μg)	56	1281
β-Cryptoxanthin (μg)	240	135
Lutein (μg)	611	1314
Zeaxanthin (μg)	176	141
Lycopene (μg)	852	631

* Energy and nutrient intakes were calculated by using the calculated data of the freely selected food items (10 % of energy) and of the analysed values of the duplicate diets (90 % of 11 MJ) and adjusted to the individual energy intake level.

elsewhere[26]. The elution of retinol was monitored at 326 nm, and carotenoids were measured at 450 nm. The samples of the vegetable batches and duplicate salad dressings were analysed for concentrations of carotenoids by HPLC[27]. All sample preparations were carried out under subdued yellow light to avoid degradation of the carotenoids.

Chemical analysis of retinol and carotenoids in serum and in faeces

Retinol and carotenoids in human serum were analysed using the HPLC method with absorbance detection described previously[28]. Briefly, to 500 µl serum, 500 µl sodium chloride (0·9 %, w/v in water) and 1·00 ml ethanol (containing retinyl acetate as an internal standard) were added, and then extracted twice with 2·0 ml portions of hexane. The hexane layers were pooled and evaporated to dryness under nitrogen at 35°C. The residue was dissolved in 250 µl methanol–tetrahydrofuran (3:1, v/v), and 25 µl were injected for each HPLC analysis. Separations were monitored at 326 nm (retinol) and 450 nm (carotenoids). Within- and between-run CV for the chemical analysis of retinol and carotenoids in serum were 1·6 and 1·9 % for retinol, 3·4 and 8·2 % for β-carotene, 4·6 and 7·0 % for α-carotene, and 3·6 and 11·4 % for β-cryptoxanthin.

Faeces samples of 72 h collection from each subject were pooled, homogenized and weighed. Sample preparation for the extraction of human faeces has been described by van Lieshout et al. [29]. Briefly, an aliquot (2 g) was extracted in duplicate with 4 g Na$_2$SO$_4$, 0·5 g CaCO$_3$, 30 ml tetrahydrofuran containing 0·01 % butylated hydroxytoluene and 1 ml of an internal standard in tetrahydrofuran–methanol (3:1, v/v) containing a known amount of retinyl acetate (about 1 µg) in a 100 ml measuring cylinder, using a Polytron. The residue was triple extracted with 30 ml tetrahydrofuran. Retinol and carotenoids in the extracts of the faeces samples were analysed by HPLC using a reversed-phase C$_{30}$ column with internal and external standards and control samples as described elsewhere[29]. All sample preparations of both serum and faeces were carried out under subdued yellow light. The recovery of β-carotene was 89, 79 and 90 % measured three times in duplicate by spiking of β-carotene standard. The reproducibility (combined within- and between-run CV) was 7·1 % based on twelve analytical runs.

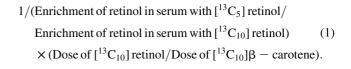### Capsule administration and measurement in serum and in faeces

The capsules contained 14·9, 20·1 or 25·7 µg [12,13,14,15,20,12′,13′,14′,15′,20′-^{13}C$_{10}$]β-carotene and 14·1, 19·1 or 23·9 µg [8,9,10,11,12,13,14,15,19,20-^{13}C$_{10}$]retinyl palmitate in oil (analysed values). The oily mixture for the capsules was prepared by dissolving the labelled β-carotene and retinyl palmitate in highly unsaturated sunflower oil (>82 % oleic acid and >9 % linoleic acid; Hozol; Contined BV, Bennekom, the Netherlands) and contained 63 µg/g [^{13}C$_{10}$]β-carotene and 60 µg/g [^{13}C$_{10}$]retinyl palmitate. all-rac-α-Tocopheryl acetate (Roche Vitamins, Deinze, the Netherlands) was added to the oil at levels of 91, 122 or 150 µg/capsule, respectively. The capsules used in the present study were made from bovine gelatin (Capsugel, Bornem, Belgium) and were filled with the oily mixture by electronic repetitive

multipipetting with 240, 320 or 400 µl. These actions were carried out under subdued light. The ^{13}C$_{10}$-labelled β-carotene and ^{13}C$_{10}$-labelled retinyl palmitate were synthesized at ARC Laboratories (Apeldoorn, the Netherlands) as described previously[30] (isotopic incorporation >99 %, isomeric purity >93 % all-trans, chemical purity >98 %). These compounds were food grade according to criteria established by the European Pharmacopoeia and the Joint FAO/WHO Expert Committee on Food Additives. The individual amount of labelled β-carotene and labelled retinyl palmitate varied from 35 to 90 µg/d as it was related to the individual's estimated daily energy intake which varied from 7 to 18 MJ/d. For example, a subject who consumed 11 MJ/d received 55 (SD 0·5) µg [^{13}C$_{10}$]β-carotene and 55 (SD 0·5) µg [^{13}C$_{10}$]retinyl palmitate each day (one capsule of 15 (SD 0·2) µg and two capsules of 20 (SD 0·3) µg [^{13}C$_{10}$]β-carotene and [^{13}C$_{10}$]retinyl palmitate). Likewise, a subject who consumed 8 MJ/d received 40 (SD 0·4) µg/d, and a subject consuming 14 MJ/d received 70 (SD 0·7) µg/d each of labelled β-carotene and labelled retinyl palmitate. Concentrations of retinol and β-carotene in the capsules were analysed by HPLC with absorbance detection[28]. There is no known health risk associated with ingestion of stable isotope-labelled β-carotene or retinyl palmitate. The degree of isotopic enrichment in serum of retinol with [^{13}C$_5$]retinol (derived from administered [^{13}C$_{10}$]β-carotene) and with [^{13}C$_{10}$]retinol and of β-carotene with [^{13}C$_{10}$]β-carotene was measured by using LC–MS with atmospheric pressure chemical ionization (APCI LC-MS) as described previously[31,32]. Signals for β-carotene were detected at mass-to-charge ratios (m/z) 537, for [^{13}C$_{10}$]β-carotene at m/z 547, for retinol at m/z 269, for [^{13}C$_5$]retinol at m/z 274 and for [^{13}C$_{10}$]retinol at m/z 279. The sample preparation, accuracy and precision of the measurement of the degree of isotopic enrichment of β-carotene and of retinol in serum and in faeces have been described in Zhu et al. [33].

Calculations

Isotopic enrichment levels of β-carotene were calculated as the signal measured by LC–MS at m/z 546 divided by the total signal at m/z 536 and 546. Enrichment levels of retinol were calculated as the signal at m/z 274 (or 279) divided by the total signal at m/z 269, 274 and 279. The vitamin A equivalency (µg) of β-carotene in oil relative to that of retinol in oil was calculated for each subject as the inverse ratio of the dose-corrected ratio of [^{13}C$_5$]retinol to [^{13}C$_{10}$]retinol in serum (by weight) as follows[34]:

$$1/(\text{Enrichment of retinol in serum with } [^{13}C_5] \text{ retinol/}$$
$$\text{Enrichment of retinol in serum with } [^{13}C_{10}] \text{ retinol}) \quad (1)$$
$$\times (\text{Dose of } [^{13}C_{10}] \text{ retinol/Dose of } [^{13}C_{10}]\beta - \text{carotene}).$$

A necessary condition for using equation 1 is to standardize strictly the daily nutrient intake during the 3-week intervention study.

A bio-efficacy of 100 % would mean that 1 µmol dietary β-carotene (537 µg) is absorbed and converted totally to retinol, thus yielding 2 µmol retinol (572 µg). Thus, the

bio-efficacy (%) of β-carotene was calculated from the vitamin A equivalency (equation 1), as follows[1,34]:

$$((537/2 \times 286)/\text{vitamin A equivalency}) \times 100. \quad (2)$$

For each diet the apparent absorption (%) of total β-carotene (both labelled and unlabelled) was calculated for each subject by subtracting the amount of β-carotene in faeces (72 h) from the amount consumed (72 h) and dividing the difference by the amount consumed multiplied by 100 as shown in Table 5. This apparent absorption multiplied by the estimated bioconversion (2 μg β-carotene in the enterocyte is equivalent to 1 μg retinol in the body) resulted in an estimated bio-efficacy and from that the estimated vitamin A equivalencies for β-carotene in the 'oil diet' and for β-carotene in the 'mixed diet'.

Statistical analysis

Data are shown as means and 95 % CI or standard deviation (in the case of descriptive measures). Data of serum concentrations and enrichments were averaged for days 0 and 1, for days 21 and 22, and for days 42 and 43 for each subject. Two-tailed t tests for independent samples were performed to evaluate differences in baseline characteristics (haematological blood values, alanine aminotransferase, creatinine, alkaline phosphatase, cholesterol) between the two groups. ANOVA was used to test period effects with diet and order as mean effects in the model. Because the order of the two diets did not significantly contribute to the model, serum retinol and serum β-carotene concentrations at baseline and after each diet were compared between the two groups with a paired t test. To test whether the percentages of apparent absorption were significantly different between both diets, two-tailed independent sample t tests were performed. All tests were two-sided, and P values < 0.05 were considered significant. The computer package SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA, USA) was used for all statistical calculations and data handling.

Results

Twenty-four subjects (aged 18–35 years) initiated and completed the study. As subjects were matched, their basic characteristics did not differ between the groups (Table 1). No significant differences were observed between both groups with respect to haematological analyses of blood, liver enzymes, creatinine, alkaline phosphatase and cholesterol. The reported and observed compliance and adherence to the dietary restrictions were very good; the observers reported 100 % consumption of the capsules during weekdays; the participants reported to have consumed 99·6 % of the capsules and 98 % of the diet. Inspection of the diaries did not reveal any deviations from the protocol, which could have affected the results.

The daily energy and nutrient content of the diets is given in Table 2 and the dietary source of β-carotene of the diets is divided into the salad dressing oil and the vegetables and fruits. Table 3 shows the serum concentrations of retinol and provitamin A carotenoids of the subjects during the study.

Table 3. Serum concentrations (μmol/l) of retinol and provitamin A carotenoids of two consecutive days of collecting fasting blood samples at baseline and after 3 weeks of controlled diets*

(Mean values and standard deviations)

	Baseline (n 24)		After 'oil diet' (n 24)		After 'mixed diet' (n 24)	
	Mean	SD	Mean	SD	Mean	SD
Retinol	1·99	0·45	1·83	0·50	1·81	0·44
Total β-carotene	0·78[a]	0·37	1·22[b]	0·59	1·01[b]	0·41
trans-β-Carotene	0·69[a]	0·35	1·15[b]	0·58	0·95[b]	0·40
cis-β-Carotene	0·09[a]	0·03	0·05[b]	0·02	0·06[b]	0·01
α-Carotene	0·10[a]	0·06	0·09[a]	0·05	0·15[b]	0·06
β-Cryptoxanthin	0·34	0·15	0·27	0·09	0·24	0·09

a,b Mean values within a row with unlike superscript letters were significantly different ($P < 0.001$).
* For details of subjects, diets and procedures, see the Subjects and methods section and Tables 1 and 2. There were no differences between groups at baseline, after the 'oil diet', and after the 'mixed diet' (paired t tests). Group 1 (n 12) followed for 3 weeks the 'oil diet' diet and consecutively for 3 weeks the 'mixed diet' and group 2 (n 12) followed for 3 weeks the 'mixed diet' and consecutively for 3 weeks the 'oil diet'.

There were no significant differences between groups in terms of serum retinol and serum β-carotene concentrations after each of the 3-week controlled diets. Compared to the baseline, the β-carotene concentrations in serum significantly increased as a result of the 'oil diet' and the 'mixed diet'. Both diets produced slight drops in serum concentrations of retinol due to the relatively low amounts of preformed vitamin A in these diets.

The isotopic enrichments of retinol and β-carotene in serum are shown in Table 4. For each subject, the vitamin A equivalency of [$^{13}C_{10}$]β-carotene in oil was calculated (Table 4). The mean vitamin A equivalency of [$^{13}C_{10}$]β-carotene in oil (the amount of β-carotene that has the same vitamin A activity as 1 μg retinol) was 3·4 μg (95 % CI 2·8, 3·9; CV 39 %) in the presence of the 'oil diet' and 3·4 μg (95 % CI 2·9, 3·9; CV 34 %) in the presence of the 'mixed diet'. Consequently, the bio-efficacy of [$^{13}C_{10}$]β-carotene in oil was 28 % (95 % CI 24, 33) in the presence of the 'oil diet' and 28 % (95 % CI 24, 32) in the presence of the 'mixed diet'.

Using data obtained with the oral–faecal balance technique, the difference in vitamin A equivalency of β-carotene in the 'oil diet' and the 'mixed diet' became clearly noticeable in the calculation of the apparent absorption (%) of β-carotene from the two diets from faeces data (Table 5); significantly more β-carotene was absorbed from the 'oil diet' (35 %; 95 % CI 24, 45) than from the 'mixed diet' (12 %; 95 % CI 1, 23) (Table 5). The apparent absorption of β-carotene from the 'oil diet' was approximately 2·9-fold higher than that of the 'mixed diet'. With the data of the oral–faecal balance and the generally assumed bioconversion of 50 % for β-carotene[2–4], the bio-efficacy of the unlabelled β-carotene was estimated from both diets. For the 'oil diet', this bio-efficacy was 18 % (0.35×0.5) by multiplying the apparent absorption of β-carotene and the estimated bioconversion, and so the estimated vitamin A equivalency of β-carotene to retinol would be 5·4:1 (95 % CI 3·8, 7·0). For the 'mixed diet', this bio-efficacy was 6 % (0.12×0.5), and so the estimated vitamin A equivalency of β-carotene to retinol would be 15·7:1 (95 % CI 1·0, 30·4). This estimation for the 'mixed diet' is rough, because of very high variation (CV 61 %) in weight of total

Table 4. Vitamin A equivalency and bio-efficacy of $[^{13}C_{10}]\beta$-carotene in oil after 3 weeks of controlled diets*

(Mean values and 95 % confidence intervals)

Symbol	Description	Oil diet (*n* 24)		Mixed diet (*n* 24)	
		Mean	95 % CI	Mean	95 % CI
$E_{5,sR}$	Enrichment of retinol in serum with $[^{13}C_5]$retinol	0·00322	0·00277, 0·00367	0·00330	0·00272, 0·00387
$E_{10,sR}$	Enrichment of retinol in serum with $[^{13}C_{10}]$retinol	0·00591	0·00511, 0·00671	0·00590	0·00500, 0·00680
$E_{10,sC}$	Enrichment of β-carotene in serum with $[^{13}C_{10}]\beta$-carotene	0·01631	0·01437, 0·01825	0·01902	0·01628, 0·02176
$E_{10,fC}$	Enrichment of β-carotene in faeces with $[^{13}C_{10}]\beta$-carotene	0·00373	0·00353, 0·00394	0·00295	0·00278, 0·00311
$A_{R,cap}$	Dose (μmol/d) of $[^{13}C_{10}]$retinol from capsules†	0·100	0·024	0·101	0·027
$A_{C,cap}$	Dose (μmol/d) of $[^{13}C_{10}]\beta$-carotene from capsules†	0·104	0·025	0·105	0·028
$AEq_{,C}$	Vitamin A equivalency (μg) of $[^{13}C_{10}]\beta$-carotene in oil‡	3·347	2·81, 3·88	3·380	2·91, 3·85
$BE_{,C}$	Bio-efficacy (%) of $[^{13}C_{10}]\beta$-carotene in oil§	28·0	24, 33	27·8	24, 32

* For details of subjects, diets and procedures, see the Subjects and methods section and Tables 1 and 2. The 'oil diet' is a diet containing vegetables low in β-carotene and supplemented with synthetic β-carotene in salad dressing oil and the 'mixed diet' is a diet containing vegetables high in β-carotene. Each subject followed both diets for 3 weeks in crossover design. All 6 weeks, each subject daily consumed capsules with $[^{13}C_{10}]\beta$-carotene and $[^{13}C_{10}]$retinol in oil.
† Values are means and standard deviations.
‡ $AEq_{,C}$ is the amount of β-carotene that has the same vitamin A activity as 1 μg retinol and was calculated as follows: $1/((E_{5,sR}/E_{10,sC}) \times (A_{R,cap} \times 286/A_{C,cap} \times 537))$.
§ $BE_{,C}$ was calculated by $((537/2 \times 286)/$Vitamin A equivalency$) \times 100$; a bio-efficacy of 100 % would mean that 1 μmol dietary β-carotene (537 μg) would yield 2 μmol retinol (572 μg).

faeces collection in 72 h (Table 5). Six of the twenty-four subjects had a negative oral–faecal total β-carotene balance for the 'mixed diet' due to the relatively high weight of total 72 h faeces collection (Table 5). Excluding these six subjects, the apparent absorption of the 'mixed diet' was 18 % (95 % CI 13, 23) and the estimated vitamin A equivalency of β-carotene to retinol would be 10·4:1 (95 % CI 5·3, 15·5). None had a negative oral–faecal total β-carotene balance for the 'oil diet'. Neither labelled retinol nor unlabelled retinol were detected in faeces. The efficiency of absorption of retinol is generally high, over 90 % in healthy subjects[2–4].

Discussion

Vitamin A equivalency of $[^{13}C_{10}]\beta$-carotene in oil using data obtained with the dual-isotope dilution technique

The present data show that in these healthy adults an amount of 3·4 μg (95 % CI 2·8, 3·9) β-carotene dissolved in oil has the same vitamin A equivalency as 1 μg retinol in oil; and thus the

bio-efficacy of β-carotene dissolved in oil was 28 %. The results were similar for both diets.

This stable-isotope method is based on the isotopic enrichment of retinol and β-carotene in serum reaching a plateau during multiple dosing with $[^{13}C_{10}]\beta$-carotene and $[^{13}C_{10}]$retinol. It is assumed that dietary β-carotene and retinol released from the food matrix and available for absorption mix completely with labelled β-carotene and labelled retinol. The degree of isotopic enrichment of β-carotene was calculated as the signal by LC–MS for $[^{13}C_{10}]\beta$-carotene divided by total unlabelled and $[^{13}C_{10}]\beta$-carotene. The degree of isotopic enrichment of β-carotene in serum with $[^{13}C_{10}]\beta$-carotene was different in both diets, which could be partially explained by the different daily dietary β-carotene intake. However, the degree of isotopic enrichment of retinol in serum with $[^{13}C_5]$retinol and with $[^{13}C_{10}]$retinol were similar in both diets. The comparable isotopic enrichment of $[^{13}C_{10}]$retinol was expected, because retinol mainly from milk and meat mix well with the labelled retinol. The comparable isotopic enrichment of $[^{13}C_5]$retinol was not expected, because the vegetable

Table 5. Apparent absorption of total β-carotene (labelled and unlabelled) from 72 h after 3 weeks of controlled diets*

(Mean values and standard deviations)

Symbol	Description	Oil diet (*n* 24)		Mixed diet (*n* 24)	
		Mean	SD	Mean	SD
C_{fC}	Total β-carotene concentration in faeces (μg/g)	29·2[a]	8·7	48·2[b]	15·0
W_f	Weight of total faeces collection in 72 h (g)	343[a]	16·1	462[b]	28·2
A_f	Faecal β-carotene (mg) ($C_{fC} \times W_f$)	9·6[a]	4·6	19·4[b]	7·2
A_d	Dietary total β-carotene (mg)	14·7[a]	3·6	22·4[b]	6·1
$A_d - A_f$	Apparent absorbed β-carotene (mg)†	5·1	4·2	3·0	7·3
AA_C	Apparent absorption (%) of β-carotene‡	34·7[a]	24·3, 45·1	11·9[b]	0·6, 23·2

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.001$; two-tailed *t* tests for independent samples).
* For details of subjects, diets and procedures, see the Subjects and methods section and Tables 1 and 2. The 'oil diet' is a diet containing vegetables low in β-carotene and supplemented with synthetic β-carotene in salad dressing oil and the 'mixed diet' is a diet containing vegetables high in β-carotene. Each subject followed both diets for 3 weeks in crossover design. All 6 weeks, each subject daily consumed capsules with a mean of 55 μg/d $[^{13}C_{10}]\beta$-carotene ($A_{C,cap}$) and 55 μg $[^{13}C_{10n}]$retinyl palmitate in oil.
† Six subjects had a negative oral–faecal total β-carotene balance for the 'mixed diet'.
‡ Values are means and 95 % CI. AA_C was calculated by oral–faecal balance (72 h) as follows: $(A_d - A_f)/A_d \times 100$.

and fruit matrices of the β-carotene were different between the 'oil diet' and the 'mixed diet'. Due to [$^{13}C_5$]retinol resulting from the central cleavage of [$^{13}C_{10}$]β-carotene, the calculation of vitamin A equivalency of β-carotene relative to that of retinol in oil only includes the isotopic enrichment of [$^{13}C_5$]retinol and of [$^{13}C_{10}$]retinol. The vitamin A equivalency of β-carotene was similar in both diets. The assumption of this labelling technique that labelled β-carotene and unlabelled β-carotene fully mix should be rejected. Thus the dietary matrix does not affect the bio-efficacy of β-carotene in oil and this means that the extrinsic dual-isotope dilution technique (adding a tracer in oil capsules to the diet) with the current calculations is not suitable for investigating the absorption of β-carotene from plant matrices.

The measured bio-efficacy of labelled β-carotene of 28 % represented the highest feasible bio-efficacy, because the β-carotene was delivered to the intestine in the most optimal form: a solution in oil in a capsule, which dissolved in the stomach. The present findings are consistent with the FAO/WHO guideline[2,3] of 28 % for β-carotene from oil (vitamin A equivalency of β-carotene to retinol is 3·3:1 μg).

Preceding studies from our group in Indonesia[1,34] showed a higher bio-efficacy of β-carotene from oil, 36 %, and vitamin A equivalency of β-carotene to retinol of 2·7:1 μg. This was in line with the guideline of the US Institute of Medicine (vitamin A equivalency of β-carotene to retinol in oil of 2:1 μg)[4]. Differences between our previous studies in a developing country and the present study in a developed country were the research population (children v. adults), diet (low in retinol and β-carotene v. low in retinol and high in β-carotene) and nutrient status (vitamin A depleted v. vitamin A sufficient). Therefore, the present results are consistent with the expectation that the efficiency of absorption and conversion of β-carotene are higher in those with higher needs[3].

Vitamin A equivalency of dietary β-carotene using data obtained with the oral–faecal balance technique

Using data obtained with the oral–faecal balance technique, we observed that supplemental β-carotene in the 'oil diet' is an approximately 2·9 times better source of β-carotene, and thus vitamin A, than β-carotene in a 'mixed diet'. The 'oil diet' was representative of a diet low in vegetables and fruits with consumption of food items fortified with retinol and/or β-carotene and/or β-carotene supplements, such as regularly consumed in industrialized societies. The estimated vitamin A equivalency of β-carotene in the 'oil diet' of 5·4:1 still contains 33 % β-carotene from vegetables and fruits (see Table 2). This approximate 6:1 factor could be calculated by 1/3 of factor 12:1 from the US Institute of Medicine for β-carotene in a mixed diet and by 2/3 of factor 3:1 for β-carotene in oil. The approximation of 3 μg β-carotene in oil can confirm the results of the dual-isotope dilution technique vitamin A equivalency of [$^{13}C_{10}$]β-carotene in oil of 3·4 μg.

The current mixed-diet guideline of the US Institute of Medicine[4] that the vitamin A activity of 1 μg retinol can be supplied by 12 μg β-carotene is slightly lower than our rough estimation for the 'mixed diet' (1 μg retinol from about 16 μg β-carotene). In the present study, the 'mixed diet' represented a healthy diet with high amounts of cooked vegetables and fruits including all necessary nutrients and fibres for optimal health.

Data from the oral–faecal balance could be overestimated by incomplete faeces collection or degradation of β-carotene by the microflora and underestimated because of excretion of endogenously secreted β-carotene together with mucosa cells which also contain β-carotene. However, oral–faecal balance data of 72 h from twenty-four subjects provided rather reliable estimations because they strictly complied with our collection instructions and were aware of the need for complete 72 h faeces collection. The average CV of total β-carotene concentration in faeces was comparable between both diets (30 %; see Table 5). However, the CV of total faeces weight for the 'mixed diet' was 61 % and for the 'oil diet' 47 %. The high variability in apparent absorption of β-carotene from the 'mixed diet' is caused mainly by the large variability in faeces weight during the 'mixed diet'.

It should be noted that the proportion of dietary β-carotene contributed by fruits in relation to vegetables described in the guidelines of the US Institute of Medicine is 1:4, which was not similar to the proportion used in the present study (fruits: vegetables was 1:8·2 in the 'mixed diet'), as the bio-efficacy of β-carotene in fruits is often higher than that of β-carotene in vegetables[7,10–13,16,34]. In the Netherlands, the most common fruits contain only low amounts of β-carotene, therefore 1:8 represents a regular ratio of β-carotene content of fruits to vegetables in a west European diet.

In most of the previous studies which quantified the bio-efficacy of β-carotene, one or more of the following factors were not standardized: the controlled diet, the energy intake and β-carotene intake. Therefore, these factors which influence the bio-efficacy of β-carotene were controlled in the present study. Also in the present study the compliance was very high. Single meal studies have to contend with even more confounding factors that affect the bio-efficacy of β-carotene. Another advantage of the present diet-controlled design was that the dietary enrichment of retinol and β-carotene was constant across all subjects, because each subject received a dose of labelled compounds proportional to their individual energy intake and unlabelled β-carotene intake.

Recently, two studies were reported that used intrinsic labelling of vegetables to quantify the bio-efficacy of β-carotene[35,36]. Tang et al.[36] showed a vitamin A equivalency of β-carotene from spinach of 21 (SD 9) μg and of β-carotene from carrot of 15 (SD 7) μg, which is consistent with our rough estimate of 16 μg β-carotene in a diet with vegetables high in β-carotene content such as carrots. The advantage of intrinsic labelling is that labelled β-carotene is contained within the food matrix instead of being added to or co-administered with food. However, intrinsic labelling has some significant limitations; these specially produced vegetables are very expensive, few subjects have been able to participate in the published studies and the bio-efficacy of β-carotene was determined after only a single meal. Furthermore, the vitamin A equivalency and bio-efficacy of β-carotene determined using intrinsic labelling might vary depending upon the plant and how it is prepared. Therefore, while the intrinsic labelling technique could provide reliable data for a few individuals eating a specific vegetable, it could not provide data for a large population consuming a varied diet. In comparison, the present study was designed to estimate the bio-efficacy of β-carotene in a western diet instead of in a single vegetable. The present results are valid for adults with an adequate

vitamin A status, and in apparently good health, consuming a varied western diet.

In conclusion, our oral–faecal balance data of twenty-four healthy adults showed that the estimated vitamin A equivalencies were 6:1 for a controlled diet with the supplemental β-carotene in salad dressing oil and 16:1 for a diet with vegetables and fruits high in β-carotene. Thus, the bio-efficacy of supplemental β-carotene in oil was approximately 2·9-fold more than β-carotene from vegetables and fruits in healthy subjects consuming a western diet. The present isotopic data showed that the vitamin A equivalency of $[^{13}C_{10}]$β-carotene in oil was 3·4 µg (95 % CI 2·8, 3·9; bio-efficacy 28 %) regardless of the presence of diets high in unlabelled β-carotene in either an oil matrix or a matrix of vegetables high in β-carotene.

Acknowledgements

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