Vitamin A equivalency of β-carotene in humans

Carolien van Loo-Bouwman

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Proefschrift

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ABSTRACT

Vitamin A equivalency of β-carotene (VEB) is defined as the amount of ingested β-carotene in μg that is absorbed and converted into 1 μg of retinol (vitamin A) in the human body. The first step of the intestinal absorption of β-carotene involves the disruption of the food matrix and the solubilisation of β-carotene within micelles (bioaccessibility) and the second step covers the entry into the intestinal cells, partial conversion into retinol, and entry into lymph and blood. Many estimates for VEB in various food matrices are reported with different methods such as oral–faecal balance, dose–response curves and isotopic labelling. The VEB is currently estimated by the US Institute of Medicine (IOM) as 12:1 in a mixed diet and 2:1 in oil and by the FAO/WHO as 6:1 in a mixed diet and 3·3:1 in oil.

β-Carotene in a Western diet contributes for approximately 14% to the vitamin A activity and β-carotene in the diet in developing countries for approximately 42%, as the latter diet is relatively high in vegetables, fruits and whole grains, and relatively low in meat and animal-derived products, which contain preformed vitamin A.

The overall aim of the thesis is to study the VEB in persons in general good health consuming a Western diet. This thesis contains a review of all published VEB values in various food matrices in which β-carotene is incorporated. Various factors and methods for estimating VEB are discussed to evaluate the reliability of the different studies. In this thesis, two diet-controlled, crossover intervention studies in adults are described. One diet contained vegetables low in β-carotene with supplemental β-carotene in oil (oil diet) and the other diet contained vegetables high in β-carotene (mixed diet). An extrinsic dual-isotope-labelling technique was used. The VEB values were calculated as the dose-corrected ratio of [$^{13}C_5$]retinol to [$^{13}C_{10}$]retinol in serum and from the apparent absorption by oral–faecal balance. Isotopic data quantify VEB in oil of 3·4:1 and 3·6:1 for both diets, respectively for the first study in 24 healthy adults and the second study in 17 ileostomy subjects. Using oral–faecal balance data and with the generally assumed conversion of 50% for absorbed β-carotene, the estimated VEB values for the oil diet were 5·4:1 and 6·7:1 and for the mixed diet 15·7:1 and 12·5:1, respectively for the first and second study. A study with an in vitro gastrointestinal model was also performed with the diets of the second in vivo study. The bioaccessibility of β-carotene was 53% from the oil diet and 28% from the mixed diet. This ratio of 1·9:1 is consistent with the 1·9-fold higher apparent absorption of β-carotene in the oil diet than in the mixed diet (30% and 16%, respectively).

For humans consuming supplemental β-carotene dissolved in oil, a VEB between 2:1 and 4:1 is feasible. For humans consuming a Western diet, a VEB in a mixed diet of 9:1 to 16:1 is realistic and encompasses the IOM VEB of 12:1.

Future research could focus on the VEB for a mixed diet in children, pregnant women and patients with fat malabsorption syndromes, who are at risk of inadequacy of vitamin A supply to maintain optimal liver storage.

ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
AUC	area under the curve
β	beta
BCMO1	β,β-carotene 15,15'-mono-oxygenase
BMI	body mass index
CI	confident interval
CV	coefficient of variance
d	day
EAR	estimated average requirement
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FFQ	food frequency questionnaire
g	gram
h	hour
Hb	haemoglobin
HPLC	high pressure liquid chromatography
IE	isotopic enrichment
Int.	international
IOM	Institute of Medicine in the United States
IS	internal standard
l	litre
LC	liquid chromatography
LC–MS	liquid chromatography mass spectrometry
m/z	mass-to-charge ratio
NA	not available
NZO	Dutch Dairy Association
RAE	retinol activity equivalents
RDI	recommended daily intake
RE	retinol equivalents
RIVM	National Institute for Public Health and the Environment
SCF	Scientific Committee on Food (Commission of the European Communities)
SD	standard deviation
TAG	triacylglycerol
t-BHQ	butylhydroquinone
THF	tetrahydrofuran
TIM-1	computer-controlled dynamic in vitro TNO gastro-Intestinal tract Model
TNO	Netherlands Organisation for Applied Scientific Research
TRL	TAG-rich lipoprotein
US	United States of America
v	volume
VEB	Vitamin A equivalency of β-carotene
WHO	World Health Organization
wt	weight

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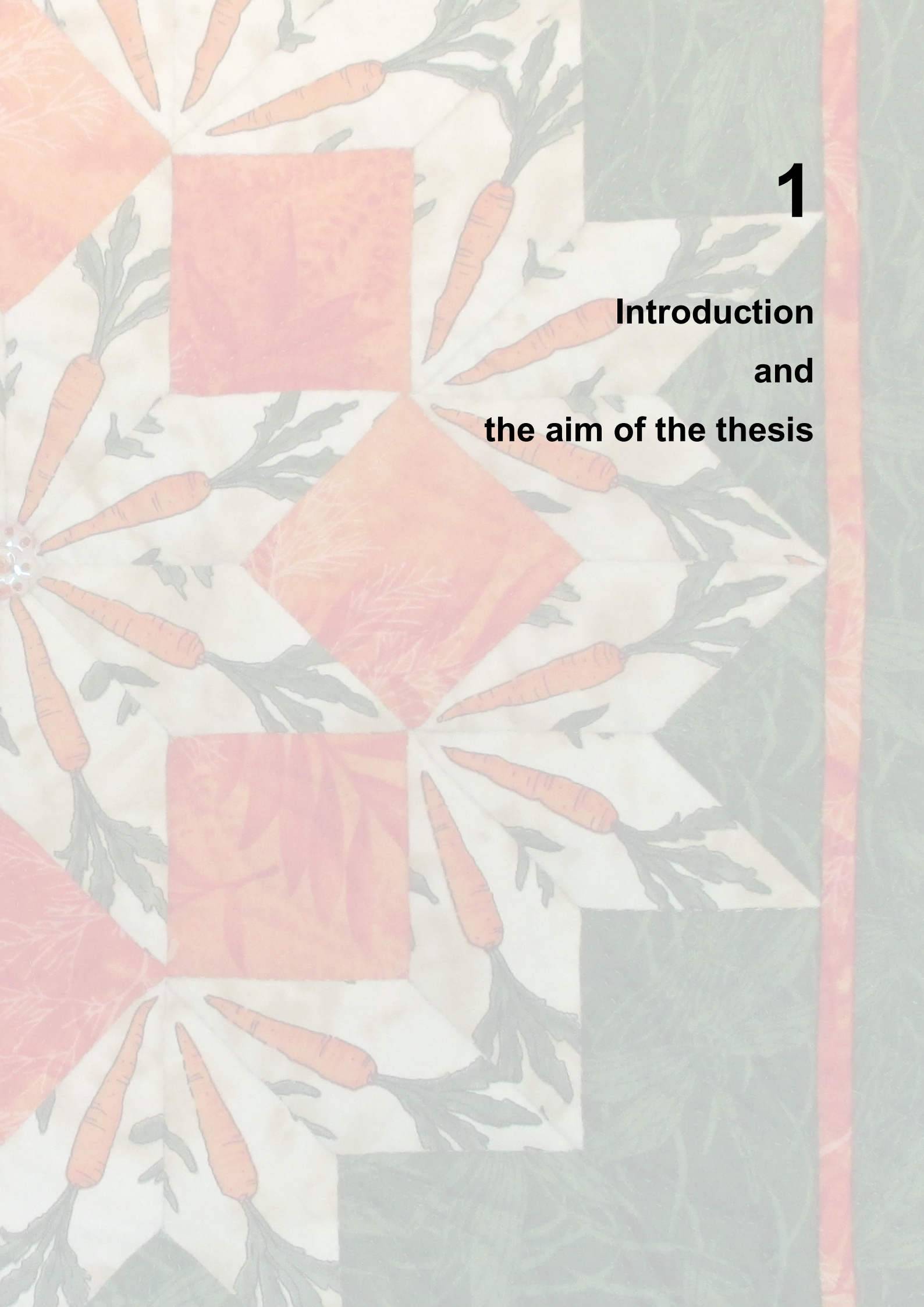

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Introduction and the aim of the thesis

INTRODUCTION

Background of the thesis

At the present time, there is considerable debate about the actual vitamin A equivalency of provitamin A carotenoids in food, from which β-carotene has the highest vitamin A activity. The discussion is about the importance of the factors, which may influence the absorption, and the bioconversion of β-carotene into retinol (vitamin A) in the human body. Reliable values of vitamin A equivalency of β-carotene in food in Western countries are needed for the dietary recommendations and the formulation of supplements. For developing countries, these reliable values are needed for the design of intervention strategies involving carotenoids and the formulation of effective policies for combating vitamin A deficiency.

Vitamin A deficiency (serum retinol level <0·70 μmol/l) affects an estimated 190 million preschool-age children and 19·1 million pregnant women globally, which corresponds to 33·3% of the preschool-age population and 15·3% of pregnant women in populations at risk of vitamin A deficiency, globally[1]. About 0·8 million (1·4%) of deaths worldwide results from vitamin A deficiency[2]. By the year 2015, two-thirds of the mortality rate among children under five years of age should be reduced as set in 2000, as one of the Millennium Development Goals by 189 United Nations Member States. For children and adults at risk for vitamin A deficiency, supplementation with vitamin A or β-carotene is one of the solutions to decrease the mortality rate, but on the long term biofortification of staple foods should be also applied. The so-called Golden Rice with a high level of β-carotene became license-free available in 2000, and in 2015 the transfer to the farmers will take place. The first Golden Rice will soon been consumed locally in some developing countries.

In this introduction chapter, a brief overview of the metabolism and the intake of vitamin A and β-carotene will be given. Also the aim of this thesis is formulated.

Vitamin A status in humans

Vitamin A (retinol) in the human body is essential for the function of the visual system, immune function, maintenance of epithelial cellular integrity, growth, and embryonic development. In the situation of depletion of the liver vitamin A stores, the serum vitamin A concentration is not a good indicator of liver vitamin A content, as the serum level remains unchanged across a wide range of liver storage levels. Moreover, there are differences in mean serum vitamin A levels between persons with high and low habitual intakes, as well as between persons in developed and developing countries. Given these discrepancies, serum vitamin A levels are not very useful as an indicator of vitamin A status unless the levels are very low, in which case the homeostatic mechanisms fail. But when these retinol levels are ≥1·05

μmol/l, equal to 30 μg/dl, they indicate adequate status and no vitamin A deficiency[3]. Toxicity of retinol in the liver may appear with high dietary retinol intake, but in most of the cases the hepatotoxicity is reversible after the withdrawal of vitamin A from the diet[4].

β-Carotene status in humans

Provitamin A carotenoids, from which β-carotene has the highest vitamin A activity, can be converted into retinol in the human body. Up to 90% of this conversion takes place in the gut mucosa. Other provitamin A carotenoids are α-carotene and β-cryptoxanthin and are present in food in much lower amounts than β-carotene.

The best condition for drawing blood samples to assess the serum β-carotene status of adults is in the fasting state. But fasting serum levels of carotenoids reflect the recent dietary intake of carotenoids. So, serum levels of β-carotene have a wide range between days and between persons, but serum levels of 0·20 to 0·80 μmol/l β-carotene are considered normal. β-Carotene levels of ≤2·2 μmol/l equal to ≤1·2 μg/ml can be regarded for sure as safe[5].

In contrast to high intake levels of vitamin A, high intake levels of β-carotene from natural foods do not cause organ toxicity. Many studies showed an association between increased intake of vegetables and fruits and higher blood concentrations of β-carotene with reduced risks for cancer and cardiovascular diseases[6,7]. These findings cannot be interpreted as a protective effect of β-carotene as the single nutrient causing this effect. Other beneficial components in vegetables and fruits or specific dietary patterns could contribute to this association.

Unexpectedly, there were findings from studies in smokers, which showed an increase of lung cancer incidence and more overall deaths with the consumption of β-carotene supplements at high doses of about 20 mg/d over a long period of time[8,9]. Therefore, the intake of β-carotene supplements in high doses should be dissuaded, despite no tolerable upper intake level for β-carotene has been set[4].

Dietary sources of vitamin A and β-carotene

The main sources of vitamin A in food are from animal-derived foods, such as dairy products, liver, eggs and fish oils. Vitamin A can also be supplied as a supplement or added in so-called fortified foods.

β-Carotene in food can be found in yellow and orange non-citrus fruits, green and yellow vegetables (including red and orange roots and tubers, such as carrots and sweet potato) as well as in several types of oils (e.g. red palm oil).

β-carotene intake

Daily intake of animal-derived food is not affordable by the majority of the world population. Therefore, β-carotene from vegetables and fruits provides most of the vitamin A activity in the diets of the majority of people in developing countries. Those persons, who consume multiple doses of fruits and vegetables per day, consume about 3 to 4 mg β-carotene. Approximately 2 mg β-carotene and 0·7 mg retinol are daily consumed by Dutch adults[10].

In the Western diet, about 66 to 80% of the habitual intake of vitamin A is preformed vitamin A and 20 to 34% intake originates from provitamin A carotenoids[11-13].

In contrast, the diet for the majority of persons in developing countries contains about 12 to 22% of preformed vitamin A and consequently they are depending for 78 to 88% of provitamin A carotenoids in the diet[3].

By assuming that at least 50% of the provitamin A carotenoids is β-carotene, dietary β-carotene contributes for approximately 14% in the Western diet and for about 42% in the diet in developing countries to the daily dietary vitamin A activity. These estimates are for persons in general good health. In addition, it is known that many factors influence the absorption and metabolism of β-carotene in humans.

Absorption and metabolism of vitamin A and β-carotene

Ingested retinol is very efficiently taken up into the intestinal mucosal cells. The dietary retinyl esters are hydrolysed in the intestine into retinol, which is then taken up either by diffusion with lipid micelles or by protein-facilitated transport[14,15]. Once in the mucosa cells, the retinol is esterified, packaged into chylomicrons and transported via the lymph to the blood.

Ingested β-carotene needs to be set free from the embedding food matrices. A large part of the β-carotene is excreted in the faeces, mainly because it is still incorporated in food matrices. The general mechanism of intestinal β-carotene absorption in humans is by passive diffusion of mixed micelles, which are formed during fat digestion in the presence of bile acids, into the intestinal mucosal cells. Once absorbed, about 50% of the β-carotene (on a weight basis) is converted into retinol in the mucosa. The newly-absorbed, unconverted β-carotene as well as the newly-formed retinol are packaged into chylomicrons, excreted into the lymph, delivered to the blood and partly taken up by the liver and in fatty tissues throughout the body[16].

The in vivo intestinal absorption of β-carotene involves several crucial steps, such as the release from the food matrix, the solubilisation into mixed lipid micelles in the lumen, and the cellular uptake by intestinal mucosal cells. Much attention has been paid to the matrix of plants, which seems to trap carotenoids, making them unavailable for absorption from the intestinal lumen. β-Carotene in foods of vegetable

origin is embedded in complex cellular structures such as the cellulose-containing matrix of chloroplasts (e.g. green leafy vegetables) or the pigment-containing portion of chromoplasts (e.g. non-citrus fruits and yellow vegetables). Disrupting the food matrix (e.g. processing, heating, mastication) has been shown to increase the bioaccessibility of β-carotene.

Habitual diet pattern and functional capacity of the intestine may explain differences in absorption of β-carotene between developing and developed countries. Additional nutrients in the diet could enhance or reduce the absorption of β-carotene, such as lutein and phytosterols. Another factor is the amount of dietary fat required to ensure maximum carotenoid absorption. This amount seems to be quite low, about 3 to 5 g per meal[17].

The acronym SLAMENGHI reflects the various factors known to affect the bioavailability and vitamin A equivalency of carotenoids. S) Denotes the species of carotenoid, L) the molecular linkage, A) the amount of carotenoids consumed in a meal, M) the matrix in which the carotenoid is incorporated, E) the effectors of absorption and bioconversion, N) the nutrient status of the host, G) the genetic factors, H) the host-related factors, and I) the mathematical interactions[18-20].

Terms used in the terminology of β-carotene metabolism

For expressing absorption, different terms are used. Therefore, the explanation of the terms is listed below.

- ✓ Vitamin A equivalency of β-carotene is the amount of ingested β-carotene in µg that is absorbed and converted into 1 µg of retinol in the human body (so, the amount of ingested β-carotene in µg with the same vitamin A activity as 1 µg retinol.)
- ✓ Bioaccessibility of β-carotene is the fraction of the ingested amount β-carotene, which is released from the food matrix (e.g. vegetables and fruits) and is available for absorption.
- ✓ Bioavailability of β-carotene is the fraction of the ingested amount β-carotene, which is absorbed and utilized for normal physiological functions or for storage. The 'relative bioavailability of β-carotene' could be assessed by comparing two or more treatments[21].
- ✓ Bioconversion of β-carotene is the fraction of absorbed β-carotene that is converted into retinol in the body.
- ✓ Bioefficacy of β-carotene is the product of the fraction of the ingested amount β-carotene, which is absorbed, and the fraction of that which is converted into retinol in the body.

Vitamin A equivalency of β-carotene

Values of vitamin A equivalency of β-carotene are needed for the dietary recommendations, the formulation of supplements, and the design of intervention strategies involving carotenoids. The concept of the retinol equivalents (RE) was introduced in 1967 by the Food and Agriculture Organization & World Health Organization (FAO/WHO) expert group[22]. They proposed that 1 μg ingested β-carotene in a mixed diet is equal to 0·167 μg RE (factor 1/6) and that 1 μg of one of the other ingested provitamin A carotenoids is equal to 0·084 μg RE (factor 1/12). So, to calculate the total RE in a diet, the amount of retinol in μg, the amount of β-carotene in μg divided by 6, the amount of α-carotene in μg divided by 12, and the amount of β-cryptoxanthin in μg divided by 12 have to be added up[3,12,22]. These equivalencies were derived from balance studies. The report of 1967 of the FAO/WHO[22] referred to a committee of the International Union of Pure and Applied Chemistry, which estimated that, in the physiological dose range, 1 μg β-carotene dissolved in oil was equivalent to 0·3 μg retinol, so 3·3 μg β-carotene in oil has the same vitamin A activity as 1 μg retinol and other provitamin A carotenoids are presumed to be 50% as active as β-carotene.

The US Institute of Medicine[13,23] (IOM) has introduced a new term, 'retinol activity equivalents' (RAE), to express the activity of carotenoids in terms of vitamin A (1 μg RAE = 1 RE of retinol (vitamin A) = 1 μg retinol (vitamin A)). The vitamin A activity of 1 μg of retinol can be supplied by 2 μg of β-carotene in oil. They calculated the vitamin A equivalency of β-carotene in a mixed vegetable diet of 1:12 from the product of 1:2 (the bioavailability of β-carotene in oil) and 1:6 (the bioavailability of β-carotene in a mixed diet). The vitamin A equivalency of other provitamin A carotenoids has been set at half this value as in the FAO/WHO recommendations.

In conclusion, two different calculations are in use at the moment for calculating the amount of retinol available for the human body after consumption.

Table 1. Overview of the vitamin A equivalencies (amount of provitamin A carotenoids with the same vitamin A activity as 1 μg retinol) according to the recommendations of the FAO/WHO (2004)[3] and the US Institute of Medicine (2001)[13].

Carotenoid and matrix	FAO/WHO (applying RE[a])	US Institute of Medicine (applying RAE[b])
β-carotene in oil	3·3:1	2:1
β-carotene in food	6:1	12:1
Other provitamin A carotenoids in food	12:1	24:1

[a]RE= retinol equivalents; [b]RAE=retinol activity equivalents.

Recommended daily intake of vitamin A

A report of the Dutch Institute for Public Health and the Environment[11] assessed that 7% of children below 4 years and 17 to 30% of the Dutch adults have an inadequate vitamin A intake to maintain sufficient vitamin A stores. The adequate daily intakes in the Netherlands are for women 800 and for men 1000 μg RE/d[24]. Dutch men should consume theoretically as solely source of β-carotene, for example 60 g of cooked carrots or 72 g of raw carrots or 417 g of average cooked vegetables or 2·7 kg of fresh citrus fruits[25].

The population reference intake of retinol for European countries is 700 μg RE/d for men and 600 μg RE/d for women[26]. The FAO/WHO assessed the recommended safe intake for adults on 600 μg RE/d[3]. Tolerable Upper Intake Level for preformed vitamin A (retinol and retinyl esters) was established on 3000 μg RE/d for adults, including women of child-bearing age. There is insufficient data to set a tolerable upper intake level for β-carotene[4].

Methods to measure the absorption and bioconversion of β-carotene

The most commonly applied methods include the assessment of the increase in plasma or serum carotenoid concentration following chronic supplementation, and the assessment of postprandial chylomicron carotenoid or retinyl ester response following a single dose of carotenoid[21]. Other methods used in the past are depletion-repletion studies, animal models, oral–faecal balance techniques, and techniques using radio- or stable isotopic tracers. Many dose–response studies have been conducted with β-carotene (for review, see Swanson *et al.*[27]) and are doubtful because of the homeostatic control of serum retinol concentrations. Change from baseline of plasma carotenoid concentration can be useful as an endpoint to estimate the relative bioavailability of carotenoids in human subjects, provided such studies are sufficiently long to result in a new steady-state condition. Animal models can be very useful for studying qualitative problems, but they have limited use for studying quantitative processes in humans, since the bioconversion of β-carotene is highly species-dependent. So, a rat converts nearly all and a cat converts nearly none of the absorbed β-carotene, apparently due to low levels of intestinal cleavage[16]. Data obtained from oral–faecal balance techniques may be inaccurate, because gastric or bacterial degradation of carotenoids in the gut may contribute to overestimation of the absorption of carotenoids. On the other hand, endogenous carotenoids from the intestinal cells or bile acids may be excreted in faeces, thus leading to underestimation of the absorption of carotenoids.

The above mentioned methods give only rough estimates of absorption of β-carotene. The most promising methods in terms of accurate measurement of β-

carotene absorption are methods, which measure newly synthesized metabolites isolated from the postprandial triglyceride-rich lipoprotein plasma fraction or chylomicron fraction.

Radioisotopes have been used reluctantly, because of the potential radiation damage. In the past two decades, the availability of stable isotope-labelled compounds increased, stable isotope techniques came available and also the analyses of these stable isotopes were improved. The use of stable isotopes in research is considered safe for human subjects and accepted by the institutional review boards. The majority of studies have been performed with extrinsically labelled food, which involves mixing with an exact dose of isotope into a food source. Applying advanced techniques, intrinsically labelled vegetables can be produced by irrigating with ^{2}H-labelled water or by supplying $^{13}CO_2$ in closed atmosphere[28]. A few studies have been conducted using intrinsically isotope-labelled vegetables for studying the bioavailability and vitamin A equivalency of β-carotene in humans. Because these specially produced vegetables are very expensive, few subjects could participate and absorption and metabolism from only one single meal was measured. With this intrinsic labelling technique, data for different single vegetables can be obtained.

In conclusion, although isotopic tracer techniques can provide quantitative data on the bioavailability and vitamin A equivalency of provitamin A carotenoids with high precision, their use has so far not yield reliable data for a complex mixed diet with a variety of vegetables and fruits for the Western population.

THE AIM OF THE THESIS

The aim of the thesis is to study the vitamin A equivalency of β-carotene in persons in general good health consuming a Western diet. The advantage of a diet-controlled study in subjects with an ileostomy is to study the apparent fractional absorption of β-carotene and to exclude the possible effect of bacterial degradation or even synthesis of β-carotene in the large bowel. Studies about foods with different food matrices show various vitamin A equivalencies of β-carotene. In the case of vegetables, the β-carotene should be released from the matrix before consumption or during digestion, otherwise it will be excreted in the faeces.

The central questions of this thesis are:

1) What is known about vitamin A equivalency of β-carotene from various food matrices in humans and which methods are used for the assessment of the vitamin A equivalency of β-carotene from various food matrices in humans?
2) What is the vitamin A equivalency of β-carotene and apparent absorption of β-carotene from a diet without and with β-carotene-rich vegetables and fruits in healthy subjects?
3) What is the vitamin A equivalency of β-carotene and apparent absorption of β-carotene from a diet without and with β-carotene-rich vegetables and fruits in healthy subjects with an ileostomy?
4) What is the bioaccessibility of β-carotene in homogenized diets?
5) In the case of extrinsic labelling, does the added labelled β-carotene and unlabelled β-carotene completely exchange during digestion?

Extensive literature research is performed. Two dietary controlled crossover intervention studies are performed with the selected labelling technique and with the oral–faecal balance technique. Two typically consumed diets are chosen: An ‘oil diet’ and a ‘mixed plant diet’ with provitamin A carotenoids content in the physiological range to study normal metabolism. A study in an in vitro gastrointestinal model is performed with the same diet as provided in the intervention study with subjects with an ileostomy for comparing in vitro and in vivo data. In this in vitro study the assumption is a complete exchange of labelled and unlabelled β-carotene in the intestine lumen is investigated. In other words, the assumption is that the supplemented labelled β-carotene and retinyl esters are digested, absorbed, converted, secreted and cleared with approximately the same kinetics as the unlabelled carotenoids and retinyl esters in the diet.

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A review of vitamin A equivalency of β-carotene in various food matrices for human consumption

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ABSTRACT

Vitamin A equivalency of β-carotene (VEB) is defined as the amount of ingested β-carotene in μg that is absorbed and converted into 1 μg of retinol (vitamin A) in the human body. The objective of the present review was to discuss the different estimates for VEB in various types of dietary food matrices. Different methods are discussed such as mass balance, dose–response and isotopic labelling. The VEB is currently estimated by US Institute of Medicine (IOM) as 12:1 in a mixed diet and 2:1 in oil. For humans consuming β-carotene dissolved in oil, a VEB between 2:1 and 4:1 is feasible. A VEB of approximately 4:1 is applicable for biofortified cassava, yellow maize and Golden Rice, which are specially bred for human consumption in developing countries. We propose a range of 9:1–16:1 for VEB in a mixed diet that encompasses the IOM VEB of 12:1 and is realistic for a Western diet under Western conditions. For a 'prudent' (i.e. non-Western) diet including a variety of commonly consumed vegetables, a VEB could range from 9:1 to 28:1 in a mixed diet.

Key words: Vitamin A equivalency: β-Carotene: Bioconversion: Human studies

Abbreviations: BCMO1, β,β-carotene 15,15'-mono-oxygenase; TRL, TAG-rich lipoprotein; VEB, vitamin A equivalency of β-carotene.

INTRODUCTION

Vitamin A equivalency of β-carotene (VEB) is defined as the amount of ingested β-carotene in μg that is absorbed and converted into 1 μg of retinol (vitamin A) in the human body. A certain amount of the ingested β-carotene is excreted in the faeces and the remaining part is absorbed, but not all of the absorbed β-carotene will be converted into retinol and enter the lymph, blood and finally the liver and other tissues.

Vitamin A can be obtained from animal-derived foods as preformed vitamin A, or from vegetables and fruit as provitamin A carotenoids, mainly β-carotene, α-carotene and β-cryptoxanthin. In the Western diet, about 20 to 34% of the habitual intake of vitamin A originates from provitamin A carotenoids[1-3]. In contrast, the majority of individuals in developing countries require >70% of provitamin A carotenoids in the diet[4]. The effect of food matrices of vegetables and fruits in which β-carotene is incorporated has been found to exert a major influence on measured VEB.

The objective of the present review was to discuss the different estimates for VEB in various types of dietary food matrices. The question is which VEB should be used for humans in generally good health.

For this purpose, first, the currently used VEB are described. Second, a brief overview of the main influencing factors on the assessment of VEB is given. Third, the habitual daily intake of dietary vitamin A and β-carotene in humans is described. Fourth, different methods for estimating VEB are discussed to evaluate the reliability of the different studies. The studies are grouped by the different types of dietary food matrices in which β-carotene is incorporated, which are the oil matrix, the complex mixed diet matrix with various vegetables and fruits and the single vegetable or fruit matrix.

Currently used vitamin A equivalency of β-carotene

Currently, two estimates of VEB for oil matrix and two estimates of VEB in mixed diet matrix are in use. In the 1967 recommendation of the FAO/WHO, the estimated VEB in the oil matrix was 3·3:1, so 3·3 μg β-carotene dissolved in oil would be required in the diet to produce 1 μg retinol in the human body[5]. According to the 1967 and 1988 recommendations of the FAO/WHO[2,5], the VEB in a mixed vegetable diet was estimated to be 6:1 and for other provitamin A carotenoids as 12:1. This VEB of 6:1 for a mixed diet is referred to as the retinol equivalents and is used in many food composition tables. The revised food composition tables have used the retinol activity equivalents, which is a VEB of 12:1 for a mixed diet and 24:1 for the other provitamin A carotenoids[1]. The term 'retinol activity equivalents' was introduced by the US Institute of Medicine (IOM)[1]. The IOM adopted a two-step process: first, examining VEB in oil, and then examining the absorption of β-carotene in vegetable foods relative to that of β-carotene dissolve in oil. The revised VEB of 12:1 in a mixed plant diet was calculated from the products of 1/2 (the intestinal conversion factor of β-carotene in oil) and 1/6 (the relative absorption of β-carotene in a mixed plant diet). The intestinal conversion factor of β-carotene in oil should be interpreted as 2 μg absorbed β-carotene in the enterocyte that is equivalent to 1 μg retinol in the human body, as has been reported in classical studies[6,7].

In the 2004 report of the FAO/WHO, the committee retained the VEB of 6:1 for a mixed diet and emphasized that the conditions that prevent carotenoids from entering enterocytes, such as food matrix, are more significant than previously thought, and that more data might support an even lower VEB for a mixed diet[4].

Main influencing factors on the assessment of vitamin A equivalency of β-carotene

There are many diet-related and host-related factors that may affect VEB. The main diet-related factors that influence VEB in humans are the food matrix in which β-carotene is incorporated, the amount ingested and the habitual diet type[8,9]. The

rupture of the food matrix by heating and homogenising promotes the release of β-carotene from the plant cells before and during digestion, and therefore it facilitates solubilisation into mixed lipid micelles in the lumen and cellular uptake by intestinal mucosal cells[10]. Cell wall structure in fruits is usually weaker than that in leaves, and therefore VEB for fruits deviates from that for vegetables[11].

VEB may be regarded as constant as long as the consumption of β-carotene is within physiological ranges and the host is in good health. With pharmaceutical doses of β-carotene, serum β-carotene levels increase, and VEB can decrease when an oral dose of β-carotene increases[2,12,13]. The habitual diet type determines the composition of the diet, and therefore various nutrient-to-nutrient interactions affect to a larger extent the absorption of β-carotene. For example, β-carotene absorption can be inhibited by lutein[14,15], when a minimum amount of about 5 g dietary fat is consumed simultaneously in a meal to ensure intestinal β-carotene uptake[16]. Also, absorption of β-carotene is reduced when dietary fibre content increases. Fibre interacts with bile acids, resulting in decreased absorption of fats and fat-soluble substances such as β-carotene[17].

Host-related factors, such as age, pregnancy, health status, immune status and treatment for worms and diarrhoea, can also affect VEB [18,19]. Intestinal helminthic infections are associated with malnutrition, and their effects are possibly mediated through impaired fat absorption and reduced vitamin absorption, particularly vitamin A[18,19]. Micronutrient malabsorption detected during intestinal parasitic infections is not easily explained or investigated, and may be caused by injury to the intestinal mucosa without invasion, mucosal invasion by parasites or bacterial overgrowth in the upper small bowel[20]. These host-related factors could have more influence on populations in developing countries than on populations in Western countries, where public health care is well organised and, in general, persons are in good health. Another host-related factor is the recently described polymorphism in the β,β-carotene 15,15'-mono-oxygenase (*BCMO1*) gene coding for the enzyme that cleaves β-carotene[21,22]. Studies have identified low responders who showed little or no response to plasma β-carotene concentration after a labelled dose of β-carotene[23-25]. The large inter-individual differences for estimates of VEB might be due to reduced enzymatic activity as a consequence of down-regulated activity of BCMO1 or polymorphisms in the *BCMO1* gene. However, in none of the studies discussed below were the genetic polymorphisms analysed, as the importance of BCMO1 was only recently realised; therefore, VEB may be more efficient than currently proposed.

The currently used VEB in a mixed diet and in oil are applicable only for individuals in general good health.

Habitual daily intake of vitamin A and β-carotene in human subjects

A Western type of diet, consumed by most people in developed countries, is relatively high in animal-derived foods (meat and dairy products), processed foods, fats and oils, and refined grains but relatively low in dietary fibres (vegetables, fruits, and whole grains)[26,27]. Milk and dairy products contribute 15 to 20% to total vitamin A intake. In the Western diet, about 66 to 80% of the habitual intake for vitamin A is preformed vitamin A in the diet, and 20 to 34% of intake is from provitamin A carotenoids[1-3]. By assuming that at least 50% of the provitamin A carotenoids are β-carotene, dietary β-carotene contributes for at least 10 to 17% in the Western diet to daily dietary vitamin A activity.

In contrast, the diet for most people living in developing countries contains only about 12 to 22% of preformed vitamin A, and consequently, they require 78 to 88% of provitamin A carotenoids in the diet[4]. By assuming that at least 50% of the provitamin A carotenoids are β-carotene, dietary β-carotene contributes for at least 39 to 44% in the diet to daily dietary vitamin A activity in developing countries. Many people residing in developing countries consume the 'prudent' diet type, which is high in fresh fruits and vegetables and whole grains (e.g. rice, maize), and low in meat[26,27]. Overall, adults in USA and Europe have a total vitamin A intake of 700–1000 μg retinol equivalents/d, and those in Southeast Asia and Africa have a total vitamin A intake of 400–800 μg retinol equivalents/d, which is particularly low in animal sources[4]. For populations that are highly dependent for their vitamin A status on the consumption of vegetables and fruits, it is important to have available the correct VEB values in various food matrices to be able to choose vegetables and fruits with the highest β-carotene amount and the highest VEB to maintain or increase their vitamin A status.

METHODS

In the past decades, various methods have been used for determination of VEB. Over the years, the chemical analyses of blood, lymph and faeces have been optimised, resulting in higher recoveries of internal standards and better reproducibility within and between samples. Many studies have been performed by measuring serum or plasma β-carotene and retinol levels after a single meal (dose–response) or after a period of depletion and/or repletion. By measuring β-carotene levels in the faeces during a controlled diet, oral–faecal mass balance can be investigated. More recently, progress in the analysis of radio- and stable isotopes in blood has made possible the application of isotope dilution methods. Extrinsic labelling involves mixing an exact dose of isotopically labelled β-carotene into a food

source. The great advantage of using isotope-labelled β-carotene is that only relatively small physiological doses need to be used to follow metabolic pathways such as true absorption and conversion into retinol by discriminating between absorbed and endogenous β-carotene. Table 1 shows the major strengths and major limitations of the methods used to study VEB in human subjects as discussed below.

Depletion–repletion method

Studies of depletion and repletion responses using a dark adaptation endpoint have been published by Hume & Krebs[28] and Sauberlich *et al*.[29]. During consumption of a vitamin A-deficient diet, subjects developed impaired dark adaptation (depletion phase). In the repletion phase of the study, the amount of retinol or β-carotene that was required to reverse the impaired dark adaptation was estimated. Such experiments are not allowed anymore because of medical ethical reasons. Moreover, the method provides only crude estimates because stepwise increased doses of β-carotene are tested. However, these experiments were the first attempts to assess VEB in oil, and they still influence the current recommendations.

Animal models

Animal models may be very useful for studying qualitative problems, but they have limited use for studying quantitative processes that represent the situation in human subjects. There is no validated way to extrapolate animal data to physiological conditions in human subjects. Monkeys, gerbils and preruminant calves convert β-carotene into vitamin A with an efficiency comparable to that of humans. However, in humans, β-carotene is mainly transported in the LDL fraction and many animals, such as ferrets and calves, have a retinyl ester metabolism different from that in humans, resulting in high levels of fasting plasma retinyl esters. Mice, rats, ferrets and chickens efficiently convert β-carotene into vitamin A, but absorb β-carotene intact only when it is provided in the diet at levels much higher than is considered physiological for humans. Not one animal model completely mimics human absorption and metabolism of β-carotene and therefore cannot be used to study the VEB in human subjects[30-32].

In vitro models

Results from *in vitro* models cannot be translated to the human situation, but can provide some predictions. For example, the available time for food processing can be influenced by various food matrices and different levels of enzymes and pH simulating infant or adult situations, as these factors influence the transit time and competition for absorption[8,9]. An *in vitro* gastrointestinal model can measure precisely measure the bioaccessible fraction, which is available for absorption by

measuring β-carotene in mixed micelles in different prepared meals of vegetable foods. Measurements in isolated human intestinal epithelial cells provide estimates of the conversion of β-carotene into retinol under various conditions.

Table 1. Overview of the methods used to study vitamin A equivalency of β-carotene in human subjects with their major strengths and major limitations

Methods	Strength	Limitation
Depletion–repletion	Crude estimates by stepwise increased doses	Ethical considerations due to depletion
Mass balance	Feasible with a controlled diet and faeces collection	Sensitive to underestimation and overestimation
Dose–response	Controlled dose measured in a relatively short time	Length of time for increased levels and for AUC calculation
Retinyl ester response	Measurement of newly absorbed and converted β-carotene	Inter-individual variation in chylomicron kinetics
Retinyl ester response with isotopes	Control for chylomicron kinetics	Sensitivity of detection
Extrinsic radio-isotope labelling	Limited number of subjects	Potential radiation damage
Extrinsic stable isotope labelling	Precise measurement of the isotopic ratio of labelled:unlabelled retinol	Assumption of the same kinetics of labelled and unlabelled β-carotene and retinol
Intrinsically isotope labelling	Same kinetics of labelled and unlabelled β-carotene in plant sources	Availability of these specially produced dietary plant sources

Mass balance method

In the mass balance method, the apparent β-carotene absorption is estimated as the difference between controlled β-carotene intake and faecal β-carotene excretion over a period of time. On the one hand, data obtained from oral–faecal mass balance method might be overestimated because of (bacterial) degradation of carotenoids in the gut and incomplete faecal collection. On the other hand, endogenously secreted carotenoids may be excreted in the faeces, thus leading to underestimation of the absorption of dietary carotenoids. Faeces collection studies in ileostomy subjects

have the advantage of excluding the possible effect of bacterial degradation or even synthesis of β-carotene in the colon, resulting in less overestimation of absorption in human subjects with an intact gut[33-37]. In spite of these limitations, oral–faecal mass balance studies may yield reasonable measurements of apparent intestinal β-carotene absorption. Together with the assumption of the FAO/WHO and IOM that half the absorbed amount of β-carotene in the intestine is converted into retinol, an acceptable estimate of VEB can be obtained using the mass balance method[33].

Dose–response method

Many dose–response studies have been conducted with β-carotene by measuring the blood level response over time after consuming a certain amount of β-carotene (for a review, see Swanson *et al.*[38]). The most commonly applied methods have included the measurement of the increase in serum or plasma β-carotene levels following chronic intervention, and the calculation of the AUC using the curves of total responses *v.* time following a single dose of β-carotene[39]. The concentration of β-carotene in serum or plasma represents a balance between intestinal absorption, breakdown, tissue uptake and release from body stores. The disadvantage of these studies is the homeostatic control of serum or plasma retinol concentrations. However, change from baseline serum or plasma β-carotene concentration can be useful as an endpoint to estimate the relative absorption of β-carotene in human subjects, provided such studies are sufficiently long to result in a new steady-state condition. Only dose–response studies with a (isotopic) reference dose can directly measure a VEB.

Retinyl ester response method

As the liver does not secrete retinyl esters, except when its storage capacity is saturated, newly absorbed and converted β-carotene can be measured by determining retinyl esters in chylomicrons. The advantage of the retinyl ester response method over the serum/plasma response method is that it accounts for intestinal conversion of β-carotene into retinyl esters. Consequently, it is theoretically possible to assess the VEB by measuring the retinyl ester response in postprandial blood. However, in practice, this is generally not feasible because of the low instantaneous concentration of chylomicron retinyl esters, the relatively low sensitivity of direct determination of retinyl ester concentration by HPLC, and the presence of large quantities of other lipids in extracted plasma or serum. In some studies, postprandial chylomicron β-carotene or retinyl ester response has been measured with a single oral dose of β-carotene[40-42]. Also, two earlier lymph recovery studies were carried out in subjects with lymph cannulation[6,7].

Interpretation of postprandial response curves of β-carotene and retinol esters in TAG-rich lipoprotein (TRL) data is limited by the lack of means to control for inter-individual variations in *in vivo* chylomicron clearance kinetics or variations in chylomicron recovery during the preparation and analysis of TRL. Consequently, use of this approach is generally restricted to comparative (between-treatment) studies, because it does not directly measure the VEB.

Retinyl ester response method with isotopes

Edwards *et al.*[43,44] adapted the TRL response model and co-administrated [$^2H_4$]retinyl acetate as an extrinsically isotope-labelled reference standard. This extrinsic reference dose controls for variations in chylomicron kinetics *in vivo* and for retinyl ester recovery during the preparation and analysis of TRL. The sensitivity and reproducibility of the detection of β-carotene in the plasma chylomicron fraction should be optimized before this approach can deliver reliable estimates of VEB[45].

Extrinsic radioisotope labelling method

The radioisotope tracer method requires a compartment model to interpret the increasing tracer curves after ingesting a single dose or constant infusion by assuming that the body is in endogenous constant steady state. Isotopic tracer techniques can provide accurate estimates of VEB with high precision, thus enabling studies with a limited number of subjects. The isotopic enrichment of labelled β-carotene in serum or plasma is corrected for the amount of labelled β-carotene consumed after some hours or days. By using isotope-labelled β-carotene, the measurement can distinguish between recently absorbed and endogenous β-carotene. Radioisotopes have been used only occasionally[6,7,46,47] because of potential radiation damage.

Extrinsic stable isotope labelling method

In the past two decades, the availability of stable isotope-labelled compounds increased and, their analyses were improved. The use of stable isotopes in research is safe for human subjects and accepted by institutional review boards. The stable isotope tracer dilution method consists of administering an oral single or multiple doses, collection of a blood sample, measurement of the plasma or serum isotopic ratio of tracer:tracee (unlabelled vitamin A), and the use of a prediction equation for calculation of the bioavailability of β-carotene or VEB. Isotopic dilution techniques can also be used to estimate the total amount of vitamin A in the body, which has been described in the review of Furr *et al.*[48].

Presently, over thirty studies have been conducted using stable isotope tracer techniques for studying the bioavailability of β-carotene and VEB in human subjects.

Because of their design, some studies could only provide qualitative data, and some other studies were performed with a limited number of subjects. Since the early 1990s, it was possible to follow absorption and biokinetics of labelled β-carotene or retinol after a single dose[49-51]. Over 20 years ago, Parker *et al.*[49] pointed out the necessity of stable isotope tracer methods for studying VEB in human subjects. The commonly used assumption is that the absorbed labelled β-carotene and retinyl palmitate are secreted and cleared with approximately the same kinetics as the unlabelled carotenoids and retinyl esters in the diet. Interpretation of data and mathematics from single-dose labelling studies are more complicated than those from multiple-dose labelling studies in which a plateau of isotopic enrichment is measured far above the threshold limit[45].

Intrinsic isotopic labelling method

Intrinsically labelled vegetables can be produced by irrigating with ^{2}H-labelled water or by supplying $^{13}CO_2$ in a closed atmosphere[52]. The advantage of the use of intrinsically labelled vegetables is that it is not necessary to assume that the labelled compound behaves in the same way as the unlabelled compound. To date, the following intrinsically isotope-labelled vegetables have been produced: biofortified yellow maize[53]; biofortified 'Golden Rice'[54,55]; carrot[56-58]; spinach[54,57-59]; collard greens[59]; kale[60,61]; tomato[62]. Because these specially produced vegetables are very expensive, few subjects could participate and only one simple single meal was measured. With this intrinsic labelling method, accurate data of VEB can be expected for specific vegetables and for human subjects with diverse nutritional status. However, data of VEB for a complex mixed diet with various vegetables and fruits are not yet available using the intrinsic labelling method.

SUMMARY OF STUDIES

By comparing data from the aforementioned studies, the method and the three main factors that influence VEB in humans (the food matrix, the amount ingested and the habitual diet type) should be mentioned. The present review focuses on the influence of the food matrix on VEB and distinguishes the results by the different types of dietary food matrices of β-carotene, which are the oil matrix, the complex mixed diet matrix with various vegetables and fruits and the single vegetable or fruit matrix.

The majority of the data that the IOM reconsidered in 2001 were obtained from children and adults in developing countries with an inadequate nutritional status and their own habitual dietary patterns. The minority of the studies that IOM reviewed were obtained from subjects in developed countries with an adequate nutritional status, consuming a Western diet. Additional studies, especially those using stable isotopes, have been published since then (see Tables 2–4).

Oil matrix: β-carotene dissolved in oil

Overall, eighteen studies are presented in Table 2, of which four[28,29,63,64] measured the VEB in oil with unlabelled β-carotene and fourteen[25,33,54,65-73] with labelled β-carotene. Of these studies, two had a depletion-repletion study design[28,29]. In the Sheffield experiment during the Second World War, Hume & Krebs[28] compared the amount of β-carotene with the amount of retinol required to reverse and prevent abnormal dark adaptation. Only two subjects achieved abnormal dark adaptation or correction of abnormal dark adaptation with β-carotene. Data for these two responding subjects suggested a VEB of 3·8:1 based on the observation that 390 μg retinol or 1500 μg β-carotene reversed abnormal dark adaptation. Many years later, a study with six subjects was performed with a validated method to confirm impaired visual function in response to vitamin A depletion[29]. The VEB was determined to be 2:1, meaning that 600 μg retinol/d or 1200 μg β-carotene/d corrected dark adaptation. This latter study was considered by the IOM to be the more reliable VEB for β-carotene dissolved in oil[1].

Furthermore, two other studies have measured the VEB in oil with dose–response study design by calculating the AUC for retinyl palmitate in the TRL fraction[63,64]. Both studies were designed to measure the VEB in biofortified maize porridge and biofortified cassava porridge. Also, the VEB of the reference dose of β-carotene in oil was measured, resulting in 2·3:1 and 2·1:1. However, although these results were obtained from a small group of subjects, they confirm the IOM recommendation of 2:1 for VEB in oil[1].

The fourteen reported VEB measured with labelled β-carotene in oil, presented in Table 2, have a wide range from 2·0:1[54] (healthy children) to 55:1[65] (in one female adult after a pharmaceutical dose of 126 mg β-carotene). In this latter study, a VEB of 3·8:1 was obtained after a labelled dose of 6 mg β-carotene given to the same female subject over 21 d[65]. In another study in one male adult, the VEB was 15·9:1 after a very high dose of 16·2 mg β-carotene over 23 d[69]. These large variations in VEB stress the importance of carrying out experiments to measure VEB using physiological doses of β-carotene.

In four labelling studies that were performed in school children, a VEB ranging from 2·0:1 to 3·2:1 was reported. In two studies in children with adequate vitamin A status in Indonesia, low retinol diets containing daily amounts of [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinol were consumed[66,67]. From measurements in plasma of the plateau enrichment of retinol with [$^{13}C_{10}$]retinol and [$^{13}C_5$]retinol, the VEB in oil was found on average to be 2·4:1 in a 10-week study[66] and 2·7:1 in a 3-week study[67]. The other two studies with children were performed in China[54,72]. In one study in China carried

out on twenty-three healthy children over 21 d, the VEB was quantified as 2·0:1[54]. The other study in China carried out over 28 d reported a VEB of 2·9:1 for a group of eight healthy children and a VEB of 3·2:1 for a group of eight vitamin A-deficient children[72].

In six labelling studies that were performed in adults, VEB values ranging from 3·4:1 to 9·1:1 were reported, which are less efficient than the reported VEB values for school children. Of these studies, two used a single dose of stable isotope-labelled [$^2H_8$]β-carotene in 'corn' oil with [$^2H_8$]retinol as a reference dose in well-nourished adults aged 55–60 years over 56 d with a low β-carotene diet[70,25]. The average VEB was 9·1:1 in twenty-two adults in the USA[70] and 9·1:1 in eleven rural Chinese adults with a diet of limited amounts of animal foods[25]. Haskell *et al.*[71] reported an estimated VEB in oil of 6·3:1 for synthetic β-carotene in fourteen young men in Bangladesh. In one short-term study, the VEB was quantified to be 5·7:1 in red palm oil after 8·5 h of a [$^2H_8$]retinyl acetate dose administered to twelve adults[68]. In two diet-controlled studies conducted in The Netherlands, a VEB of 3·4:1 was quantified in twenty-four healthy young adults[73] and a VEB of 3·6:1 in seventeen ileostomy subjects[33] using the same dual-isotope dilution technique as used by van Lieshout *et al.*[66,67].

In conclusion, at low physiological doses, a VEB in oil of approximately 3:1 was obtained; however, at very high doses, the VEB decreases, as already mentioned in the introduction.

Vegetable matrix: β-carotene in a diet with multiple vegetables and fruits

Table 3 presents five diet-controlled studies with a duration of 2 to 10 weeks with multiple vegetables and fruits. The study design of the study in school children in Indonesia[11] and of the study in breast-feeding women in Vietnam[74] were similar and comprised four dietary groups: low-retinol, low-carotenoid (negative control); dark-green leafy vegetables (also carrots in the Indonesian study); yellow and orange fruits; a retinol-containing diet (positive control). The increase in serum retinol concentrations was measured over the 9 to 10 weeks, mainly diet-controlled period. For the dark-green leafy vegetables, the VEB was estimated to be 26:1 in the Indonesian study[11] and 28:1 in the Vietnamese study[74], while for the fruits, it was 12:1 in both studies. In an intervention study in Chinese kindergarten school children, a VEB of 27:1 was calculated for a diet with green and yellow vegetables[75]. In two completely diet-controlled studies in The Netherlands with a diet containing β-carotene-rich vegetables, a VEB of 15·7:1 was obtained in twenty-four healthy young adults[73] and a VEB of 12·5:1 in seventeen ileostomy subjects[33]. The same two studies crossed-over with another controlled diet for 2 to 3 weeks. That diet contained β-carotene-poor vegetables and fruits with consumption of food items fortified with β-carotene and/or β-carotene supplements, such as those regularly consumed in industrialized societies. For this complex β-carotene supplemented and vegetable matrix, the VEB was found to be 5·4:1[73] and 6·7:1[33].

In conclusion, the seven reported VEB for a diet with multiple vegetables are lower than the IOM recommendation of 12:1 for VEB in a mixed plant diet. For the fruit matrix, the VEB of 12:1 is realistic.

Table 2. Overview of the studies with unlabelled and extrinsically isotope-labelled β-carotene in oil to quantify vitamin A equivalency of β-carotene in oil

Reference	Food matrix	Amount ingested	Country (diet type)*	Subjects	Study design (duration)	Result (μg β-carotene : μg retinol)	SD or 95% CI
Hume & Krebs[28]	β-Carotene in oil	< 2 mg/d	UK (deficient diet)	2 (one for repletion with β-carotene; 32 years)	Depletion–repletion study (14 months of depletion; 6 months of repletion)	3·8:1	NA
Sauberlich *et al.*[29]	β-Carotene in oil	150–2400 μg/d	USA (deficient diet)	6 (four for repletion with β-carotene; 32–43 years)	Depletion–repletion study (12-25 months of depletion; 1-15 months of repletion)	2:1	NA
Li *et al.*[63]	β-Carotene in oil added to white maize porridge	595 μg (reference dose)	USA (Western diet)	6 women (18–30 years)	Dose–response study; AUC of the TRL response (9 h)	2·34:1	1·61
Liu *et al.*[64]	β-Carotene in oil added to white cassava porridge	537·6 μg (reference dose)	Colombia (prudent diet)	8 women	Dose–response study; AUC of the TRL response (9 h)	2·11:1	0·81
Tang *et al.*[65]	[$^{2}H_{8}$]β-Carotene capsule in 'corn' oil	6 mg (reference dose [$^{2}H_{8}$]retinyl acetate)	USA (Western diet)	1 women	Single dose–response study; AUC (21 d)	3·8:1	NA
Tang *et al.*[65]	I[$^{2}H_{8}$]β-Carotene capsule in 'corn' oil	126 mg	USA (Western diet)	1 women	Single dose–response study; AUC (2·5 years apart)	55:1	NA
van Lieshout *et al.*[66]	[$^{13}C_{10}$]β-Carotene in oil capsule	160 μg/d for 10 weeks (reference dose [$^{13}C_{10}$]retinol)	Indonesia (prudent diet)	35 (nineteen boys; sixteen girls) (average 9 years)	Multiple-dose plateau study with a low β-carotene diet (10 weeks)	2·4:1	2·1, 2·7
van Lieshout *et al.*[67]	[$^{13}C_{10}$]β-carotene in oil capsule	89 μg/d for 6 weeks (reference dose [$^{13}C_{10}$]retinol)	Indonesia (prudent diet)	77 (thirty-nine boys; thirty-eight girls) (average 9·5 years)	Multiple-dose plateau study with a low β-carotene diet (6 weeks)	2·7:1	2·5, 2·8
You *et al.*[68]	β-Carotene in refined red palm oil	2·37 mg (reference dose [$^{2}H_{4}$]retinyl acetate)	USA (Western diet)	12 (six women, six men)	Single-dose study; TRL response (8·5 h)	5·7:1	NA
Hickenbottom *et al.*[69]	[$^{2}H_{6}$]β-Carotene in olive oil	16·2 mg (reference dose [$^{2}H_{6}$]retinyl acetate)	USA (Western diet)	1 man (36 years)	Single-dose study; AUC (23 d)	15·9:1	NA

Table 2. *Continued*

Reference	Food matrix	Amount ingested	Country (diet type)*	Subjects	Study design (duration)	Result (μg β-carotene : μg retinol)	SD or 95% CI
Tang *et al.*[70]	[$^2H_8$]β-Carotene in 'corn' oil	6 mg (reference dose [$^2H_8$]retinyl acetate)	USA (Western diet)	22 (ten men; twelve women) (average 60 years)	Single dose–response study; AUC (56 d)	9·1:1 (range: 2·4–20·2)	5·8
Wang *et al.*[25]	[$^2H_8$]β-Carotene in 'corn' oil	6 mg (reference dose [$^2H_8$]retinyl acetate)	Rural China (prudent diet)	11 (seven men; four women) (average 55 years)	Single dose–response study; AUC (56 d)	9·1:1 (range: 3·8–22·8)	5·3
Haskell *et al.*[71]	Synthetic β-carotene in 'corn' oil capsule	2·25 mg for 60 d (reference dose [$^2H_4$]retinyl acetate)	Bangladesh (prudent diet)	14 men (average 22·6 years)	Multiple dose–response study (60 d)	6·3:1	NA
Li *et al.*[72]	Pure β-carotene in oil capsule	200 μg/d for 7 d (reference dose [$^{13}C_{10}$]retinyl acetate)	China (Western diet)	8 (normal vitamin A status) (7–9 years)	Single dose–response study (28 d)	2·9:1	NA
Li *et al.*[72]	Pure β-carotene in oil capsule	200 μg/d for 7 d (reference dose [$^{13}C_{10}$]retinyl acetate)	China (Western diet)	8 (vitamin A-deficient) (7–9 years)	Single dose–response study (28 d)	3·2:1	NA
Van Loo-Bouwman *et al.*[73]	[$^{13}C_{10}$]β-Carotene in oil capsule	Average 55 μg/d (reference dose [$^{13}C_{10}$]retinyl palmitate)	The Netherlands (Western diet)	24 (ten men; fourteen women) (average 22 years)	Multiple-dose plateau study with a controlled diet (21 d)	3·4:1	2·8, 3·9
Van Loo-Bouwman *et al.*[33]	[$^{13}C_{10}$]β-Carotene in oil capsule	Average 190 μg/d (reference dose [$^{13}C_{10}$]retinyl palmitate)	The Netherlands (Western diet)	17 (five men; twelve women) (average 49 years)	Multiple-dose plateau study with a controlled diet (14 d)	3·6:1	2·8, 4·6
Tang *et al.*[54]	[$^2H_8$]β-Carotene in 'corn' oil capsule	0·5 mg (reference dose [$^{13}C_{10}$]retinyl acetate)	Rural China (prudent diet)	23 (thirteen boys; ten girls) (6–8 years)	Single dose–response study; AUC (21 d)	2·0:1	0·9

NA, not available; TRL, TAG-rich lipoprotein

* The 'prudent' diet type is high in fresh fruits and vegetables and whole grains (e.g. rice, maize), and low in meat.

Table 3. Overview of the studies with unlabelled β-carotene in a mixed diet from multiple vegetables and fruits to quantify vitamin A equivalency of β-carotene in a mixed plant diet

Reference	Food matrix	Amount ingested	Country (diet type)*	Subjects	Study design (duration)	Result (μg β-carotene:μg retinol)	SD or 95% CI
De Pee *et al.*[11]	Orange fruits (papaya, mango, squash, pumpkin)	4·3 mg/d	Indonesia (prudent diet)	45 (twenty-five boys; twenty girls) (average 11 years)	Dose–response study with mainly a controlled diet (9 weeks)	12:1	6, 29
De Pee *et al.*[11]	Dark-green leafy vegetables (cassava leaves, water spinach, spinach) and carrots	4·1 mg/d	Indonesia (prudent diet)	49 (twenty-nine boys; twenty girls) (average 11 years)	Dose–response study with mainly a controlled diet (9 weeks)	26:1	3, 76
Khan *et al.*[74]	Orange and yellow fruit (e.g. papaya, mango)	4·8 mg/d	Vietnam (prudent diet)	69 breast-feeding women (average 26 years)	Dose–response study with mainly a controlled diet (10 weeks)	12:1	8, 22
Khan *et al.*[74]	Dark-green leafy vegetables	5·6 mg/d	Vietnam (prudent diet)	73 breast-feeding women (average 26 years)	Dose–response study with mainly a controlled diet (10 weeks)	28:1	17, 84
Tang *et al.*[75]	Dark-green and yellow vegetables	4·7 mg/d	Rural China (prudent diet)	22 (nine boys; thirteen girls) (average 6 years)	Dose–response study with mainly a controlled diet (10 weeks)	27:1 (estimated)	19, 48
Van Loo-Bouwman *et al.*[73]	Mixed vegetables high in β-carotene (broccoli, green peas, endive, carrot savoy cabbage)	6·8 mg/d	The Netherlands (Western diet)	24 (ten men; fourteen women) (average 22 years)	Multiple-dose plateau study with a controlled diet; mass balance (21 d)	15·7:1 (10·4:1 if excluding six subjects with negative oral–faecal balance)†	1·0, 30·4; 5·3, 15·5
Van Loo-Bouwman *et al.*[73]	Supplemental β-carotene and mixed vegetables low in β-carotene (e.g. white cabbage, cauliflower)	3·3 mg/d (supplemented); 1·6 mg/d (vegetables)	The Netherlands (fortified Western diet)	24 (ten men; fourteen women) (average 22 years)	Multiple-dose plateau study with a controlled diet; mass balance (21 d)	5·4:1	3·8, 7·0
Van Loo-Bouwman *et al.*[33]	Mixed vegetables high in β-carotene	7·6 mg/d	The Netherlands (Western diet)	17 (five men; twelve women) (average 49 years)	Multiple-dose plateau study with a controlled diet; mass balance (14 d)	12·5:1	NA
Van Loo-Bouwman *et al.*[33]	Supplemental β-carotene and mixed vegetables low in β-carotene	2·6 mg/d (supplemented); 0·4 mg/d (vegetables)	The Netherlands (fortified Western diet)	17 (five men; twelve women) (average 49 years)	Multiple-dose plateau study with a controlled diet; mass balance (14 d)	6·7:1	NA

NA, not available.* The 'prudent' diet type is high in fresh fruits and vegetables and whole grains (e.g. rice, maize), and low in meat.
† Due to the relatively high weight of total 72 h faeces collection.

Vegetable matrix: β-carotene in a single vegetable or fruit matrix

Of the thirteen studies presented in Table 4, three were performed with a single unlabelled vegetable by measuring the TRL response over 9 h in women. A study with maize porridge determined a VEB of 6·48:1[63]. A study with biofortified cassava porridge in Colombia reported a VEB of 2·80:1[64]. Another study with biofortified cassava porridge in the USA determined a VEB of 4.2:1 when provided with added oil and a VEB of 4·5:1 when provided without added oil[76].

Overall, three β-carotene-labelled studies, which used [$^{2}H_{4}$]retinyl acetate as the reference dose, are presented in Table 4. Parker *et al.*[39] reported a VEB of 13:1 for raw carrot in one adult. Edwards *et al.*[43] estimated a VEB of 23:1 for raw carrot as well as raw spinach in three adults. A study with daily supplementation by Indian spinach and sweet potato in fourteen Bangladeshi men for 60 d quantified VEB values of 9·5:1 and 13·4:1, respectively[71].

In total, seven studies have been published, which quantified the VEB for intrinsically labelled spinach, carrot, maize, or Golden Rice (see Table 4). Tang *et al.*[57] produced two ^{2}H-labelled vegetables and quantified a VEB of 21:1 for spinach and a VEB of 15:1 for carrot over 36 d compared with [$^{13}C_{8}$]retinyl acetate as the reference dose. In two Chinese studies with $^{2}H_{10}$-labelled spinach, a VEB of 9·0:1 was presented for male adults[77], 10·1:1 for healthy school children and 10·3:1 for vitamin A-deficient school children[72]. A 72 h short-term study in the UK in four adults provided an approximate VEB of 77:1 for raw carrot and a VEB of 11·6:1 for the same intrinsically isotope-labelled carrot, but consumed after stir-frying in groundnut oil[56]. Muzhingi *et al.*[53] produced ^{2}H-labelled yellow maize β-carotene and determined a VEB of 3·2:1 in eight men. In two studies by Tang *et al.*[54,55], different measured VEB have been reported using intrinsically labelled Golden Rice with high levels of β-carotene that could be explained by the target group and duration of measuring the AUC. The first study[55] determined a VEB of 3·8:1 in five adults in the USA measured over 36 d and the second study[54] obtained a VEB of 2·3:1 for twenty-three healthy children in China measured over 21 d. The latter study in twenty-two other healthy Chinese children, a VEB of 7·5:1 was reported for intrinsically labelled spinach[54].

In conclusion, the type of vegetable matrix plays a dominant role in determining the VEB as demonstrated by the VEB reported above. The intrinsic labelling method is very helpful to quantify the values of major vegetables consumed, but it is very time-consuming and expensive and will not directly quantify a value for a mixed vegetable diet.

Table 4. Overview of the studies with unlabelled or extrinsically isotope-labelled or intrinsically isotope-labelled β-carotene in a diet from a single vegetable or fruit to quantify vitamin A equivalency of β-carotene in a single vegetable or fruit

Reference	Food matrix	Amount ingested	Country (diet type)*	Subjects	Study design (duration)	Result (μg β-carotene: μg retinol)	SD or 95% CI
Li *et al.*[63]	Maize porridge (unlabelled)	527 μg	USA (Western diet)	6 women (18–30 years)	Dose–response study; AUC of TRL response (9 h)	6·48:1	3·51
Liu *et al.*[64]	β-Carotene-biofortified cassava porridge (unlabelled)	1097·5 μg	Colombia (prudent diet)	8 women	Dose–response study; AUC of TRL response (9 h)	2·80:1	1·77
La Frano *et al.*[76]	β-Carotene-biofortified cassava porridge (unlabelled)	2 mg with added oil of 20 g	USA (Western diet)	12 women (average 29 years)	Dose–response study; AUC of TRL response (9·5 h)	4·2:1	3·1
La Frano *et al.*[76]	β-Carotene-biofortified cassava porridge (unlabelled)	2 mg without added oil	USA (Western diet)	12 women (average 29 years)	Dose–response study; AUC of TRL response (9·5 h)	4·5:1	3·1
Parker *et al.*[39]	Raw carrot (extrinsically isotope labelled)	5·25 mg (reference dose [$^{2}H_{4}$]retinyl acetate)	USA (one undefined meal)	1	Dose–response study; AUC (7 h)	13:1	NA
Edwards *et al.*[43]	Raw carrot or raw spinach (extrinsically isotope-labelled)	6 mg (reference dose [$^{2}H_{4}$]retinyl acetate)	USA (one meal)	3 (two men; one women) (25–35 years)	Single-dose study; TRL response (8·5 h)	23:1	NA
Haskell *et al.*[71]	Indian spinach (extrinsically isotope-labelled)	4·5 mg/d for 60 d (reference dose [$^{2}H_{4}$]retinyl acetate)	Bangladesh (prudent diet)	14 men (average 22·6 years)	Multiple dose–response study (60 d)	9·5:1	NA
Haskell *et al.*[71]	Sweet potato (extrinsically isotope-labelled)	4·5 mg/d	Bangladesh (prudent diet)	14 men (average 22·6 years)	Multiple dose–response study (60 d)	13·4:1	NA
Tang *et al.*[57]	Spinach [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	11 mg (reference dose [$^{13}C_{8}$]retinyl acetate)	USA (Western diet)	14 (seven men; seven women) (average 57 years)	Single dose–response study; AUC (36 d)	20·9:1 (range: 10·0–46·5)	9·0
Tang *et al.*[57]	Carrot [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	11 mg	USA (Western diet)	7 women (average 56 years)	Single dose–response study; AUC (36 d	14·8:1 (range: 7·7–24·5)	6·5
Wang *et al.*[77]	Spinach [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	12 mg (reference dose [$^{13}C_{10}$]retinyl acetate)	China (Western diet)	10 men (43–56 years)	Single dose–response study; AUC (56 d)	9·0:1	4·5

Table 4. *Continued*

Reference	Food matrix	Amount ingested	Country (diet type)*	Subjects	Study design (duration)	Result (μg β-carotene : μg retinol)	SD or 95% CI
Li *et al.*[72]	Spinach [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	230 μg/d for 7 d (reference dose [$^{13}C_{10}$]retinyl acetate)	China (Western diet)	8 (normal vitamin A status) (7-9 years)	Single dose–response study (28 d)	10·1:1	NA
Li *et al.*[72]	Spinach [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	230 μg/d for 7 d (reference dose [$^{13}C_{10}$]retinyl acetate)	China (Western diet)	8 (vitamin A-deficient) (7–9 years)	Single dose–response study (28 d)	10·3:1	NA
Tang *et al.*[55]	Golden Rice [$^{2}H_{10}$]β-carotene with butter (intrinsically isotope-labelled)	0·99–1·53 mg (reference dose [$^{13}C_{10}$]retinyl acetate)	USA (Western diet)	5 (two men; three women) (41–70 years)	Single dose–response study; AUC (36 d)	3·8:1 (range: 1·6–6·4)	1·7
Muzhingi *et al.*[53]	Yellow maize [$^{2}H_{9}$]β-carotene porridge with butter (intrinsically isotope-labelled)	1·2 mg (reference dose [$^{13}C_{10}$]retinyl acetate)	Zimbabwe (prudent diet)	8 men (average 48 years)	Single dose–response study; AUC (36 d)	3·2:1 (range: 1·5–5·3)	1·5
Ghavami *et al.*[56]	Stir-fried carrot [$^{2}H_{10}$]β-carotene in groundnut oil (intrinsically isotope-labelled)	3·79 mg (reference doses [$^{13}C_{20}$]β-carotene and [$^{2}H_{4}$]retinyl acetate)	UK (Western diet)	4 (two men; two women) (average 45 years)	Single dose–response study; AUC (72 h)	11·6:1	NA
Ghavami *et al.*[56]	Raw carrot [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	3·79 mg (reference doses [$^{13}C_{20}$]β-carotene and [$^{2}H_{4}$]retinyl acetate)	UK (Western diet)	4 (two men; two women) (average 45 years)	Single dose–response study; AUC (72 h)	77:1	NA
Tang *et al.*[54]	Spinach [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	1·4 mg (reference dose [$^{13}C_{10}$]retinyl acetate)	Rural China (prudent diet)	22 (fifteen boys; seven girls) (6–8 years)	Single dose–response study; AUC (21 d)	7·5:1	0·8
Tang *et al.*[54]	Golden Rice [$^{2}H_{10}$]β-carotene (intrinsically isotope-labelled)	0·6 mg (reference dose [$^{13}C_{10}$]retinyl acetate)	Rural China (prudent diet)	23 (twelve boys; eleven girls) (6–8 years)	Single dose–response study; AUC (21 d)	2·3:1	0·8

TRL, TAG-rich lipoprotein; NA, not available.

* The 'prudent' diet type is high in fresh fruits and vegetables and whole grains (e.g. rice, maize), and low in meat.

DISCUSSION

Discussing the methods

In summary, dose–response methods cannot discriminate between absorbed and endogenous β-carotene, while the isotopic labelling methods can discriminate between absorbed and endogenic β-carotene. In small physiological doses, isotopically labelled β-carotene is measurable in blood. Intrinsic isotopic labelling methods provide reliable data on VEB in various plant sources of β-carotene. Stable isotope labelling methods measure the proportion of β-carotene ingested, which is absorbed and converted into vitamin A, but cannot distinguish between the degree of absorption and the degree of conversion. There are methods to measure VEB directly without steps in between. Therefore, in many studies the absorption of β-carotene from various matrices is compared with β-carotene in oil. Depletion–repletion studies can provide the VEB dissolved in oil.

In conclusion, as much data as possible should be collected to identify the impact of the three main factors that influence the VEB in healthy human subjects: the amount ingested; the habitual diet type; the food matrix in which the β-carotene is incorporated. Furthermore, the known polymorphisms in the *BCMO1* gene[21,22] should be measured in the participants for clarification of possible variations in estimates for VEB. Stable isotope labelling studies appear to be the best approach to collect data for large groups (healthy or specific disease) by collection of blood samples after a controlled meal or diet. Validation studies of the stable isotope labelling methods are necessary to estimate and understand the reproducibility of data.

Next, we suggest the most appropriate VEB for a Western diet and for a 'prudent' diet based on current evidence.

Applicability of vitamin A equivalency of β-carotene in an oil matrix for a Western diet and for a 'prudent' diet

The current recommendations of the FAO/WHO is 3·3:1 and that of the IOM is 2:1 for VEB in oil. Fortified foods and dietary supplements are available, which contain physiological dose of β-carotene in oil[2,4] to complete an inadequate diet pattern. In four studies with unlabelled β-carotene, the IOM recommendation of 2:1 for VEB in oil has been confirmed[28,29,63,64]. In seven studies with labelled compounds, a VEB in oil ranging from 3·4:1 to 9·1:1 has been reported in adults, which was less efficient than that reported by the four studies for VEB in oil of 2·0:1 to 3·2:1 in school children. As has already been established[12,65], the studies using higher amounts of ingested β-carotene resulted in higher reported VEB. By only considering the studies with amounts of β-carotene that were lower than 6 mg, a VEB in oil for a Western diet

ranged from 2·3:1 to 5·7:1 and for a 'prudent' diet from 2:1 to 6·3:1. Furthermore, by only considering the studies with amounts of ingested β-carotene that were lower than 2 mg, a VEB in oil for a Western diet ranged from 2·3:1 to 3·6:1 and for a 'prudent' diet from 2:1 to 3·8:1.

For humans consuming β-carotene dissolved in oil, a VEB between 2:1 and 4:1 is feasible in a Western diet as well as in a 'prudent' diet. The down-regulation mechanism of expression of BCMO1 by high doses and genetic polymorphisms in *BCMO1* gene might explain the observed variation in VEB in oil.

Applicability of vitamin A equivalency of β-carotene in the plant matrix for a Western diet and for a 'prudent' diet

The current recommendations of the FAO/WHO is 6:1 and that of the IOM is 12:1 for VEB in a mixed diet. Only five studies directly investigated the mixed plant matrix with multiple vegetables, of which three studies reported a VEB of 26:1 [11], 27:1 [75] and 28:1 [74] using a 'prudent' diet, and two studies reported a VEB of 15·7:1 [73] and 12·5:1 [33] using a Western diet. These three studies were performed in developing countries in children[11,75] and breast-feeding women[74], where the subjects may have different health status and nutritional needs from those of healthy adults participating in the two studies with the Western diet[33,73].

To determine which VEB should be used for humans in generally good health, all studies with single vegetables and single fruits were considered. Only two studies investigated the fruit matrix and both reported a VEB of 12:1[11,74]. In two studies using a fortified Western diet a VEB of 5·4:1 and 6·7:1 was obtained, respectively[33,73]. The results of the latter two studies reflect a combination of VEB in oil and VEB in a mixed plant matrix.

The overall VEB of approximately 9:1 for spinach (range 7·5:1–10·3:1) is assumed on the basis of four studies [52,69,70,74], and excludes two studies with a reported VEB of 23:1 [43] and 21:1 [57] with non-physiological doses of 6 and 11 mg β-carotene, respectively. The overall VEB of approximately 13:1 for carrot (range 11·6:1–14·8:1) is assumed on the basis of three studies[39,56,57] and excludes the reported VEB of 77:1 in raw carrots [56], which were very minimally processed before ingestion. Only one study was performed with sweet potato, which reported a VEB of 13:1 [71]. The overall VEB for three biofortified crops are approximately 4:1 for cassava[64,74], approximately 3:1 to approximately 6:1 for yellow maize[53,63] and approximately 2·5:1 to approximately 4:1 for Golden Rice[54,55]. A β-carotene-rich alga is available as a food supplement and has a VEB that is similar to biofortified crops, namely 4·5:1[78]. Specially bred cassava, maize and Golden Rice are intended to be consumed in developing countries. Currently, spinach, carrots and sweet potato are widely available for consumption.

So, for the Western diet including a variety of fruits, leafy vegetables such as spinach, and root vegetables such as carrots and sweet potato, a VEB for a mixed diet of 9:1 to 16:1 is feasible.
For a 'prudent' diet including a variety of commonly consumed and specially bred vegetables, a VEB for a mixed diet could range from 4:1 to 28:1. These VEB values were obtained from human subjects in apparently normal health in developing countries. Data are not available on VEB in a mixed diet applicable to malnourished children or pregnant women. Furthermore, less favourable host-related factors, such as parasites and gastrointestinal infections, should be taken into account. In addition, individuals in developing countries have a diet relatively low in animal-derived foods, and their diet should contain as much as possible carotenoids-rich vegetables and fruits to maintain or gain adequate vitamin A status.
In conclusion, the proposed range for VEB in a mixed diet of 9:1 to 16:1 includes the IOM VEB of 12:1 and is realistic for a Western diet and Western conditions. For a 'prudent' diet including a variety of commonly consumed vegetables, a VEB in a mixed diet could range from 9:1 to 28:1. Large inter-individual variations in the estimates of VEB are reported, possible due to genetic polymorphisms in the *BCMO1* gene and the degree of regulation of the expression of BCMO1 in response of vitamin A status.

Different reported vitamin A equivalency of β-carotene in children and in adults
A closer examination of some studies suggests that there is an indication that children can convert β-carotene into retinol more effectively than adults, as shown by a lower VEB in children than in adults. For instance, four studies used the same dual-isotope dilution technique and quantified a VEB in oil of 2·4:1 and 2·7:1 in children[66,67] and a VEB in oil of 3·4:1 and 3·6:1 in adults[33,73]. Furthermore, two studies with intrinsically labelled Golden Rice used the single dose–response method and quantified a VEB of 2·3:1 in children[54] and a VEB of 3·8:1 in adults[55]. As another example, two studies with intrinsically labelled spinach by Tang *et al.*[57] used the single dose–response method and quantified a VEB of 7·5:1 in children[54] and a VEB of 20·9:1 in adults. However, these different measured VEB could be explained by the provided doses 1·4 and 11 mg in children and adults, respectively. The VEB of 7·5:1 for spinach in children[54] could also be compared with the estimated VEB of 9·5:1 for spinach in adults[71] obtained with a multiple dose–response method with a reference dose of labelled retinyl acetate. However other host-related factors (e.g. health status, immune status, treatment for worms and diarrhoea, BCMO1 expression) could explain the differences in VEB. However, two studies using the same method indicated that the VEB in children is not always higher than in adults; those studies with intrinsically labelled spinach used the single dose–response

method and reported a VEB of 10·1:1 in children[72] and a VEB of 9·0:1 in adults[77]. When more studies are performed in children, more precise data will be available for determining whether there should be separate VEB values for children and adults.

Future studies

Studies with isotope-labelled β-carotene in fruits and vegetables in habitual diets, such as the Western diet, measured over a long period have not yet been performed. The intrinsic labelling method could be used to compare labelled β-carotene in plant sources with labelled β-carotene in oil in a common diet to provide an estimate of VEB in a complex mixed total diet. However, as has already been mentioned, the preparations will be very time-consuming and expensive, and a representative target group should be recruited. There is a need for more studies to be carried out in populations in developing countries as well as in Western populations to see whether and to what extent the VEB is influenced by the nutritional status, age and other factors that might differ between developed and developing countries, such as genetic variability and polymorphisms in *BCMO1* gene across different ethnic groups. Future isotopic labelling studies should be carried out to obtain more accurate and precise data for various factors influencing the VEB. Populations in developing countries should consume carotenoid-rich vegetables, which are processed whenever possible for optimal disruption of the food matrix to release β-carotene.

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3

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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ABSTRACT

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 precise the vitamin A equivalency of the β-carotene in oil capsules.

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

INTRODUCTION

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 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 administrated [$^{13}C_{10}$]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 this 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/m^2, 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.

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/m^2)	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 x10^{12}/l)	4·7	0·4	4·6	0·3	4·2–5·6	3·7–5·0
Leukocytes (cells x10^9/l)	7·5	1·1	6·9	1·2	4·0–10·0	
Thrombocytes (cells x10^9/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	

* Group 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).

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 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 by 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 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 Kjehldahl 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 ½; 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 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.

Table 2. Composition of 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.

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 of 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 was 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 of Na_2SO_4, 0·5 g of $CaCO_3$, 30 ml of 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 time 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 at 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]

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

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 with 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'.

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 Table 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' 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'.

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. 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 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 relative 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].

Table 4. Vitamin A equivalency and bio-efficacy of [$^{13}C_{10}$]-β-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 [$^{13}C_{10}$]-β-carotene	0·01631	0·01437, 0·01825	0·01902	0·01628, 0·02176
$E_{10,fC}$	Enrichment of β-carotene in faeces with [$^{13}C_{10}$]-β-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 [$^{13}C_{10}$]-β-carotene from capsules †	0·104	0·025	0·105	0·028
$AEq_{,C}$	Vitamin A equivalency (μg) of [$^{13}C_{10}$]-β-carotene in oil ‡	3·347	2·81, 3·88	3·380	2·91, 3·85
$BE_{,C}$	Bio-efficacy (%) of [$^{13}C_{10}$]-β-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 [$^{13}C_{10}$]-β-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 ×286)/Vitamin A equivalency) ×100; a bio-efficacy of 100% would mean that 1 μmol dietary β-carotene (537 μg) would yield 2 μmol retinol (572 μg).

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 or 95% confidence intervals)

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 [$^{13}C_{10}$]-β-carotene ($A_{C,cap}$) and 55 μg [$^{13}C_{10}$]-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: $(A_d - A_f) / A_d \times 100$.

DISCUSSION

Vitamin A equivalency of [$^{13}C_{10}$]β-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 [$^{13}C_{10}$]β-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 [$^{13}C_{10}$]β-carotene divided by total unlabelled and [$^{13}C_{10}$]β-carotene. The degree of isotopic enrichment of β-carotene in serum with [$^{13}C_{10}$]β-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 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 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 is of β-carotene to retinol in oil is 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 'oil diet' is 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 an industrialized societies. The estimated vitamin A equivalency of β-carotene in the 'oil diet' of 5·4:1 contains still 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 an 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.

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4

Vitamin A equivalency and apparent absorption of β-carotene in ileostomy subjects using a dual-isotope dilution technique

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ABSTRACT

The objective was to quantify the vitamin A equivalency of β-carotene in two diets using a dual-isotope dilution technique and the apparent β-carotene absorption as measured by the oral–faecal balance technique. Seventeen healthy adults with an ileostomy completed the 4-week diet-controlled, cross-over intervention study. Each subject followed both diets for 2 weeks: a diet containing vegetables low in β-carotene content with supplemental β-carotene in salad dressing oil ('oil diet'; mean β-carotene intake 3·1 mg/d) and a diet containing vegetables and fruits high in β-carotene content ('mixed diet'; mean β-carotene intake 7·6 mg/d). Daily each subject consumed a mean of 190 μg [$^{13}C_{10}$]β-carotene and 195 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. Apparent absorption of β-carotene was determined with oral–faecal balance. Isotopic data quantified a vitamin A equivalency of [$^{13}C_{10}$]β-carotene in oil of 3·6:1 (95% CI 2·8, 4·6) regardless of dietary matrices differences. The apparent absorption of (labeled and dietary) β-carotene from the 'oil diet' (30%) was 1·9-fold higher than from the 'mixed diet' (16%). This extrinsic labelling technique can measure precisely the vitamin A equivalency of β-carotene in oil capsules, but it does not represent the effect of different dietary matrices.

Key words: β-Carotene; Vitamin A equivalency; Stable isotopes; Ileostomy subjects

Abbreviation: LC, liquid chromatography.

INTRODUCTION

The vitamin A equivalency of β-carotene is defined as the amount of β-carotene which is required in the diet to produce 1 μg retinol in the body. According to the current views, 6 μg[1,2] or 12 μg[3] of β-carotene in a mixed diet are equivalent to 1 μg dietary retinol. For supplemental β-carotene in oil, the current views are that 3·3 μg[1,2] or 2 μg[3] of β-carotene are equivalent to 1 μg retinol.

Data concerning the vitamin A equivalency of β-carotene from various dietary sources are inconsistent. The way β-carotene is incorporated in dietary matrices (plant foods or dissolved in oil) influences the degree of absorption. Uptake into the enterocyte is the critical step in the bioconversion of β-carotene, since it is generally assumed that 2 μg β-carotene in the enterocyte is equivalent to 1 μg retinol in the body[2,3]. In order to quantify how much β-carotene enters the enterocyte, stable-isotope techniques have been developed in the last decade[4,5]. However, these

techniques and the studies in which they have been applied have reported conflicting results[6-9].

Absorption studies performed with healthy subjects with an ileostomy have the advantage of excluding the possible effect of bacterial degradation or even synthesis in the large bowel of the nutrient under study, allowing accurate measurement of its apparent fractional absorption.

Unfortunately, absorption studies alone cannot quantify the vitamin A equivalency of β-carotene. Therefore in the present investigation, the extrinsic labelling technique and the classical oral–faecal balance technique were combined. In addition to the administered [$^{13}C_{10}$]retinol, [$^{13}C_5$]retinol, formed by central cleavage of administered [$^{13}C_{10}$]β-carotene, was measured in serum[10,11]. The efficiency of absorption of retinol is generally expected to be over 90% in healthy subjects[2,3].

The aim of the present 14 d cross-over diet-controlled study was to quantify the vitamin A equivalency of β-carotene and to measure the apparent β-carotene absorption in healthy adults with an ileostomy with an adequate vitamin A status. Subjects were given two types of controlled Western diets: an 'oil diet' which contained mainly supplemental β-carotene in oil as the source of β-carotene and a 'mixed diet' which contained mainly β-carotene from vegetables and fruits.

SUBJECTS AND METHODS

Recruitment of subjects

Subjects with an ileostomy aged 23–75 years from four hospitals in the surroundings of Nijmegen in The Netherlands were selected for participation. Their general health status was checked in their medical dossiers. The screening examination included a health and lifestyle questionnaire, a FFQ[12], weight (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: routine clinical chemistry abnormalities, chronic diseases, ileal resection of >15 cm, medication suspected of interfering with fat-soluble-vitamin absorption, pregnancy, BMI <18 or >30 kg/m^2, abnormal dietary pattern, excessive alcohol consumption (>40 g/d), and consumption of carotenoids, vitamin, or mineral supplements 6 weeks before or during the study. A total of eighteen volunteers participated in the screening and were selected to form two groups, which were matched for sex, age, BMI and habitual energy intake. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Committee on Research Involving Human Subjects, Region Arnhem-Nijmegen, The Netherlands. Written informed consent was obtained from all subjects.

Table 1. Characteristics of the seventeen ileostomy subjects at baseline *
(Mean values and standard deviations)

	Group 1 (*n* 9)		Group 2 (*n* 8)		Laboratory references	
	Mean	SD	Mean	SD	Male	Female
Sex (*n*)						
Male	3		2			
Female	6		6			
Age (years)	47·1	12·2	51·5	13·9		
BMI (kg/m^2)	26·0	2·9	26·0	2·1		
Habitual energy intake (MJ)	8·9	1·2	9·8	1·6		
Smoking (*n*)						
Never	3		2			
Yes	0		3			
Stopped	6		3			
Alcohol (*n*)						
Never	1		3			
Yes	8		5			
Consumption (glasses/week)	5	–	8	–		
Medication (*n*)						
No	2		1			
Yes	7		7			
Diagnosed disease (*n*)						
Crohn's disease	3		5			
Ulcerative colitis	1		2			
Various	5		1			
Hb (g/l)	142	18	135	10	131–172	118–156
Packed cell volume (litres/litres)	0·42	0·05	0·42	0·03	0·39–0·51	0·34–0·46
Erythrocytes (cells x10^{12}/l)	4·7	0·7	4·7	0·6	4·4–5·6	3·7–5·2
Leukocytes (cells x10^9/l)	7·5	1·1	6·9	1·2	4·0–10·0	–
Thrombocytes (cells x10^9/l)	274	58	284	54	120–350	–
Creatinine (µmol/l)	80	14	80	13	60–110	50–90
Alanine aminotransferase (IU/l)	34	13	22	9	<45	–
Alkaline phosphatase (U/l)	82	22	74	25	<120	–
Cholesterol (mmol/l)	5·3	0·8	4·5	0·8	<6·5	–

* Group 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). Medication that is suspected of interfering with fat-soluble-vitamin absorption, was not allowed and not used.

Study design

The study was designed as a cross-over intervention with two controlled diets in eighteen healthy ileostomy subjects. Each subject followed both diets for 2 weeks each. The subjects consumed capsules each day for 4 weeks during both diets. The capsules contained a mean of 190 μg/d [$^{13}C_{10}$]β-carotene and 195 μg/d [$^{13}C_{10}$]retinyl palmitate in oil relative to their daily energy intake. The extrinsic dual-isotope-labelling technique is based on attaining a plateau of isotopic enrichment of β-carotene and retinol in serum during prolonged daily intake of capsules containing low doses of labelled β-carotene and labelled retinol[10,11]. The plateau has been reported to be reached by day 21[10,11], and in preliminary investigations for the present study, it was shown that the plateau was reached by day 14. On days 0, 1, 14, 15, 27 and 28 fasting blood samples of 13 ml 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 defined as not consuming any food or energy-containing drinks for 12 h before the blood sampling. On days 13 and 14 and also on days 27 and 28, complete 48 h ileostomy effluent (faeces) was collected at home, stored on dry ice in plastic containers, and then transported to the –80°C freezer. The concentrations of carotenoids and retinol in duplicate diets, serum and in faeces were measured by HPLC, and the isotopic enrichments of serum and faecal retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of β-carotene with [$^{13}C_{10}$]β-carotene were measured by using liquid chromatography–MS (LC–MS).

Diets and compliance

One diet contained vegetables low in β-carotene content with supplemental β-carotene in salad dressing oil (‘oil diet’) and the other diet contained vegetables high in β-carotene content (‘mixed diet’). The ratio of β-carotene provided by fruits to vegetables was 1 to 2·2 in the ‘oil diet’ and 1 to 8·4 in the ‘mixed diet’ as estimated from the Dutch food database[13]. Low-β-carotene fruits included a mix of orange, apple, kiwi and banana. Menus were designed for ten levels of energy intake ranging from 7 to 16 MJ/d. The subjects were allocated to an energy intake level close to their habitual energy intake, which was estimated from a FFQ[12]. Both diets were designed according to the Dutch guidelines for a healthy diet [14] and provided 90% of energy intake. 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, and which had to be recorded in a diary. The diaries were inspected 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. The food had to be reheated in a microwave oven at home. With each hot meal, a salad with

salad dressing was provided. 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, Basle, Switzerland). The margarine (Unilever, Rotterdam, The Netherlands) was not supplemented with retinol or β-carotene. Subjects kept a diary for monitoring compliance to the diet, to the intake of the capsules, to fasting instructions, and to ileostomy effluent collection. In the diary, illnesses, medication and the daily choice of low-fat food items had to be recorded.
Diets were homogenised, extracted and analysed in duplicate by HPLC as described previously[11].

Table 2. Total daily intake of energy, macronutrients, fibres, retinol and provitamin A carotenoids of two controlled diets during the 4-week cross-over intervention study*

	Oil diet	Mixed diet
Energy (MJ)	9·5	9·9
Fat (g)	72	78
Protein (g)	80	81
Carbohydrates (g)	304	292
Fibres (g)	26	29
Alcohol (g)	9	8
Retinol (μg)	192	180
Total β-carotene in salad dressing oil (μg)	2647	<1
Total β-carotene in vegetables and fruits (μg)	427	7635
trans-β-Carotene (μg)	3007	6859
cis-β-Carotene (μg)	67	776
α-Carotene (μg)	49	1121
β-Cryptoxanthin (μg)	180	115

* Results are based on the analysis of typical 11 MJ duplicate diets and the calculated composition[13] of the consumed free items, which did not contain carotenoids or retinol.

Chemical analysis of retinol and carotenoids in serum and in faeces

Retinol and carotenoids in serum were analysed using the HPLC method with absorbance detection as described previously[11]. Within- and between-run CV for the quantitative analysis of retinol in serum were 1·1 and 3·8% and were 6·1 and 9·2% for the quantitative analysis of β-carotene.
Ileostomy effluent of 48 h from each subject were pooled, homogenised and weighed. Sample preparation was carried out in duplicate and has been described previously[11]. The recovery of β-carotene was 86 (SD 6) % (*n* 3) which was measured

by spiking faeces samples with β-carotene. Within- and between-run CV for the chemical analysis of β-carotene in faeces were 3·9 and 6·2%.

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 2 weeks of controlled diets*
(Mean values and standard deviations)

	Baseline (*n* 17)		After 'oil diet' (*n* 17)		After 'mixed diet' (*n* 17)	
	Mean	SD	Mean	SD	Mean	SD
Retinol	2·18	0·45	2·00	0·43	1·92	0·47
Total β-Carotene	0·26	0·16	0·44	0·31	0·33	0·16
trans-β-Carotene	0·24	0·31	0·42	0·31	0·31	0·16
cis-β-Carotene	0·02	0·01	0·02	0·01	0·02	0·01
α-Carotene	0·03	0·03	0·03	0·02	0·04	0·03
β-Cryptoxanthin	0·12	0·08	0·09	0·04	0·09	0·04

* There were no differences between the groups at baseline, after the 'oil diet', and after the 'mixed diet' and nu differences between the measurement time points (paired *t* tests). Group 1 (*n* 9) followed for 2 weeks the 'oil diet' and consecutively for 2 weeks the 'mixed diet' and group 2 (*n* 8) followed for 2 weeks the 'mixed diet' and consecutively for 2 weeks the 'oil diet'.

Capsule administration and measurement of isotopic enrichment in serum and in faeces

Three capsules were prepared to meet the proportion of labelled compounds to the daily level of energy intake. The capsules contained 65·1, 82·8 or 98·0 μg [12,13,14,15,20,12',13',14',15',20'-$^{13}C_{10}$]β-carotene and 61·7, 83·2 or 102·1 μg [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate in oil (analysed values). The preparation of the capsules and β-carotene analyses were performed as described previously[11]. The [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinyl palmitate were synthesised at ARC Laboratories (Apeldoorn, The Netherlands) as described previously[15]. The ratios of unlabelled retinol, [$^{13}C_5$]retinol, [$^{13}C_{10}$]retinol, unlabelled β-carotene and [$^{13}C_{10}$]β-carotene in serum and in faeces were measured using LC–atmosphere pressure chemical ionisation–MS assay (LC–APCI–MS)[16,17]. β-Carotene and [$^{13}C_{10}$]β-carotene were monitored in negative ion mode at *m/z* 536 and *m/z* 546, respectively[17]. Retinol, [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol were measured as [MH–H_2O]$^+$ ions in positive ion mode at *m/z* 269, *m/z* 274 and *m/z* 279, respectively[17,18].

Calculations

The 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. The isotopic 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 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 wt as compared with 1 μg retinol) as follows[19]:

1 / (Enrichment of retinol in serum with [$^{13}C_5$]retinol / enrichment of
retinol in serum with [$^{13}C_{10}$]retinol)
x (dose of [$^{13}C_{10}$]retinol / dose of [$^{13}C_{10}$]β-carotene).

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 (48 h) from the amount consumed (48 h) and dividing the difference by the amount consumed multiplied by 100. A necessary condition to use this calculation is to standardise strictly the daily nutrient intake during the 2-week intervention study.

Design and statistical analysis

A cross-over design was chosen to eliminate between-subject variation. A carry-over effect was unlikely, because in preliminary investigations, it was shown that isotopic enrichment levels were under the detection limit after 7 d after intervention. Based on previous data[11], a sample size of seventeen subjects had a 80% power to detect a difference in vitamin A equivalency between treatments of 0·27 with a significance level (α) of 0·05 (two-sided). Data are shown as means and 95% CI or standard deviations (in the case of descriptive measures). Data of serum concentrations and enrichments were averaged for days 0 and 1, for days 14 and 15, and for days 27 and 28 for each subject. To evaluate differences in baseline blood values between the two groups, *t* tests for independent samples were performed. ANOVA was used to test order effects. Because the order of the two diets did not significantly contribute to the model (P=0·23), serum retinol and serum β-carotene concentrations at baseline and after each diet were compared between the two dietary treatments with a paired *t* test. To test whether the percentages of apparent absorption were significantly different between both diets, paired *t* tests were performed. All tests were two-sided, and P values <0·05 were considered significant. The computer package SPSS 12·0 (SPSS Inc., Chicago, IL, USA) and Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA, USA) were used for all statistical calculations and data handling.

RESULTS

The baseline characteristics of the seventeen subjects who completed the study are shown in Table 1. One individual dropped out because of not following the diet. No significant differences were observed between both groups with respect to haematological analyses of blood, liver enzymes, creatinine, alkaline phosphatase and cholesterol. The reported compliance to the diets was very good: 99·4% of the capsules and 97% 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 contents of the diets are given in Table 2, and the dietary sources of β-carotene in the diets are 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. There were no significant differences between the groups in terms of serum retinol and serum β-carotene concentrations after each diet period compared with baseline. No significant post-intervention inter-diet differences for either retinol or β-carotene concentrations in serum were found.

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. The mean vitamin A equivalency of [$^{13}C_{10}$]β-carotene in oil was 3·71 (95% CI 2·79, 4·63) μg (CV 49%) in response to the 'oil diet' and 3·56 (95% CI 2·86, 4·26) μg (CV 40%) in response to the 'mixed diet'.

Oral–faecal balance data demonstrate an apparent fractional absorption of β-carotene of 1·9-fold higher from the 'oil diet' (30%) than from the 'mixed diet' (16%) (Table 5). With the oral–faecal balance data and the generally assumed bioconversion of 50% for absorbed β-carotene[2,3], the vitamin A equivalency of the unlabelled β-carotene was estimated as 6·7:1 (1/(0·30 x 0·5)) for the 'oil diet' and as 12·5:1 (1/(0·16 x 0·5)) for the 'mixed diet'. Neither labelled retinol nor unlabelled retinol was detected in faeces.

Table 4. Vitamin A equivalency and bio-efficacy of [$^{13}C_{10}$]-β-carotene in oil after 2 weeks of controlled diets*
(Mean values and 95% confidence intervals)

Symbol	Description	Oil diet (*n* 17)		Mixed diet (*n* 17)	
		Mean	95% CI	Mean	95% CI
$E_{5,sR}$	Enrichment of retinol in serum with [$^{13}C_5$]-retinol	0·00635	0·00520, 0·00750	0·00653	0·00507, 0·00799
$E_{10,sR}$	Enrichment of retinol in serum with [$^{13}C_{10}$]-retinol	0·01551	0·01172, 0·01930	0·01475	0·01242, 0·01708
$E_{10,sC}$	Enrichment of β-carotene in serum with [$^{13}C_{10}$]-β-carotene	0·04153	0·03672, 0·04633	0·04902	0·04211, 0·05593
$E_{10,fC}$	Enrichment of β-carotene in faeces with [$^{13}C_{10}$]-β-carotene	0·03467	0·03108, 0·03773	0·01415	0·01126, 0·01704
$A_{R,cap}$	Dose (μmol/d) of [$^{13}C_{10}$]-retinol from capsules				
	Mean		0·374		0·377
	SD		0·059		0·058
$A_{C,cap}$	Dose (μmol/d) of [$^{13}C_{10}$]-β-carotene from capsules				
	Mean		0·362		0·361
	SD		0·058		0·059
$AEq_{,C}$	Vitamin A equivalency (μg) of [$^{13}C_{10}$]-β-carotene in oil †	3·7	2·8, 4·6	3·6	2·9, 4·3

* The 'oil diet' is a diet containing vegetables low in β-carotene content supplemented with synthetic β-carotene in salad dressing oil and the 'mixed diet' contained vegetables high in β-carotene content. Each subject followed both diets for 2 weeks in cross-over design. All 4 weeks, each subject daily consumed capsules with [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinol in oil.

† $AEq_{,C}$ is the amount of β-carotene that has the same vitamin A activity as 1 μg retinol and was calculated as follows: $1/ (E_{5,sR}/E_{10,sC}) \times (A_{R,cap} \times 286/ A_{C,cap} \times 537))$.

Table 5. Apparent absorption of total β-carotene (labelled and unlabelled) from 48 h after 2 weeks of controlled diets†
(Mean values and standard deviations)

Symbol	Description	Oil diet (*n* 17)		Mixed diet (*n 17*)	
		Mean	SD	Mean	SD
C_{fC}	Total β-carotene concentration in faeces (μg/g)	2·6	0·9	7·4*	2·0
W_f	Weight of total faeces collection (g/48 h)	1759	692	1809	735
A_d	Dietary total β-carotene (mg/48 h)	6·1	0·8	15·2*	2·9
A_f	Faecal β-carotene (mg/48 h) ($C_{fC} \times W_f$)	4·3	1·3	12·8*	3·9
A_d-A_f	Apparent absorbed β-carotene (mg/48 h) ‡	1·7	1·0	2·4*	2·9
AA_C	Apparent absorption of β-carotene (%) §				
	Mean	29·6		15·7*	
	95% CI	23, 37		6, 24	

* Mean values was significantly different from that on the oil diet (P<0·001; paired t-test).
† The 'oil diet' is a diet containing vegetables low in β-carotene and content supplemented with synthetic β-carotene in salad dressing oil and the 'mixed diet' contained vegetables high in β-carotene. Each subject followed both diets for 2 weeks in cross-over design. Daily for 4 weeks, each subject consumed capsules with labelled [$^{13}C_{10}$]-β-carotene in oil.
‡ Two subjects had a negative oral–faecal total β-carotene balance for the 'mixed diet'.
§. AA_C was calculated by oral–faecal balance (48 h) as follows: (A_d – A_f) / A_d × 100.

DISCUSSION

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

Our data in these healthy adults with an ileostomy show that the average vitamin A equivalency of extrinsic labelled β-carotene in oil is 3·6:1 regardless of dietary matrices differences. This finding is consistent with the vitamin A equivalency of β-carotene of 3·3:1 of FAO/WHO[1,2]. The vitamin A equivalencies of β-carotene were similar for both diets, mainly because the enrichment of retinol in serum with [$^{13}C_5$]retinol and with [$^{13}C_{10}$]retinol were similar for both diets (Table 4). It appears that labelled β-carotene added to the diet in oily capsules did not exchange with β-carotene in plant cells in the intestinal lumen. As a consequence, the assumption of this method, that labelled and unlabelled β-carotene fully mix, should be rejected. This isotopic technique cannot show that the dietary matrix affects the vitamin A equivalency of β-carotene; however, the oral–faecal balance technique does show that the dietary matrix affects the vitamin A equivalency of dietary β-carotene. This indicates that the extrinsic dual-isotope dilution technique (adding a tracer in oil capsules to the diet) with the current calculation is not suitable to investigate the absorption of β-carotene from plant matrices. Thus, the measured vitamin A equivalency of labelled β-carotene of 3·6:1 represented the highest feasible vitamin A equivalency, 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.

A preceding study with twenty-four healthy adults in The Netherlands showed a very similar vitamin A equivalency of β-carotene to retinol of 3·4:1[11], which is comparable with the equivalency in the present study. This indicates that the vitamin A equivalency of β-carotene in healthy ileostomy subjects does not differ from that of healthy adults with an intact gastrointestinal tract.

Two other studies in Indonesian children[10,19] with the same isotope technique showed vitamin A equivalencies of β-carotene to retinol of 2·7:1 and 2·4:1, respectively. These studies in a developing country were not diet-controlled, and the children did not have an optimal vitamin A status. Therefore, the present results are consistent with the expectation that the vitamin A equivalency of β-carotene could be lower in those with lower needs[1].

In 2005, two studies were published that used intrinsic labelling of vegetables to quantify the vitamin A equivalency of β-carotene[20,21]. Novotny *et al.*[20] produced complete ^{13}C-labelled kale and calculated 28 d serum areas under the curve but did not estimate the vitamin A equivalency of β-carotene. Tang *et al.*[21] produced two ^{2}H-labelled vegetables and showed a vitamin A equivalency of β-carotene from spinach of 21:1 and of β-carotene from carrot of 15:1 calculated from 35 d serum areas under

the curve, which are lower than we found. However, our isotope technique measured the plateau at 2 weeks after a diet with a mix of vegetables and fruits.

Strengths and limitations of the extrinsic labelling technique

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 labeling has some significant limitations; these specially produced vegetables are very expensive, few subjects have been able to participate in the published studies and the vitamin A equivalency of β-carotene was determined after only a single meal. Furthermore, the vitamin A equivalency 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 cannot provide data for a large population consuming a varied diet. Our extrinsic labelling technique can measure the vitamin A equivalency of the labelled β-carotene in oil capsules accurately but not the effect of dietary matrices.

Estimated 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 1·9 times better source of β-carotene (and thus vitamin A) than β-carotene in a 'mixed diet'. We cannot exclude the possibility that the lower apparent absorption in the 'mixed diet' is attributable to the higher absolute amount of β-carotene ingested. The 'oil diet' was representative of a diet high in β-carotene-poor vegetables and fruits with consumption of food items fortified with β-carotene and/or β-carotene supplements, such as regularly consumed in industrialised societies. The 'oil diet', which still contained about 15% β-carotene from vegetables and fruits (Table 2), had an estimated vitamin A equivalency of β-carotene of 6·7:1.

According to the current mixed-diet guideline of the US Institute of Medicine[3], the vitamin A equivalency of β-carotene is 12:1, which is similar to our estimation for the 'mixed diet' (12·5:1). In the present study, the 'mixed diet' represented a healthy diet with amounts of cooked vegetables and fruits high in β-carotene content and including all necessary nutrients and fibres for optimal health.

When using the oral–faecal balance technique, the apparent absorption of β-carotene in healthy adults with an intact large bowel will probably always be overestimated because of bacterial degradation of β-carotene in the large bowel. The apparent absorption of β-carotene from both diets in these seventeen ileostomy

subjects approaches apparent absorption of β-carotene in healthy adults, even with the shorter gastrointestinal transit time in the small intestine resulting from the surgical removal of the ileocaecal valve. Because of this fast transit, the collected ileostomy effluent over 48 h is representative for the dietary intake during this period, which is in contrast to adults with an intact gastrointestinal tract because of a variable residence time in the large bowel. Bacterial degradation in the ileostomy bag might still occur; however, the subjects were instructed to empty the bag into a box containing dry ice to optimise the integrity of β-carotene in the effluent.

Three studies have been published involving ileostomy subjects that all used mass balance over 24 h for measurement of apparent absorption of β-carotene. Faulks *et al.*[22] showed an apparent absorption of 90% (range 74–97%) in five ileostomy subjects after the consumption of ^{13}C-labelled β-carotene in oil (dose 10 mg). Faulks *et al.*[23] showed an apparent absorption of β-carotene of 25% (range 4–41%) in seven ileostomy subjects after the consumption of spinach meals (β-carotene intake 10 mg). Livny *et al.*[24] showed an apparent absorption of β-carotene of 65% (SD 7) from cooked carrot meals and 41% (± 7) from raw carrot meals in eight ileostomy subjects (β-carotene intake 15 mg). In the present study, in which seventeen ileostomy subjects participated, the daily intake of β-carotene was either 3·1 or 7·6 mg, and ileostomy effluent collections were over 48 h. Our data showed an apparent absorption of β-carotene of 30% (range 11–53%) in the 'oil diet' and 16% (range 5–42%) in the 'mixed diet'. In each study, the inter-individual apparent absorption of β-carotene was highly variable. Nevertheless, these results indicate clearly that the vitamin A equivalency of β-carotene from various dietary matrices is dependent on the release of β-carotene from vegetable foods. A factor of 1·9 between the intestinal absorption of β-carotene dissolved in oil and β-carotene in vegetable foods appears to be realistic. An earlier study of twenty-four healthy adults with similar diets showed a factor of 2·9 between apparent absorption of β-carotene in oil and β-carotene in vegetable foods[11]. The difference can be explained by intra-individual variation in faecal β-carotene and a much higher degree of bacterial degradation in the adults with an intact gastrointestinal tract.

Serum β-carotene concentrations

The serum β-carotene concentrations in these ileostomy subjects were three times lower than in the healthy adults in the previous study[11]. Also serum α-carotene and serum β-cryptoxanthin concentrations in the ileostomy subjects were strikingly low. It should be noted that the serum retinol concentrations in these subjects were much greater than 1·07 μmol/l, which excludes vitamin A deficiency.

An explanation of the low serum β-carotene concentrations at baseline in these ileostomy subjects could be the somewhat lower absorption of fat because of faster transit compared with subjects with an intact gastrointestinal tract and the relatively low consumption of vegetables and fruits because of the possibility of temporary blockage of the ileostomy. Based on the FFQ, which were completed before the present study to assess their daily habitual energy intake, these ileostomy subjects consume a mean of 2·7 servings of vegetables and 1·1 pieces of fruit. Their daily fibre consumption was 3·5 g lower than the habitual fibre consumption of the average Dutch adult (mean of 18·1 *v.* 22·5 g, taking into account the age group and sex)[25]. During the present study, the subjects consumed about 28 g fibre (Table 2), and indeed, their serum β-carotene concentrations increased compared with baseline by consumption of more vegetable foods than their habitual intake. The influence of serum β-carotene concentrations (which also decrease with ageing) on the vitamin A equivalency of β-carotene should be assessed in future research.

Conclusion

Our oral–faecal balance data of seventeen healthy ileostomy subjects consuming a Western diet showed that the apparent absorption from supplemental β-carotene in oil was approximately 1·9-fold higher than of β-carotene from vegetables and fruits. Our isotopic data showed that the vitamin A equivalency of [$^{13}C_{10}$]β-carotene in oil was 3·6:1 regardless of a high amount of unlabelled β-carotene either in an oil or vegetable matrix.

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CLB, TN and CW designed the study and HD, ES, PH, FR & GS provided significant advice and consultation. RB and DZ performed the enrichment analyses and PH was responsible for the carotenoid analyses. HD and ES coordinated the preparation and distribution of the diets. CLB was in charge of the data collection and analysed the data. TN, FR, and GS assisted with the calculations. CLB wrote the manuscript and all authors, except CW (deceased), provided a critical review of the manuscript.

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5

Food matrix effects on bioaccessibility of β-carotene can be measured in an in vitro gastrointestinal model

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ABSTRACT

Since the food matrix determines β-carotene availability for intestinal absorption (bioaccessibility), food matrix effects on the bioaccessibility of β-carotene from two diets were investigated in vitro and compared with in vivo data. The "mixed diet" consisted of β-carotene-rich vegetables, and the "oil diet" contained β-carotene-low vegetables with supplemental β-carotene. The application of extrinsically labeled β-carotene was also investigated. The bioaccessibility of β-carotene was 28 μg/100 μg β-carotene from the mixed diet and 53 μg/100 μg β-carotene from the oil diet. This ratio of 1·9:1 was consistent with in vivo data, where the apparent absorption was 1·9-fold higher in the oil diet than in the mixed diet. The labeled β-carotene was not equally distributed over time. In conclusion, the food matrix effects on bioaccessibility of β-carotene could be measured using an in vitro model and were consistent with in vivo data. The application of extrinsically labeled β-carotene was not confirmed.

Key words: bioaccessibility; β-carotene; food matrix; in vitro digestion

Abbreviations: TIM-1, TNO gastro-Intestinal tract Model; t-BHQ, butylhydroquinone; THF, tetrahydrofuran; IS, internal standard; IE, isotopic enrichment.

INTRODUCTION

The absorption of β-carotene by humans comprises two steps[1,2]. The first step involves the disruption of the food matrix and the solubilization of β-carotene within micelles (micellarization). This step determines the availability of β-carotene for absorption (bioaccessibility). The second step covers the entry into the intestinal cells, partial conversion into vitamin A (retinol) and entry into lymph and finally into the blood. The first step (bioaccessibility) can be studied in vitro, since micelles are readily absorbed in vivo. True absorption is determined by the first step and entry into the intestinal cells. This could be calculated from classical labor-intensive in vivo studies by using data from dietary intake and collected stools. The combined first and second steps can only be studied in vivo, by analyzing blood samples.

Thus, there is a need for a simple, quick and realistic in vitro model to measure the food matrix effects on β-carotene absorption. The food matrix is an important determinant of the bioaccessibility of β-carotene. Factors such as food processing and the amount and type of vegetable and fruit cause food matrix effects[3,4].

The computer-controlled dynamic in vitro TNO gastro-Intestinal tract Model (TIM-1)[5] was selected for quantifying the bioaccessibility of β-carotene from different food matrices in this study. Compared to alternative in vitro models, TIM-1 mimics the dynamic conditions in the human gastrointestinal tract with high reproducibility,

especially for fat-soluble compounds, and facilitates the study of bioaccessibility of food components in a controlled and standardized manner[6-8].

TIM-1 has been validated for the digestion and bioaccessibility of various nutrients in comparison with in vivo studies. This include the bioaccessibility of, e.g. fats[8,9], water-soluble vitamins[10,11], and fat-soluble vitamins[12,13].

In this study, the food matrix effects on the bioaccessibility of β-carotene from two types of diets were investigated by using TIM-1 and then compared with the results with those of an in vivo study. The diets consisted of a "mixed diet" containing β-carotene-rich vegetables and an "oil diet" containing β-carotene-low vegetables with supplemental β-carotene. In an in vivo study using these diets, the apparent absorption of β-carotene was 1·9-fold higher in the oil diet than in the mixed diet[14].

This in vitro study provided an opportunity to evaluate how effectively extrinsically labeled β-carotene could be used as a tracer for measurement of absorption during in vivo studies. Ideally, the extrinsic label (administered by a capsule) should mix and equilibrate with the intrinsic β-carotene. This means that the isotopic enrichment of β-carotene should become homogeneous within the feeding sample and be equal to that observed in the bioaccessible fractions in TIM-1.

MATERIALS AND METHODS

Dynamic in vitro gastrointestinal model

TIM-1, a dynamic in vitro gastrointestinal model, was originally described by Minekus *et al*.[5,6]. For a schematic diagram of TIM-1, see Verwei *et al*.[15]. This gastric small-intestinal model comprises four connected compartments that represent the stomach, duodenum, jejunum, and ileum, respectively. Each compartment consists of a glass outer wall with a flexible inner wall. The flexible wall is surrounded by water at 37°C which is used to squeeze the flexible walls and mix the chyme, simulating peristaltic movements in the gastrointestinal tract. The jejunal and ileal compartments are connected with semipermeable hollow-fiber membranes to remove digested products and especially fat-soluble compounds that were incorporated into mixed micelles[6-8]. The pH values, as well as the gastric emptying and small-intestinal passage of the food, are computer-controlled according to preset curves based on information on human in vivo conditions[6].

TIM-1 experimental design

Two diets that had already been tested in vivo[14] were each evaluated using TIM-1. The difference between the two diets was the food matrix where the β-carotene was incorporated; with one diet containing vegetables high in β-carotene (mixed diet) and the second diet containing vegetables low in β-carotene but supplemented with β-

carotene in salad dressing oil (oil diet). The frozen diets were defrosted at 4°C for ~16 h before the experiment and were homogenized to mimic the process of chewing by human teeth, to increase experimental consistency and to minimize mixing errors as only a limited amount could be introduced in the gastric compartment. A portion of the diet was put into the gastric compartment of TIM-1. During digestion, the total filtrate (bioaccessible fraction) was collected in 30-min fractions during the first 2 h and hourly fractions until the end of the experiment at 6 h. The total ileal efflux was collected hourly during the 6 h experiment. At the end of the experiment, the residues from the gastric, jejunal and ileal compartments were collected. The ileal efflux and residues were analyzed to determine the non-bioaccessible fraction. Possibly present β-carotene content in the bile and pancreatic solutions was also analyzed. The samples were stored at –20°C until β-carotene analysis by HPLC.

Table 1. Values of energy, macronutrients, fiber, retinol, and carotenoids of two diets per 100 g wet weight [a]

	Mixed diet	Oil diet
Energy (kJ)	584	570
Fat (g)	4·7	4·3
Protein (g)	4·9	4·8
Carbohydrates (g)	17·5	18·2
Fiber (g)	1·7	1·6
Retinol (μg)	12	13
Total β-carotene in salad dressing oil (μg)	<1	156
Total β-carotene in vegetables and fruits (μg)	415	28
trans-β-Carotene (μg)	373	180
cis-β-Carotene (μg)	42	4
α-Carotene (μg)	75	3
β-Cryptoxanthin (μg)	8	12
Lutein (μg)	84	37
Zeaxanthin (μg)	8	8

[a] Values of energy and carbohydrates were calculated. The mixed diet contained vegetables high in β-carotene, and the oil diet contained vegetables low in β-carotene supplemented with synthetic β-carotene in salad dressing oil. For the in vitro study, the feeding sample consisted of 120·0 g of the homogenized diet.

Feeding sample

The feeding sample consisted of 120·0 g of the homogenized diet and was introduced into the gastric compartment together with 50·0 g gastric juice, 5 g amylase, and 37 g water[6,8]. The 120·0 g of the mixed diet contained 498 μg β-carotene (90% as *trans*-β-carotene) and 120·0 g of the oil diet contained 220 μg β-carotene (98% as *trans*-β-carotene) (Table 1). A capsule containing 47·0 μg [$^{13}C_{10}$]β-carotene was introduced into TIM-1 immediately after putting the feeding sample in the gastric compartment.

The β-carotene in the oil diet originated from supplemental β-carotene (85%) and from vegetables and fruits (15%). The supplemental β-carotene in the oil suspension was added to the salad dressing for the oil diet (all-*trans* β-carotene, 30% suspension in vegetable oil, Hoffmann-La Roche, Switzerland). 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). Both diets were prepared as duplicate diets during a previously conducted diet-controlled study in humans.[14] Each of the duplicate diets was pooled, homogenized, mixed thoroughly with 2·5 ml of 20% butylhydroquinone (t-BHQ) per kilogram of food, and stored at –20°C until analysis.

Chemical analysis of β-carotene in the feeding sample

Samples of each homogenized diet (4·0 g each) were extracted in duplicate using a mixture of methanol, tetrahydrofuran (THF), and dichloromethane (45:45:10 v/v/v). The resulting extract was saponified at room temperature overnight using a 1·5 mol/l potassium hydroxide ethanolic solution supplemented with sodium ascorbate, disodium sulfide, and glycerol. Then the mixture was extracted using diisopropyl ether, which was subsequently washed three times with water. Part of the resulting extract was evaporated to dryness and redissolved in HPLC mobile phase. The resulting solution was analyzed for β-carotene using reversed-phase HPLC with diode array detection[16,17]. These analyses were performed for quality control at two laboratories (Food Analysis Laboratory at TNO, Zeist and at Human Nutrition, Wageningen University). Results from both analyses were similar.

Chemical analysis of β-carotene in jejunal and ileal filtrates

Samples of 1000 μl each were vortex mixed with an equal volume of ethanol containing tocol as an internal standard (IS). This mixture was extracted with a 2-fold volume of hexane using vortex mixing for 2 min. Part of the resulting extract was evaporated to dryness, and redissolved in eluent. The resulting solution was analyzed for β-carotene and IS using reversed-phase HPLC-diode array detection[16].

The limit of precision of the known value of IS was 5%. These analyses were performed at the Food Analysis Laboratory at TNO, Zeist.

Chemical analysis of β-carotene in ileal efflux and residues

The ileal efflux and residues had more complex and less dissoluble matrices than the filtrates and consisted of supernatants and pellets. The concentrations of carotenoids in the ileal efflux and residues were measured at the division of Human Nutrition, Wageningen University. The supernatants of both ileal efflux and residues were treated according to the procedure described by Khan *et al*.[17]. Briefly, the pellets of both ileal efflux and residues were extracted in duplicate in the presence of anhydrous sodium sulphate (4·0 g) and calcium carbonate (0·5 g), with 20 ml of THF containing 0·01% t-BHT after using a tissue grinder. The extract was filtered through a glass funnel fitted with Whatman paper no. 54. The residue was twice re-extracted with 20 ml of THF. The extract was evaporated to dryness, redissolved in methanol/THF (1:1 v/v %) containing 0·01% t-BHT, transferred into a 25·0 ml volumetric flask and made up to volume. An amount of 25 μl was injected into the HPLC system for analysis. Within-run and between-run coefficients of variation for β-carotene were 2·6% and 7·1%, respectively.

Labeled β-carotene in the capsule and measurement of isotopic enrichment in filtrates

The concentration of β-carotene in the capsules was analyzed by HPLC with absorbance detection according to the method described by Khan *et al*.[17]. The capsule contained 47·0 (±0·8) μg [12,13,14,15,20,12',13',14',15',20'-$^{13}C_{10}$]-β-carotene in sunflower oil (>82% oleic acid and >9% linoleic acid; Hozol; Contined BV, Bennekom, The Netherlands). The $^{13}C_{10}$-labeled β-carotene was synthesized at ARC Laboratories (Apeldoorn, The Netherlands) as described previously[18] (isotopic incorporation >99%, isomeric purity >93% *all-trans*, chemical purity >98%). The capsules were designed for oil solutions and were made from bovine gelatin (Capsugel, Bornem, Belgium).

The first experiment of each diet was analyzed for isotopic enrichment in the filtrates. The degree of isotopic enrichment of β-carotene with [$^{13}C_{10}$]β-carotene in filtrates was measured by using liquid chromatography-atmospheric pressure chemical ionization–mass spectrometry (APCI LC–MS) as described previously[19,20]. β-Carotene and [$^{13}C_{10}$]β-carotene were monitored in negative ion mode at mass-to-charge ratios (*m/z*) 536 and *m/z* 546, respectively[20]. Isotopic enrichment of β-carotene in the filtrates was the proportion of labeled β-carotene to total β-carotene. These analyses were performed at the University of Illinois.

Calculations

The β-carotene bioaccessibility, the amount of bioaccessible β-carotene in μg per 100 μg of β-carotene available for digestion, was expressed as a fraction of β-carotene feeding and calculated by the formula

((μg β-carotene $_{filtrates}$) / (μg β-carotene $_{feeding}$ + β-carotene $_{endogenous}$)) x 100

where β-Carotene $_{filtrates}$ is the total β-carotene in the jejunal and ileal filtrate, β-carotene $_{feeding}$ is the β-carotene in the feeding sample and comprises the β-carotene in 120·0 g of the diet and a capsule with 47 μg labeled β-carotene, and β-carotene$_{endogenous}$ is the β-carotene content in the bile and pancreatic solutions secreted into the duodenal compartment.

The recovery (%) (mass balance) of β-carotene was calculated by the formula

((β-carotene $_{filtrates}$ + β-carotene $_{ileal\ efflux}$ + β-carotene $_{residues}$) /
(β-carotene $_{feeding}$ + β-carotene $_{endogenous}$)) x 100

where β-carotene $_{ileal\ efflux}$ is the β-carotene content in the total material delivered from the ileal compartment, and β-carotene $_{residues}$ is the total β-carotene in the residues of stomach, duodenum and ileum.

The proportion of isotopic enrichment (IE) of β-carotene with [$^{13}C_{10}$]β-carotene was calculated by IE in filtrates divided by IE in feeding sample and multiplied by 100.

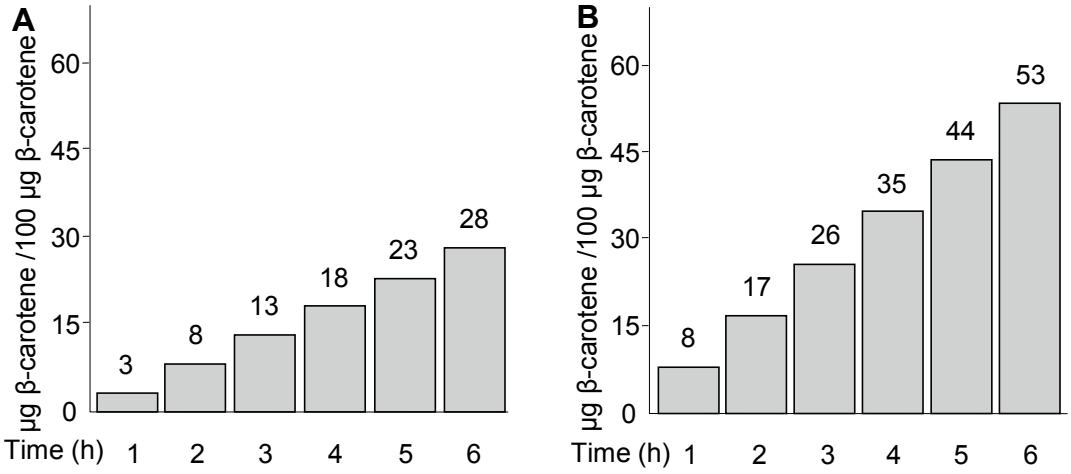

Figure 1. Cumulative β-carotene in filtrates expressed as a percentage of feeding to time after the runs (*n* = 3) with the mixed diet (A) and with the oil diet (B) after the runs (*n* = 2) in TIM-1 during 6 h.

RESULTS

The endogenous β-carotene content of the bile and pancreatic juice used in TIM-1 was below the detection limit in all runs. There were no other sources of β-carotene than the feeding samples. The results with the mixed diet are based on triplicate experiments. The results with the oil diet are based on duplicate experiments since one experiment failed due to a defective hollow-fiber membrane unit. Because of the high recovery in both oil diet runs and similar bioaccessibilities, these duplicate runs were regarded as representative.

The bioaccessibility of β-carotene, determined by collection of all the filtrates, was 28·2 μg/100 μg β-carotene from the mixed diet and 53·4 μg/100 μg β-carotene from the oil diet (Table 2 and Figure 1). The recovery of β-carotene was 92% and 105% in the two runs with the oil diet and 88%, 94%, and 104% in the three runs with the mixed diet (Table 2).

Table 2. β-Carotene amounts, the bioaccessibility of β-carotene and the recovery of β-carotene with the two diets in TIM-1 over 6 h [a]

	β-carotene in mixed diet			β-carotene in oil diet	
	run 1	run 2	run 3	run 1	run 2
Feeding					
Diet (μg)	498	498	498	220	220
Capsule (μg)	47	47	47	47	47
Endogenous (μg)	nd	nd	nd	nd	nd
Filtrates					
Jejunal filtrate (μg)	95·6	83·5	80·3	76·3	80·5
Ileal filtrate (μg)	73·7	73·0	55·5	65·4	62·8
Ileal efflux (μg)	264·2	312·0	233·6	34·8	44·9
Residues					
Residue of stomach and duodenum (μg)	31·7	27·4	67·9	44·0	62·9
Residue of jejunum and ileum (μg)	48·2	69·7	40·1	25·8	29·7
Bioaccessibility (μg β-carotene/100 μg β-carotene)[b]	31·1	28·7	24·9	53·1	53·7
Recovery (%)[c]	94·2	103·8	87·6	92·3	105·2

[a] The mixed diet contained vegetables high in β-carotene, and the oil diet contained vegetables low in β-carotene supplemented with synthetic β-carotene in salad dressing oil. nd= not detected.

[b] The β-carotene bioaccessibility is the amount bioaccessible β-carotene in μg per 100 μg β-carotene available for digestion and is calculated as (μg β-carotene in filtrates) / (μg β-carotene from feeding + endogenous) x 100.

[c] Recovery (%) = (β-carotene in filtrates + ileal efflux + residues) / (β-carotene from feeding + endogenous) x 100.

Table 3. Proportion of isotopic enrichment of β-carotene with [$^{13}C_{10}$]β-carotene in filtrates compared with that of the feeding sample for the two diets in TIM-1 over 6 h

%	Mixed diet (%)	Oil diet (%)
Jejunal filtrate 0–3 h	85	106
Jejunal filtrate 3–6 h	111	122
Ileal filtrate 0–3 h	112	79
Ileal filtrate 3–6 h	139	85
Weighted average filtrate 0–6 h [a]	113	99

[a] The measured β-carotene levels in jejunal filtrates were slightly higher than in ileal filtrates (Table 2).

The isotopic enrichments of β-carotene in the feeding sample compared with the isotopic enrichment of β-carotene in the filtrates varied over time from 85% to 122% in the jejunal filtrates and from 79% to 139% in the ileal filtrates (Table 3).
Averaged over 6 h, the isotopic enrichment of β-carotene in the filtrate for the oil diet was closer to the theoretical value of 100% than for the mixed diet (99% v 113%). This difference in isotopic enrichment was expected because the oil diet with supplemental β-carotene was more comparable with the matrix of labeled β-carotene in the feeding sample than was the β-carotene in vegetables in the mixed diet. In the TIM-1 run with the mixed diet, the isotopic enrichment in the jejunal filtrate was lower than in the ileal filtrate, which indicates that micellarization of labeled β-carotene in the jejunal compartment lagged behind that in the ileal compartment. In contrast, the opposite was seen in the TIM-1 run with the oil diet, where micellarization of labeled β-carotene was faster in the jejunal compartment than in the ileal compartment. The isotopic enrichment increased over time in both jejunal and ileal filtrates, which means that the labeled β-carotene was not equally distributed over time in the bioaccessible fractions. Labeled β-carotene either did not fully equilibrate with the β-carotene in the food matrix or else followed a different micellarization pattern.

DISCUSSION

This in vitro gastrointestinal model appears useful for determination of the effects of food matrices on the bioaccessibility of β-carotene from foods, especially when fat-soluble nutrients compete for uptake in mixed micelles.

Comparing the bioaccessibilities of β-carotene from both diets

The mixed diet represented a healthy diet with high amounts of cooked vegetables and fruits. The oil diet was representative of a diet low in vegetables and fruits but high in food items fortified with β-carotene, such as those regularly consumed in industrialized societies (e.g. margarine). The oil diet and the mixed diet contained β-carotene in the physiological dose range and 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). The β-carotene amount in the oil diet was not the same as in the mixed diet; however, this was also the situation in the in vivo study[14]. The absorption efficiency of β-carotene is regarded constant as long as the β-carotene consumption is within physiological ranges[1,2,21]. Furthermore, the mixed diet contained a higher amount of β-carotene than the oil diet but had a lower amount bioaccessible β-carotene compared to the oil diet; in contrast, the oil diet contained a lower amount of β-carotene than the mixed diet but had a higher amount bioaccessible β-carotene compared to the mixed diet.

Bioaccessibility of β-carotene was 28 μg/100 μg β-carotene (or 28%) from the mixed diet and 53 μg/100 μg β-carotene (or 53%) from the oil diet. So, the bioaccessibility of (labeled and dietary) β-carotene in the oil diet was 1·9-fold higher than in the mixed diet. This 1·9-fold enhancement in apparent absorption between two diets was also found in the in vivo study[14].

Although in absolute terms, results of β-carotene bioaccessibility using TIM-1 are not strictly comparable with in vivo experiments, the differences in β-carotene bioaccessibilities between food matrices are still comparable with absorption estimates in humans. Thus, TIM-1 cannot be used for predicting absorption in vivo, but the system is a useful tool for measuring differences in bioaccessibility of β-carotene between various food matrices.

Comparing the bioaccessibilities of β-carotene measured using other models

Bioaccessibility of β-carotene in various meals, fruits and vegetables have been reported using other in vitro digestion models. Garrett *et al.*[22] developed an in vitro digestion model based on the method of Miller *et al.*[23], which simulates human gastric and pancreatic digestion as static duo-compartments. Our result of 28% bioaccessibility of β-carotene from the mixed diet is slightly higher than the reported bioaccessibility of β-carotene from meals. For example, the micellarization of β-carotene was 12–18% from a baby food meal of carrots, spinach and chicken[22], 16% from a meal of mango and chicken[24], 16% from stir-fried meal of spinach,

carrots and tomato paste[25], and 18% from a salad meal of spinach, tomato, carrot and lettuce[26].

Several other studies have used the same in vitro method for single fruits or vegetables. From orange and kiwi, the micellarization of β-carotene was 34% and 47%, respectively[27]. From mango, the micellarization of β-carotene was 25–39%; from mango with milk and sugar, 37–48%; from papaya, 31–34%; and from papaya with milk and sugar, 41–44%[28]. The micellarization of β-carotene from carrot was 14% for carrot juice[29], 17% from processed carrots[30], 20% from raw carrots[31], and 33% from stir-fried carrots[31], From other vegetables, the micellarization of β-carotene was 17% from maize[32],17% from boiled spinach[29], 30% from spinach puree[33], 30% from spinach[27], 29% from tomatoes[34], 30% from boiled cassava[35,36], 45% from sweet potato[27], 54% from broccoli[27], and 57% from courgette[34]. Our result of 28% is comparable to these as our study included a mix of different cooked vegetables and fruits.

Hedrén *et al*.[37] developed a similar static duo-compartmental in vitro model, which estimates the maximum amount of carotenoids released, but is not necessarily micellarized. Using this other in vitro model, the percentages of accessible β-carotene in homogenized, raw carrots were 21% and 30% and from cooked carrots were 27% and 39%, without and with addition of oil, respectively[37]. Another study by Hedrén *et al*.[38] showed accessible β-carotene was 39% from sweet potato, 64% from pumpkin and 47% from cassava, which were all cooked with sunflower oil. Three other studies with this method showed a bioaccessibility of β-carotene of 19% from boiled pumpkin[39], 74% in boiled carrot[39], and when cooked with vegetable oil, 11–22% from various processed sweet potato[40], 21% from sweet potato, and 39% from pumpkin[41]. Our results of 28% and 53% are also comparable with the results from this in vitro method.

Although these other models are temperature- and pH-controlled, they are not representative of the continuously changing variables during passage through the stomach and the small intestine. For general prediction of micellarization of β-carotene, these models are adequate. However, TIM-1 allows for the closest simulation to date of in vivo dynamic physiological processes occurring within the lumen of the stomach, duodenum, jejunum, and ileum of humans. Furthermore, TIM-1 has a major advantage that the model allows for the collection of samples during the process of digestion without disturbing or temporarily stopping the experiment and thereby allows studying the digestion process in time.

Extrinsic labeled β-carotene vs time of both diets

When using any extrinsic labeling technique in absorption studies, the assumption must be made that the label fully mixes with the compound and equally distributes in

the lumen of the small intestine. This means that in each time period of the digestion process, the isotopic enrichment of β-carotene in the filtrates should be the same as in the diet. However, during the 6 h of the TIM-1 experiment, the isotopic enrichments of β-carotene in the ileal filtrate in mixed diet runs increased and in oil diet runs decreased compared with the jejunal filtrate, which indicates discrimination for micellarization. This enrichment pattern may partly be explained by retarded equilibration in the mixed diet run, where the labeled β-carotene is bound and captured by the complex matrix and available later in time for digestion than in de oil diet. The isotopic enrichment of β-carotene with [$^{13}C_{10}$]β-carotene in filtrates was not constant over time. This implies that the labeled β-carotene behaved differently from the β-carotene incorporated into the matrices of vegetables and fruits high in β-carotene and behaved differently from the matrix of supplemental β-carotene in oil. The labeled β-carotene dissolved in oil was not equally distributed over time in the bioaccessible fractions, which means that during the experiment, it did not fully mix with dietary β-carotene. As suggested by the average filtrate over 6 h for the oil diet, which was 99%, the extrinsic labeling technique could be used to measure absorption in vivo if the matrix of the homogenized diet were comparable with the oily matrix of extrinsic labeled β-carotene. However, the different isotopic enrichments of β-carotene in the ileal filtrate and jejunal filtrate in the oil diet suggested different micellarization patterns for dietary β-carotene in oil and labeled β-carotene in oil.
Additional experiments are required to confirm and explain this result, which may be influenced by dissolving of the capsule, and to investigate the reproducibility of the measured isotopic enrichments in these samples with very low amounts of labeled and unlabeled β-carotene.
The advantage of this TIM-1 dynamic model is that it can address mixing effects and partitioning effects of labeled fat-soluble compounds during the digestion progress. Therefore, this in vitro model could provide significant value for checking assumptions in distribution of labeled compounds in in vivo labeling studies.
In summary, the food matrix effects on bioaccessibility of β-carotene could be measured using TIM-1 and were consistent with in vivo data. The labeled β-carotene either did not fully equilibrate with the β-carotene in the food matrix or else followed a different micellarization pattern. The application of extrinsically labeled β-carotene was not confirmed.

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6

A pilot study to assess the required study duration with daily intake of labelled β-carotene and retinol for measurement of a plateau of isotopic enrichment in serum

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ABSTRACT

The aim was to determine how many days are needed to reach a plateau of isotopic enrichment of the ratio of enrichment of retinol with [$^{13}C_5$]retinol ($E_{5,sR}$) and the enrichment of retinol with [$^{13}C_{10}$]retinol ($E_{10,sR}$). That ratio represents the dilution of [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinyl palmitate with the unlabelled retinol. Two subjects consumed daily a capsule of ~55 μg labelled β-carotene and retinol. The diet was not controlled, however β-carotene-rich food items were avoided. Serum samples were collected at day 7, 14 and 21. We conclude that 14 days is sufficient to reach a plateau of isotopic enrichment of β-carotene and retinol in serum. Therefore, the duration of the first study in 24 healthy adults was 21 days and that of the second study could be reduced to 14 days.

INTRODUCTION

In the first study[1] performed in healthy adults, an extrinsic dual-isotope-labelling technique was used, which is based on reaching a plateau of isotopic enrichment of β-carotene and retinol in serum in time during daily intake of capsules with low doses of [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinyl palmitate. The extrinsic dual-isotope-labelling technique can quantify the vitamin A equivalency of β-carotene. For this quantifying, the necessary condition is to standardize strictly the daily nutrients intake during the intervention.

The duration of this study was 21 days, as two studies[2,3] proved that the plateau was reached by day 21. In the first study in children[2], the collection of blood samples was set for group 1 (n=12) on day 21, and 28, for group 2 (n=12) on day 21, 42, and 49, and for group 3 (n=11) on day 21, 63 and 70. They made this conclusion on basis that the coefficient of the regression of the vitamin A equivalency of β-carotene in oil was not significantly different among groups and that for all groups pooled, the regression coefficient was not significantly different from zero. In the second study in children[3], blood samples were collected of a sub-sample of 6 children on day 8 and 21, and it was calculated that the coefficient of the regression of vitamin A equivalency of β-carotene in oil on day 8 and 21 differed significantly from zero. Thus in these children isotopic enrichment of retinol and β-carotene in serum reached a plateau between 8 and 21 days. The open question is how many days between 8 and 21, should the duration of daily capsule intake last to reach a plateau.

Therefore, this investigation was performed with collection of blood samples on day 7, 14 and 21 during daily capsule intake. The diet was not controlled as the aim was to judge isotopic enrichments of retinol and the aim was not to measure vitamin A equivalency of β-carotene. The aim was to determine how many days are needed to reach a plateau of isotopic enrichment of the ratio of enrichment of retinol with

[$^{13}C_5$]retinol ($E_{5,sR}$) and the enrichment of retinol with [$^{13}C_{10}$]retinol ($E_{10,sR}$). That ratio represents that [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinyl palmitate are diluted with the unlabelled retinol.

METHODS

Study design

The screening comprised the analyses of a blood sample for haemocytometry, cholesterol, liver enzymes, creatinine, and alkaline phosphatase to confirm the general good health of the subjects. Two subjects were selected and signed the informed consent form. The subjects consumed capsules each day for 21 days. On days 0, 7, 14, and 21 fasting blood samples were obtained. The dietary guidelines were not to consume the β-carotene-rich food items on the provided list during the 21 days of the study and the preceding 14 days. This list included liver, liver products, eggs, and any vitamin supplement.

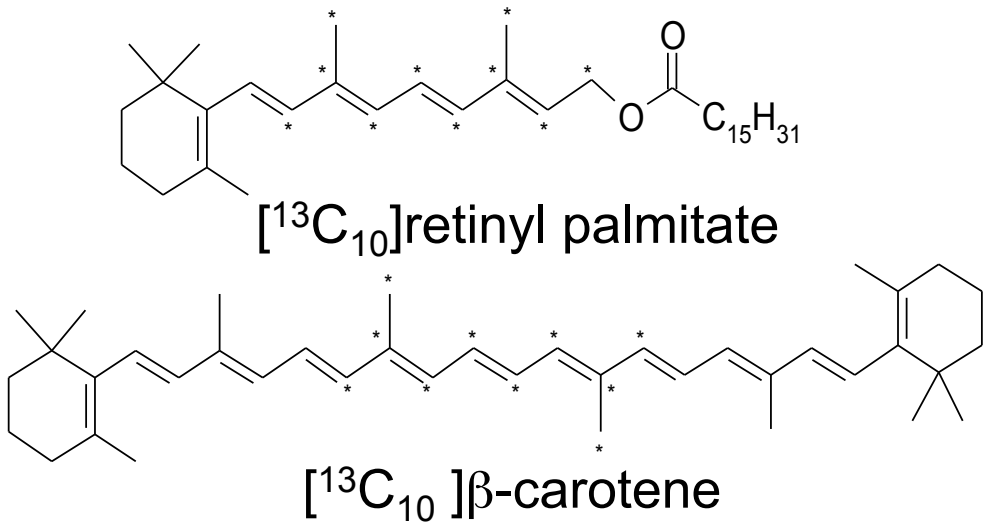

Figure 1. Molecular structure of synthesized [12,13,14,15,20,12',13',14',15',20'-$^{13}C_{10}$]β-carotene and [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate. The asterisks indicate the positions of the ^{13}C labels.

Capsule administration and measurement in serum

The capsules were identical to the capsules used in the preceding study[1]. The capsules contained 14·9, 20·1 or 25·7 μg [$^{13}C_{10}$]β-carotene and 14·1, 19·1 or 23·9 μg [$^{13}C_{10}$]retinyl palmitate in oil (Figure 1 shows the molecular structure). The individual assigned daily intake of labeled β-carotene and retinol is related to the individual's estimated daily energy intake and show in Table 1.

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[4,5]. The sample preparation, accuracy and precision of the measurement of the degree of isotopic enrichment of β-carotene and of retinol in serum have been described in Zhu *et al.*[6].

Table 1. Daily intake of labeled β-carotene and retinol on basis of the estimated habitual energy intake of the two subjects.

	unit	Subject 1	Subject 2
Number of capsules per day	#	3	2
Estimated habitual energy intake	(MJ)	11	10
[$^{13}C_{10}$]β-carotene	(μg/dag)	55 [a]	51 [b]
[$^{13}C_{10}$]retinyl palmitate	(μg/dag)	52 [c]	48 [d]

[a] This comprises one capsule of 14·9 μg and two capsules of 20·1 μg labeled β-carotene.
[b] This comprises two capsules of 25·7 μg labeled β-carotene.
[c] This comprises one capsule of 14·1 μg and two capsules of 19·1 μg labeled retinol.
[d] This comprises two capsules of 23·9 μg labeled retinol.

Table 2. Blood values of the two subjects at screening.

	unit	Subject 1	Subject 2	Laboratory references male	female
Retinol	μmol/l	2·4	2·5	>1·05	
β-Carotene	μmol/l	0·24	0·28	NA	
Creatinine	μmol/l	81	80	60-110	50-90
Cholesterol	mmol/l	5·2	3·7	4·7-6·5	
Alkalische fosfatase	U/l	41	60	<120	
Alanine aminotransferase	U/l	32	23	<45	
Hb	mmol/l	8·7	8·5	8·1-10·7	7·3-9·7
Haemotocrit	l/l	0·40	0·41	0·39-0·51	0·34-0·46
Erytrocytes	10^{12}/l	4·60	4·65	4·4-5·6	3·7-5·2
Mean Cel Hemoglobine	mmol/l	21·9	21·0	19·0-22·5	
Mean Cel Volume	fl	86	87	80-98	
Mean Cel Hemoglobine	fmol	1·89	1·83	1·73-2·15	
Redcell distribution width	%	12·7	11·8	11·8-15·0	
Trombocytes	10^9/l	241	300	120-350	
Leucocytes	10^9/l	4·3	7·1	3·5-11·0	

NA = not available

RESULTS

The subjects were in general good health as assessed by the blood values (Table 2). The subjects had retinol levels of ≥1·05 μmol/l, equal to 30 μg/dl. They consumed an adequate diet for total vitamin A. They reported in the diary that they complied with the dietary guidelines for not consuming the β-carotene-rich food items on the provided list during the 21 days and preceding two weeks.

Table 3 shows the isotopic enrichments of retinol and the ratio of enrichment of retinol with [$^{13}C_5$]retinol and the enrichment of retinol with [$^{13}C_{10}$]retinol for both subjects at day 7, 14 and 21. Figure 2 is a graphical representation of these data.

Table 3. Isotopic enrichment of retinol in serum of 2 subjects at day 7, 14 and 21.

Subject	$E_{5,sR}$	$E_{10,sR}$	$E_{5,sR}$ / $E_{10,sR}$
#1 at day 7	0·003000	0·011000	0·272727
#2 at day 7	0·002833	0·011667	0·242857
#1 at day 14	0·002333	0·010500	0·222222
#2 at day 14	0·003333	0·010667	0·312500
#1 at day 21	0·002333	0·011500	0·202899
#2 at day 21	0·004500	0·016667	0·270000

$E_{5,sR}$ = enrichment of serum retinol with [$^{13}C_5$]retinol.
$E_{10,sR}$ = enrichment of serum retinol with [$^{13}C_{10}$]retinol.
$E_{5,sR}$ / $E_{10,sR}$ = ratio of enrichment of retinol with [$^{13}C_5$]retinol ($E_{5,sR}$) and the enrichment of retinol with [$^{13}C_{10}$]retinol ($E_{10,sR}$).

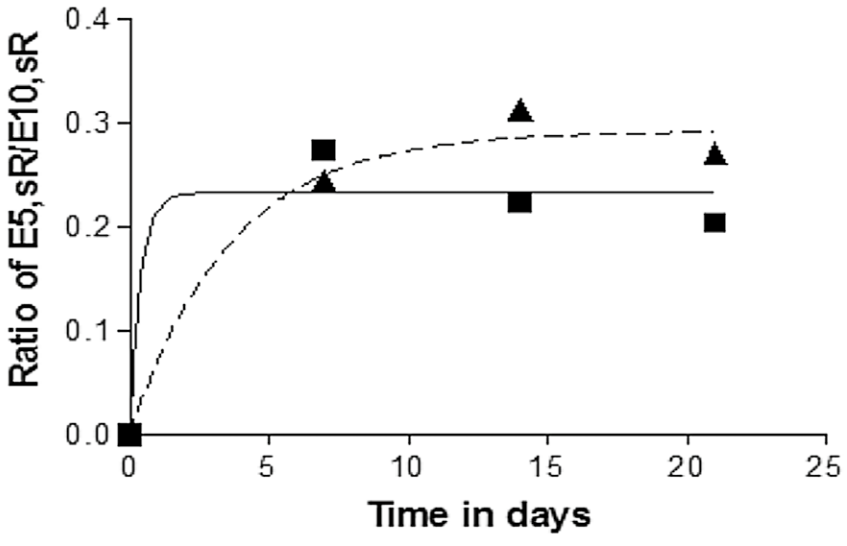

Figure 2. Plateau of isotopic enrichment of retinol on basis of the ratio of enrichment of retinol with [$^{13}C_5$]retinol ($E_{5,sR}$) and the enrichment of retinol with [$^{13}C_{10}$]retinol ($E_{10,sR}$) for 2 subjects on day 7, 14 and 21 (lines are based on nonlinear curve-fitting; one-phrase exponential association).

DISCUSSION

In the present investigation, the plateau of isotopic enrichment of β-carotene and retinol in serum in time was measured using daily intake of low doses of labelled β-carotene and retinol. At day 7, the enrichments were similar to the measurement on day 14 and 21. The presented data was collected from only 2 subjects who did not consume a controlled diet. Under more strictly controlled circumstances 7 to 13 days shall be sufficient to measure the plateau of isotopic enrichment of retinol.

In a future study, it is important that participants should get used to the controlled circumstances for full compliance to the diet, the capsules and the diary. Another influencing factor is the diet before start of the intake of the capsules and the controlled diet. If the preceding diet contains high or very low amounts of β-carotene, the fasting serum level of β-carotene reflects the recent dietary intake of β-carotene. So, every day the ingested and absorbed labeled β-carotene dilutes with the serum β-carotene.
In the case of dietary retinol, if this preceding diet contains high or very low amounts of retinol, this will not influence the dilution, because retinol levels are homeostatically controlled by the body. The subjects had an adequate vitamin A status as the serum retinol level was far above the threshold level.

Furthermore, the plateau is depending on the dilution with the body pool of retinol, which is depending on the habitual diet and the nutritional status of each subject. The variation in the ratio of enrichment of retinol with [$^{13}C_5$]retinol and the enrichment of retinol with [$^{13}C_{10}$]retinol between these two subjects is higher (CV 25%) than the variation within these two subjects, where the averaged ratio is 0·2326 SD 0·036 and 0·2751 SD 0·035 resulting in CV within subject of 16% and 13%, respectively. These CV are regarded as normal as the diet was not controlled.

In conclusion, it appears that 14 days is sufficient to reach a plateau of isotopic enrichment of β-carotene and retinol in serum. Seven days may also be sufficient for reaching the plateau in controlled conditions. However, subjects have to get used to the strictly controlled diets. Therefore it is uncertain that everyone could reach the plateau at day 7. So, the duration of the study in 17 ileostomy subjects[7] was set at 14 days for each diet.

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7

General discussion and summary

Introduction

Information on the content and the vitamin A equivalency of provitamin A carotenoids, such as β-carotene, in plant foods is of great interest due to the widespread vitamin A deficiency in developing countries. The effect of food matrices of vegetables and fruits, in which the β-carotene is incorporated, has been found to exert a major influence on measured vitamin A equivalency of β-carotene. Dietary reference intakes for all vitamins are set for various age groups. Especially in the last decade, the food composition tables are updated with analysed data of provitamin A carotenoids in various foods, including biofortified staple foods (like maize, cassava and rice), complementary foods and supplements. However, the food composition tables do not all use the same factors for vitamin A equivalency of β-carotene.

Research on vitamin A equivalency of β-carotene

The first research question of this thesis focuses on what is known about the vitamin A equivalence of β-carotene (VEB) from various food matrices in humans and which methods are used to assess the VEB. In the past decades, various methods have been used for determination of the VEB. In table 1 of Chapter 2[1], the major strengths and major limitations of the methods used in human subjects are shown. The classical methods such as depletion-repletion, mass balance and dose–response curves are available for decades. The last two decades, the application of isotopic dilution methods was possible as the isotopic enrichments analysis became available with high precision and high reproducibility. Very different models are used to obtain data on VEB, with very variable doses of β-carotene, which were incorporated into different matrices and ingested in various meals.

In conclusion, data concerning the VEB from various dietary sources are inconsistent. This is because so many diet-related and person-related factors have an influence on the obtained data. Indeed it is very challenging to review all available data and to set a compromised conversion factor by official health authorities, which should be applicable to the general population. On the basis of the overview in Chapter 2[1] for persons in general good health consuming a Western diet, we propose a range for the VEB of 9:1 to 16:1 in a mixed diet. So, the 12:1 of IOM is indeed a realistic VEB in a mixed diet with various vegetables and fruits. So, the current VEB of IOM from 2001[2] is justified for use in USA, Canada and European countries.

For countries in Africa and Asia the VEB could range from 9:1 to 28:1 in a mixed diet, taking into account less favourable host-related factors, such as parasites and gastrointestinal infections. Furthermore, their habitual diet contains much more vegetables and fruits. Intensive processing of the meals will improve the bioaccessibility of β-carotene and consequently their vitamin A status can become

adequate. Especially, this should be the case with the biofortified staple foods, such as cassava, yellow maize and Golden Rice. The so-called Golden Rice will soon be consumed locally in some developing countries. A bowl of 100 to 150 g cooked Golden Rice (50 g dry weight) can provide approximately 60% of the Chinese Recommended Nutrient Intake of vitamin A for 6–8 years old children[3].

Nutritional composition of the diets used in the intervention studies

As seen worldwide, more and more biofortified foods and dietary supplements are on the market and a clear increase is observed in the consumption of foods fortified with micronutrients. In the Netherlands in 2003 about 40% of the young adults aged 19–30 years consumed a fortified product on one or both of the survey days, whereas for the 2007–2010 survey this was about 75%[4,5]. The most frequently consumed fortified products were dairy products and non-alcoholic beverages[5].

For both human studies, it was a convinced decision to investigate the vitamin A equivalency of β-carotene from a complex diet with different matrices. Many studies, which are summarized in Chapter 2[1], report a VEB from a single vegetable or fruit, however, the 'average healthy person' does not consume a single fruit or a simple meal, but a diet varying in multiple vegetables and fruits and fortified products. With this knowledge, the both diets composed for the diet-controlled intervention in healthy adults in Chapter 3[6] and in healthy ileostomy subjects in Chapter 4[7] are realistic examples of diets in a Western country. The one diet contained commonly consumed vegetables and fruits high in β-carotene content and the other diet contained commonly consumed vegetables and fruits not that high in β-carotene content and the salad dressing oil was supplemented with synthetic β-carotene in oil. In both studies, the diet with β-carotene-rich vegetables appeared much higher in β-carotene than calculated. These calculations were made with data from a previous food composition table, where the RE of 6:1 for β-carotene in a mixed diet was used. However, the badges of various vegetables which were bought for the both human studies contained more β-carotene. As the diets were composed according to the Dutch guidelines for 'good nutrition', it was not ethical to provide less vegetables and consequently, also less other micronutrients and fibres. So, the 6·8 mg and 7·6 mg total β-carotene in the 11 MJ diet are contents that are realistic for the summer season in the Netherlands. Each subject consumed every day two pieces of fruit, cooked vegetables and also a salad, however this is in general practise not the case. In the 2007–2010 Dutch consumption survey, the median daily consumption of fruit varied between 61 and 145 g for adults, and the median daily consumption of vegetables was 103 to 140 g for adults, while the food based dietary guideline for vegetables is 200 g for adults[5].

Results of our intervention studies

The used extrinsic labelling technique in the two human studies can measure precisely the VEB in an oily matrix. By comparing the VEB of 3·4:1 and 3·6:1 obtained in the two studies, the conclusion is that this extrinsic labelling technique is valid in healthy adults consuming a Western diet, only when it concerns β-carotene in oil. These estimated VEB values in oil are most probably applicable to other adults in Western countries.

It should be stressed that the used extrinsic labelling technique cannot measure VEB from a diet with vegetables and fruits as source of β-carotene, since the extrinsic labelled β-carotene does not behave in the same way as the intrinsic β-carotene. This was investigated in the digestion model TIM-1 in Chapter 5[8].

Nevertheless, it was possible to estimate the VEB from a diet with β-carotene-rich vegetables and fruits and a diet without β-carotene-rich vegetables and fruits. Using data obtained with the oral–faecal balance technique, the difference in VEB 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; significantly more β-carotene was absorbed from the 'oil diet' (first study: 35%, 95%CI 24–45; second study: 30%, 95%CI 23–37) than from the 'mixed diet' (first study: 12%, 95%CI 1–23; second study: 16%, 95%CI 6–24) [6,7]. With the generally assumed conversion of 50% for absorbed β-carotene, the estimated VEB for the 'oil diet' are 5·4:1 and 6·7:1 and for the 'mixed diet' 15·7:1 (or 10·4:1 by deleting six adults with negative oral–faecal balance) and 12·5:1, respectively for the first and second study[6,7].

By comparing the β-carotene apparent absorption for each diet, the influence of bacterial degradation on the β-carotene in the colon, which is mainly in the non-disrupted food matrices is minimal. Therefore, faeces samples, beside blood samples, in dietary controlled interventions with assessments of β-carotene absorptions should be stimulated, since they add valuable results.

Vitamin A equivalency of β-carotene: children vs. adults

As mentioned in the discussion of Chapter 2[1], when more studies are performed in children, more precise data will be available for the consideration of having a vitamin A equivalency of β-carotene applicable for children and one applicable for adults. By comparing US adults with the Chinese children, the children converted Golden Rice β-carotene to vitamin A (2·3:1) [3] more efficiently than did US adults (3·8:1) [9]. This might have been related to the age and/or the differences in vitamin A status because these children have a relatively lower vitamin A status than US adults have. In the same study with Chinese children a VEB of 7·5:1 for spinach was quantified[3]. Using the same method, the spinach in US adults has a VEB of 20·9:1 [10]. However

these differences in values could partly be explained by the provided dose, respectively 1·4 mg in the children and 11 mg in the adults. The suggestion for the consideration of having a VEB applicable for children and one applicable for adults, is also based on studies performed in children in Indonesia[11,12] and the studies in adults presented in Chapter 3 and 4[6,7]. All four studies used the same dual-isotope dilution technique and quantified a VEB in oil of 2·4:1 and 2·7:1 in schoolchildren[11,12] and a VEB in oil of 3·4:1 and 3·6:1 in Dutch healthy adults[6,7]. However, all investigated children were living in non-Western countries, so partly the results concealed from a higher requirement to 'compensate' for infections and lower health status. Besides, the genetic variability and polymorphisms in the recently described β,β-carotene 15,15'-monoxygenase (BCMO1) gene coding for the enzyme that cleaves β-carotene, across different ethnic groups could explain partly the higher values for VEB for children in non-Western countries compared with adults in Western countries.

In figure 1, the sixteen VEB in oil from Table 2 of Chapter 2 are expressed for the number and average age of the subjects. The two highest VEB of both on average 9·1:1, both in corn oil performed with a reference dose [$^2H_8$]retinyl acetate had a broad range, which is likely caused by the not optimal health status of these adults with average age of 55 and 60 year. It seems likely that age influences the total absorption and/or rate of absorption. In conclusion, a VEB in oil between 2:1 and 4:1 is feasible for children and healthy adults.

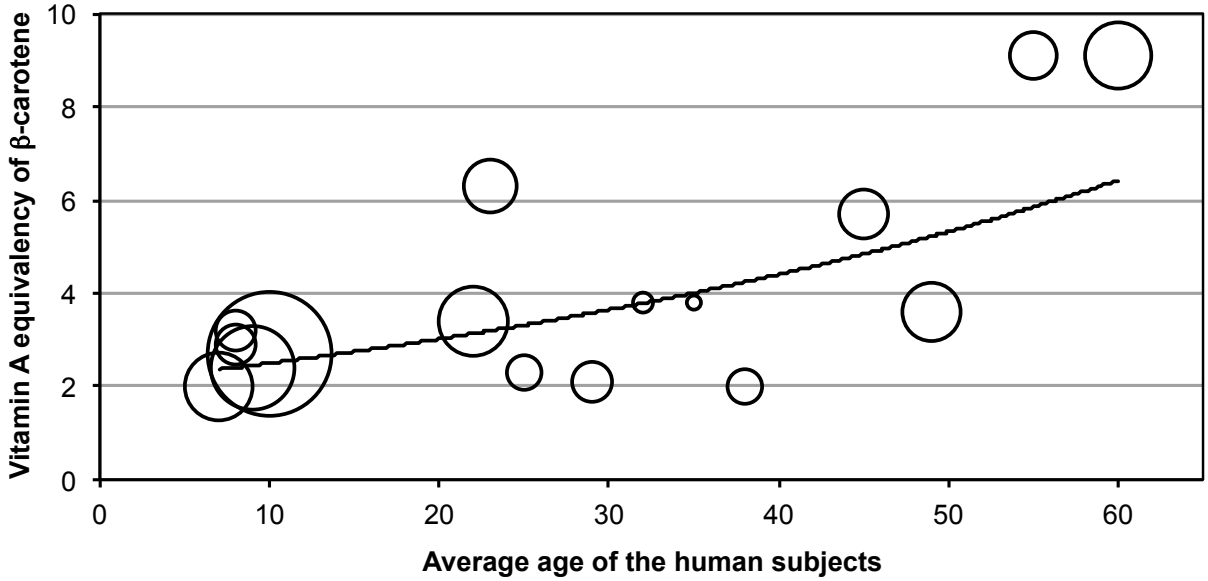

Figure 1. Vitamin A equivalency of β-carotene in oil in μg to 1 μg retinol in oil in sixteen studies expressed against the average age in years of the human subjects. The size of the circle expressed the number of participating human subjects.

Food matrix effect studies in in vitro model

On the basis of the overview in Chapter 2[1], a VEB in oil between 2:1 and 4:1 is feasible and a VEB in a mixed diet between 9:1 and 16:1. β-Carotene in foods of vegetable origin is embedded in complex cellular structures such as the cellulose-containing matrix of chloroplasts (e.g. green leafy vegetables) or the pigment-containing portion of chromoplasts (e.g. non-citrus fruits and yellow vegetables). This illustrates that the most influencing factor in human β-carotene absorption is the disruption of the food matrix before the possible incorporation of β-carotene in micelles and entry into the intestinal cells. This bioaccessibility can be studied in more detail in in vitro. The chosen digestion model TIM-1 in Chapter 5[8] is validated for fat absorption in healthy adults. For ideal comparison and validation with in vivo data, the two homogenized diets of the second human study were investigated. The bioaccessibility of β-carotene was 53% for the 'oil diet' and 28% for the 'mixed diet', while the β-carotene content was higher in the 'mixed diet'. Supplemental β-carotene in the 'oil diet' was thus 1·9-fold more bioaccessible than β-carotene in the 'mixed diet' derived from β-carotene-rich vegetables. This 1·9-fold in apparent fractional absorption between two diets was also found in the in vivo study. This confirmed the results from a diet-controlled experiment in humans. Thus, TIM-1 is a useful tool for measuring differences in bioaccessibility of β-carotene between various food matrices and processing methods of the vegetables.

The in vitro model provided a great opportunity to investigate the generally used assumption that labelled β-carotene fully mixes and equilibrates with the unlabelled β-carotene in the intestinal lumen. This means that the isotopic enrichment of β-carotene should be the same in both the feeding sample and the bio-accessible fractions in the gastro-intestinal model. As already suggested in the human studies, this assumption could not be confirmed. The labelled β-carotene was not equally distributed over time in the bio-accessible fractions, because the isotopic enrichment increased over time in both jejunal and ileal filtrates. This means that labelled β-carotene did not fully mix with the β-carotene in the food matrix or follows a different micellarization pattern. This is of major importance for the use of extrinsically labelled compounds in studies.

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. However the extrinsic dual-isotope dilution technique can be used for determining the vitamin A equivalency of β-carotene dissolved in oil, as seen in the 'oil diet', where the supplemental β-carotene has the same oily matrix as the labelled β-carotene in the capsules.

Future research

The most urgent factors to investigate in the future are the nutrient status, including children versus adults, and genetic factors, influenced by the BCMO1 activity or polymorphisms in the BCMO1 gene. For the Western situation, it is of interest to investigate the subpopulations who are at risk of inadequacy of vitamin A supply to maintain optimal liver storage, for instance children, pregnant women, patients with fat malabsorption syndromes, and subjects with extreme habitual diet patterns, such as those avoiding margarines and products used for baking and frying. Inadequacy of vitamin A supply is based on the estimated average requirement (EAR) and the recommended daily intake (RDI) for vitamin A. More research on the health effects associated with low vitamin A intake is recommended, as well as nutritional status research based on body storages.

In Western countries like the Netherlands, the consumption of dairy products is relatively high compared to other countries. Therefore, research to quantify the VEB in milk, cheese, and other dairy products will be of interest, as the milk has a complex dietary matrix with many nutrients involved. The relevant question here is whether β-carotene in dairy behaves as β-carotene in oil. Currently, the VEB for a mixed diet is used for dairy products, however it is likely that the VEB for dairy products is more close to the VEB in oil because of the oily matrix of dairy products.

In developing countries, the VEB of mixed vegetables and fruits in their habitual diet should be investigated. In currently published studies in developing countries, only the VEB from a single frequently consumed vegetable was investigated.

Advice for adequate intake of total vitamin A

Since, the 'true' total vitamin A content obtained by healthy humans from their diet is not clear, the following dietary guidelines can be applied. For the general population in Western countries, the dietary recommendation would be to consume the recommended daily amounts of vegetables and fruits. In the Netherlands, this recommendation concerning adults for vegetables is 200 g, for dairy products is 450 ml, for meat products, fish and eggs is 100–125 g, and for baking oils and margarine is 45 g. As adults in Western countries consume various plant foods imported from all over the world, they could easily maintain their vitamin A status with the different levels of provitamin A carotenoids in the plant foods and with the preformed retinol in meat products, fish, eggs and dairy products.

For the general population in developing countries, the dietary recommendation would be to cook, heat or mash the vegetables before consumption for disrupting the food matrix and to choose the more productive crops with high level of provitamin carotenoids, as they consume mostly locally produced vegetables and fruits. For the vulnerable groups in developing countries, like pregnant women, children and elderly,

the dietary recommendation would be to consume vegetables, fruits, fortified foods (e.g. fortified biscuits, fortified sugar and salt) or supplements (e.g. in-home fortification with micronutrient powders), which are in many situations supplied for free by local health promoting initiatives.

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8

Samenvatting

Vitamine A komt in twee vormen voor in de voeding, namelijk als voorgevormd vitamine A (retinol) en als provitamine A carotenoïden, waarvan beta-caroteen het meest voorkomt in de voeding en ook de hoogste vitamine A activiteit heeft. Voorgevormd vitamine A zit in voedingsmiddelen van dierlijke oorsprong, zoals borstvoeding, lever, melk, eieren en wordt in vele landen ook toegevoegd aan de margarine en halvarine. Plantaardige voedingsmiddelen bevatten geen vitamine A, maar zijn wel een belangrijke bron van provitamine A carotenoïden. Voorbeelden zijn palmolie, donkergroene bladgroenten, geel en oranje fruit, oranje wortelen en zoete aardappelen. In Nederland dragen wortelen, spinazie, andijvie en boerenkool het meest bij aan de consumptie van beta-caroteen. Ook melk bevat naast vitamine A, provitamine A carotenoïden.

Dit proefschrift gaat over de vitamine A equivalentie van beta-caroteen. Met ‘vitamine A equivalentie van beta-caroteen’ wordt de hoeveelheid beta-caroteen bedoeld die nodig is in de voeding om omgezet te worden in 1 microgram vitamine A (retinol) in het lichaam.

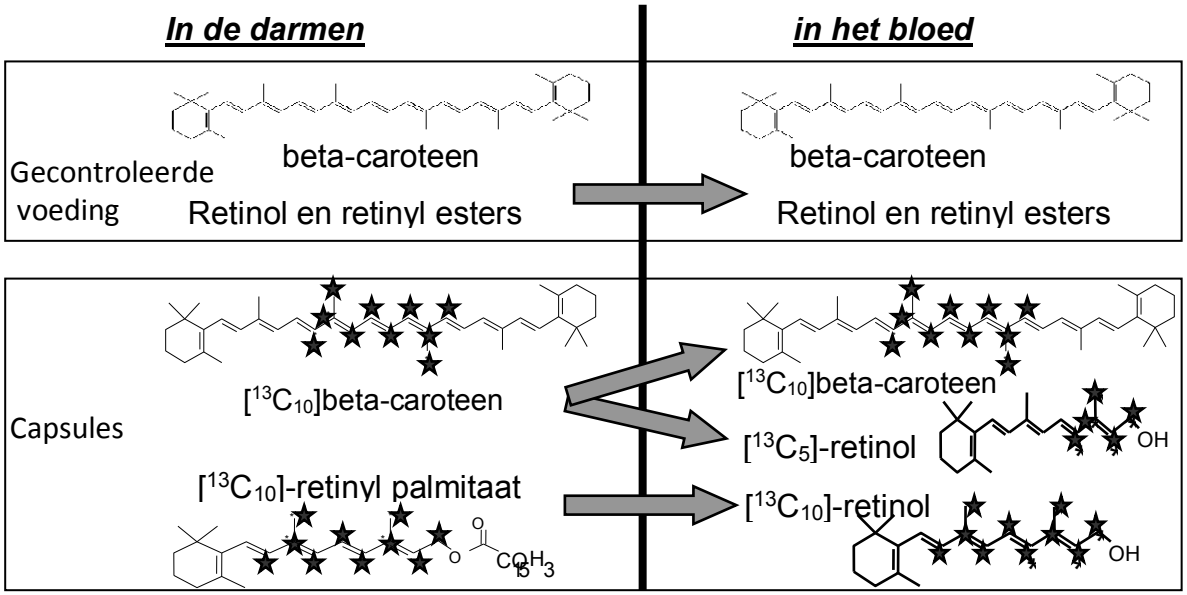
Vele factoren hebben invloed op de absorptie en gedeeltelijke omzetting van beta-caroteen in vitamine A. Dit kunnen maaltijd-gerelateerde factoren zijn, zoals het type voedsel, de hoeveelheid beta-caroteen, de aanwezigheid van vet en vezels. Ook persoon-gerelateerde factoren spelen een rol, zoals gezondheidstoestand en leeftijd. Het is bekend dat koken en pureren de hoeveelheid beta-caroteen die vrijkomt uit de plantencellen verhoogt. Ook behandeling van darmparasieten, bijvoorbeeld in kinderen in ontwikkelingslanden, helpt aanzienlijk om de absorptie van beta-caroteen te verhogen. Verder blijkt uit recent onderzoek dat de omzetting van het geabsorbeerde beta-caroteen in vitamine A verlaagd kan zijn, als een persoon een bepaalde mutatie in het gen heeft, die dat enzym aanmaakt. Ondanks deze factoren, zijn er aanbevelingen opgesteld voor deze vitamine A equivalentie van beta-caroteen. Deze aanbevelingen zijn nodig voor het berekenen van de totale hoeveelheid beschikbare vitamine A in een voeding, zoals die berekend kunnen worden uit voedingsstoffentabellen.

Volgens de huidige opvattingen zijn 12 microgram (volgens de Amerikaanse gezondheidsraad) en 6 microgram (volgens de Wereldgezondheidsorganisatie) beta-caroteen in een gemengde voeding gelijkwaardig aan 1 microgram retinol in de voeding. Voor toegevoegde beta-caroteen in olie zijn de huidige opvattingen dat 2 microgram (volgens de Amerikaanse gezondheidsraad) en 3·3 microgram (volgens de Wereldgezondheidsorganisatie) beta-caroteen gelijkwaardig zijn aan 1 microgram retinol.

De resultaten van studies in de literatuur over vitamine A equivalentie van beta-caroteen zijn zeer uiteenlopend. Betrouwbare gegevens over de vitamine A equivalentie van beta-caroteen zijn nodig voor voedingsaanbevelingen voor bepaalde leeftijdsgroepen, voor de maximale hoeveelheid beta-caroteen in voedingssupplementen, en ook voor effectief beleid om vitamine A tekorten tegen te gaan, bijvoorbeeld in ontwikkelingslanden.

Een bloedwaarde van retinol onder een bepaalde waarde geeft aan dat het lichaam een lage voorraad van vitamine A heeft en dat de huidige voeding niet deze voorraad kan aanvullen. Bloedwaarden van retinol boven deze waarde geeft geen goed beeld van de vitamine A voorraad. Met andere woorden zegt een bloedwaarde van retinol weinig over de vitamine A status van een persoon.

In het onderzoek beschreven in dit proefschrift, wordt gebruikt gemaakt van een methode om geconsumeerde beta-caroteen en vitamine A te volgen in het lichaam (zie figuur 1). Dit wordt gedaan door ze iets 'zwaarder' te maken dan de beta-caroteen en retinol in de rest van de voeding en in het lichaam. Hierdoor kunnen ze goed gemeten worden in het bloed. Dit heet ook wel isotoop-labelen. In de darmwand zit een stofje die de iets zwaardere beta-caroteen ([$^{13}C_{10}$]beta-caroteen) door midden 'knipt' en het wordt dan het iets zwaardere retinol ([$^{13}C_5$]retinol). De verhouding van de iets zwaardere beta-caroteen ([$^{13}C_{10}$]beta-caroteen) en vitamine A en de normale beta-caroteen en vitamine A in het bloed in een berekening geeft dan de vitamine A equivalentie van beta-caroteen.



Figuur 1. De absorptie van beta-caroteen en vitamine A uit de voeding en de capsules en het meten van ongelabelde en gelabelde beta-caroteen en vitamine A in het bloed. Het sterretje geeft aan waar de beta-caroteen en retinol iets zwaarder is gemaakt.

Het hoofddoel was het meten van de vitamine A equivalentie van beta-caroteen in een ‘olie voeding’ (groenten en fruit laag in beta-caroteen gehalte en toegevoegd synthetische beta-caroteen in salade dressing olie) en in een ‘gemengde voeding’ (groenten en fruit hoog in beta-caroteen gehalte). Beide voedingen zijn haalbaar in een Westerse voeding. Sommige mensen consumeren een voeding laag in groenten en fruit en nemen daarbij voedingssupplementen, die beta-caroteen in olie bevatten. Andere mensen consumeren een voeding volgens richtlijnen Goede Voeding van het Voedingscentrum, die veel diverse groenten en fruit aanbevelen.

De uitkomst van onze eerste voeding-gecontroleerde studie met 24 gezonde volwassenen (Hoofdstuk 3) was dat de vitamine A equivalentie van beta-caroteen in olie 3·4:1 was en in de tweede studie met 17 volwassenen met een ileostoma (Hoofdstuk 4) dat de vitamine A equivalentie van beta-caroteen in olie 3·6:1 was. Daarnaast waren er ook geanalyseerde hoeveelheden van beta-caroteen in de voedingen en in de ontlasting per volwassene beschikbaar. Hiermee kon de schijnbare absorptie (%) van beta-caroteen per volwassene worden berekend. Deze getallen maakten het verschil in vitamine A equivalentie van beta-caroteen in de ‘olie voeding’ en de ‘gemengde voeding’ duidelijk zichtbaar. Significant meer beta-caroteen was geabsorbeerd van de ‘olie voeding’ (eerste studie 35%; tweede studie 30%) dan van de ‘gemengde voeding’ (eerste studie 12%; tweede studie 16%). Met de algemeen aangenomen omzetting van 50% voor geabsorbeerd (opgenomen) beta-caroteen, waren de geschatte vitamine A equivalenties voor de ‘olie voeding’ 5·4:1 en 6·7:1, en voor de ‘gemengde voeding’ 15·7:1 en 12·5:1, respectievelijk voor de eerste en tweede studie (Hoofdstuk 3 en 4).

Uit deze twee voedingsonderzoeken kwam duidelijk naar voren dat een grote hoeveelheid beta-caroteen niet vrij komt uit de plantencellen of niet beschikbaar komt voor absorptie en daardoor wordt teruggevonden in de ontlasting. Bij de gezonde volwassenen kwam 65 tot 88% en bij de volwassenen met een ileostoma kwam 70 tot 84% in de ontlasting terecht.

De beschikbaarheid voor absorptie (in het Engels: bioaccessibility) van beta-caroteen is het gedeelte van de geconsumeerde hoeveelheid beta-caroteen, die vrijkomt uit de voedingsmatrix (zoals groenten en fruit) en beschikbaar is voor absorptie. Dit belangrijke proces voor het beschikbaar komen van beta-caroteen voor absorptie, kan bestudeerd worden in een model in een laboratorium. Voor het onderzoek beschreven in dit proefschrift, is gebruikt gemaakt van het dynamisch, computergestuurd verteringsmodel bij de Nederlandse Organisatie voor toegepast-

natuurwetenschappelijk onderzoek (TNO) in Zeist (Hoofdstuk 5). Dezelfde voedingen als in de tweede studie werden ook in dit model aan vertering blootgesteld. De beschikbaarheid voor absorptie was 53% van de 'olie voeding' en 28% van de 'gemengde voeding'. Deze verhouding van 1·9 tot 1 is hetzelfde als de schijnbare absorpties van de 'olie voeding' en de 'gemengde voeding' in de 17 volwassenen met een ileostoma, namelijk 30% ten opzichte van 16%. Dit model is zeer bruikbaar om verschillen in beta-caroteen absorptie in verschillende voedingsmatrices en effecten van behandelingen van voedingsmatrixen op absorptie te meten.

Deze studie in dit verteringsmodel gaf ook de mogelijkheid om de iets zwaardere beta-caroteen en retinol te bestuderen. Gedurende de 6 uur dat dit model bezig is met verteren, kunnen er fracties worden verzameld zonder verstoring van het verteringsproces. De aanname bij het gebruik van de iets zwaardere beta-caroteen en retinol is dat ze mengen met de normale beta-caroteen en retinol en dat het proces van opneming in de vetbolletjes voor absorptie in de darmen, geen onderscheid maakt. Echter, er was in de fracties niet een constante verhouding tussen de iets zwaardere en de normale beta-caroteen te zien. Dit duidt erop dat er niet een volledige menging was. Aanvullende studies in dit model moeten worden uitgevoerd om het effect van andere voedingsstoffen en matrices op het proces van opname in de vetbolletjes verder te onderzoeken. Dit model kan daarmee van grote waarde zijn voor het beoordelen en valideren van de aannames in de absorptie van iets zwaardere voedingsstoffen in voedingsstudies.

Een overzichtsartikel is opgenomen in dit proefschrift met alle gepubliceerde vitamine A equivalenties van beta-caroteen van diverse voedingsmatrixen en gemeten met diverse methoden (Hoofdstuk 2). Een aantal methoden wordt veel gebruikt, zoals de 'uitputting-aanvulling', 'voeding-ontlasting' balans en dosis-bloed-respons curves omdat bepalingsmethoden voor beta-caroteen en retinol in bloed en ontlasting al beschikbaar waren in de afgelopen decennia. De laatste twee decennia worden ook de methoden met isotoop-labelen gebruikt omdat deze metingen met hoge precisie en reproduceerbaarheid kunnen worden uitgevoerd. De studies werden opgesplitst naar het type voedingsmatrix waarin de beta-caroteen ligt 'opgeslagen', namelijk de olie-achtige matrix, de complexe matrix van een gemengde voeding met verschillende groenten en fruit en de matrix van een enkele groente of fruit. Heel uiteenlopende hoeveelheden beta-caroteen zijn gebruikt in de studies. Achttien studies rapporteerden een vitamine A equivalentie van beta-caroteen in een olie-achtige matrix. Vijf studies rapporteerden negen getallen voor de complexe gemengde voeding. Negentien vitamine A equivalenties van beta-caroteen van een enkele groente werden gevonden in dertien studies. Wortelen, spinazie en zoete

aardappel geven getallen tussen 9:1 tot 16:1, echter de recente biologisch gefortificeerde groenten, zoals cassave, gele mais en gele rijst laten een vitamine A equivalentie van beta-caroteen rondom 4:1 zien. Deze biologisch gefortificeerde groenten zijn nog niet beschikbaar voor de lokale bevolking. De studies uitgevoerd in niet-Westerse landen laten bij hun huidige voedingspatroon een vitamine A equivalentie van beta-caroteen tussen 9:1 en 28:1 zien.

De resultaten van de hoofdstukken 2, 3 en 4 laten zien dat voor mensen die beta-caroteen opgelost in olie (zoals bij supplementen of gefortificeerde voedingsmiddelen) consumeren, een vitamine A equivalentie van beta-caroteen tussen 2:1 en 4:1 haalbaar en realistisch is. Voor mensen die een Westers voedingspatroon hebben, is een vitamine A equivalentie van beta-caroteen in een gemengde voeding van 9:1 tot 16:1 realistisch. Deze uitkomsten ondersteunen dat 2 microgram toegevoegde beta-caroteen in olie en 12 microgram beta-caroteen in een gevarieerde voeding rijk aan groenten en fruit gelijkwaardig zijn aan 1 microgram retinol uit de voeding, zoals de huidige aanbeveling van de Amerikaanse gezondheidsraad. Lang niet alle voedselconsumptietabellen in diverse landen houden deze getallen aan.

De aanbevelingen voor de vitamine A equivalentie van beta-caroteen in olie en in gemengde voeding moeten geregeld worden herzien, aangezien ze tot nu toe vooral gebaseerd zijn op een aantal studies die gedaan zijn in gezonde volwassenen. Er komen steeds meer studies die de vitamine A equivalentie van beta-caroteen onderzoeken in groepen die een mogelijk risico lopen op het ontwikkelen van vitamine A tekort, zoals zwangeren, kinderen en patiënten met malabsorptie. Het is mogelijk dat in de toekomst wordt gekozen voor verschillende vitamine A equivalenties van beta-caroteen voor bepaalde groepen afhankelijk van hun gezondheidstoestand.

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LIST OF PUBLICATIONS

Peer-reviewed papers

1. **Van Loo-Bouwman CA**, Naber THJ, Schaafsma G (2014) A review of vitamin A equivalency of β-carotene in various food matrices for human consumption. *Br J Nutr* 111, 2153–2166. doi:10.1017/S0007114514000166.
2. **Van Loo-Bouwman CA**, Naber TJH, Minekus M, van Breemen RB, van Roekel-Jansen T, Hulshof PJM, Schaafsma G (2014) Food matrix effects on bio-accessibility of β-carotene can be measured in an in-vitro gastrointestinal model. *J Agric Food Chem* 62, 950–955. doi:10.1021/jf403312v.
3. **Van Loo-Bouwman CA**, Naber TJH, van Breemen RB, Zhu D, Dicke H, Siebelink E, Hulshof P, FGM Russel, Schaafsma G, West CE (2010) Vitamin A equivalency and apparent absorption of β-carotene in ileostomy subjects using a dual-isotope dilution technique. *Br J Nutr* 103, 1836–1843. doi:10.1017/S0007114509993849.
4. **Van Loo-Bouwman CA**, West CE, van Breemen RB, Zhu D, Siebelink E, Versloot P, Hulshof PJM, van Lieshout M, FGM Russel, Schaafsma G, Naber AHJ (2009) Vitamin A equivalency of β-carotene in healthy adults: limitation of a dual-isotope dilution technique to measure matrix effect. *Br J Nutr* 101, 1837–1845. doi:10.1017/S0007114508131762.
5. Zhu D, Wang Y, Pang Y, Liu A, Guo J, **Bouwman CA**, West CE, van Breemen RB (2006) Quantitative analysis of β-carotene and retinol in serum and feces in support of clinical bioavailability studies. *Rapid Comm Mass Spectr* 20, 2427–2432. doi:10.1002/rcm.2601.
6. Roos de N, Steenhagen E, **Bouwman CA**, CJ van Laarhoven, WA van Staveren (2006) Sources and severity of self-reported food intolerance after ileal-anal anastomosis. *J Am Diet Ass* 106, 1459–1462. doi:10.1016/ j.jada.2006.06.013.

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1. **Van Loo-Bouwman CA**, Naber THJ, Minekus M, van Breemen RB, van Roekel-Jansen T, Hulshof PJM, Schaafsma G (2010) Food matrix effects on bio-accessibility of β-carotene can be measured in an in-vitro gastrointestinal model. *Programme and abstract book NVGE meeting March*, p 145.
2. **Bouwman CA**, Regelink M, van Nispen L, Siebelink E, Naber AHJ (2008) Sodium and liquid balance in ileostomy subjects. Programme and abstract book NVGE meeting March 2007, p 44. *Eur J Gastroenterol Hepatol*, volume 20 (7), p A45–A77 doi:10.1097/01.meg.0000324107.71685.22.
3. **Bouwman CA**, West CE, van Breemen RB , Zhu D, van Lieshout M, Siebelink E, Versloot P, Naber AHJ (2006) Vitamin A equivalency of β-carotene in oil in healthy

Dutch adults measured using specifically ^{13}C-labelled β-carotene and retinol. Programme and abstract book NVGE meeting March 2005, p 47. *Eur J Gastroenterol Hepatol*, volume 18 (1), p A7–A8.

4. **Bouwman CA**, West CE, van Breemen RB, Zhu D, van Lieshout M, Siebelink E, Hulshof P, Naber AHJ (2005) Vitamin A equivalency and bioefficacy of β-carotene in oil in healthy adults measured using ^{13}C-labeled β-carotene and retinol. Abstract of the papers presented at the 14th International Symposium on Carotenoids. *Carotenoid Science* July, volume 9, p 6.
5. Zhu D, Wang Y, Pang Y, **Bouwman CA**, West CE, van Breemen RB (2005) Quantitative analysis of β-carotene and retinol in serum and feces: bioavailability of stable isotopically labeled β-carotene. *Report of the 53rd ASMS Conference of American Society for Mass Spectrometry*, p 92.
6. **Bouwman CA**, West CE, van Breemen RB, Zhu D, van Lieshout M, Siebelink E, Versloot P, Naber AHJ (2005) Vitamin A equivalency of β-carotene in oil in healthy Dutch adults measured using specifically ^{13}C-labelled β-carotene and retinol. *Report of the XXII International Vitamin A Consultative Group meeting*. Washington, DC: ILSI, M24, p 69.
7. Steenhagen E, **Bouwman CA**, van Staveren WA, van Laarhoven CJHM (2004) Nutrition related complaints of patients after ileal pouch-anal-anastomosis. *Report of the XIV International Congress of Dietetics*. Chicago, IL: International confederation of Dietetic Associations.

Other publications

1. Lietz G, Furr HC, Gannon BM, Green MH, Haskell M, Lopez-Teros V, Novotny JA, Palmer A, Russell RM, Tanumihardjo SA, **Van Loo-Bouwman CA** (2015) Current capabilities and limitations of stable isotope techniques and applied mathematical equations in determining whole body vitamin A status. *Food and Nutrition Bulletin* (submitted)
2. Souverein OW, de Vries JHM, Freese R, Watzl B, Bub A, Miller III ER, Castenmiller JJM, Pasman WJ, van het Hof K, Chopra M, Karlsen A, Dragsted LO, Winkels R, Itsiopoulos C, Brazionis L, O'Dea K, **Van Loo-Bouwman CA**, Naber THJ, van der Voet H, Boshuizen HC (2015) Prediction of fruit and vegetable intake from biomarkers using individual participant data of diet-controlled intervention studies. *Br J Nutr* (accepted)
3. Farfan JA, Rodriguez-Amaya DB, **Bouwman CA**, van Lieshout M, Vinodkumar M, Wieringa FT, Dijkhuizen M, El Mougi M, Passi SJ, Solano L, Benn CS, Mahy L, Cusack G, Chowdhury T (2005) Poster presentations, XXII IVACG meeting, 15-17 November 2004, Lima, Peru. *Sight and Life Newsletter* 1, 27–37.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline meetings

2014 3rd Int. conference on food digestion, Wageningen
2013 6th National nutrition congress, Ede
2011 11th European nutrition conference, FENS, Madrid
2010 5th Dutch growth study symposium, Rotterdam
2009 Wageningen nutritional sciences forum, Arnhem
2007 10th European nutrition conference, FENS, Paris
2007 40th European society gastroenterology & nutrition, ESPGHAN, Barcelona
2007 1st Micronutrient int. symposium, Int. Life Sciences Institute (ILSI), Istanbul
2005 14th Int. symposium on carotenoids, Int. Carotenoid Society, Edinburgh
2005, 2006 research day Dutch Dairy Association (NZO), Wageningen
2004 XXII Int. meeting, Int. Vitamin A Consultative Group (IVACG), Lima
2004 Int. symposium, Int. Nutritional Anemia Consultative Group (INACG), Lima
2004 Int. symposium, Int. Zinc Nutrition Consultative Group, (IZINCG) Lima
2004 Gordon conference on carotenoids, Ventura, California
2004-2009, 2013 NZO symposium, Utrecht and Ede
2004-2007, 2010 meetings Dutch Society of Gastroenterology (NVGE), Veldhoven
2002-2003, 2005 meetings NWO days nutrition (Dutch Scientific Research) Arnhem
2002 Symposium ready-to-eat industrial-prepared meals, NVVL, Wageningen

Discipline courses

2007 EU Claim Legislation, Food industry, Maarssen
2006 Regulation of food intake and its implications for obesity, VLAG, Wageningen
2005 Eco-physiology of the gastrointestinal tract, VLAG, Wageningen
2004 Clinical nutrition, European Society Clinical Nutrition (ESPEN), Maastricht
2004 Tracer methodology in metabolism, ESPEN, Maastricht
2002 Pharmacokinetics, Leiden /Amsterdam Center Drug Research (LACDR), Oss
2002 Pharmacokinetic and pharmacodynamic modelling, LACDR, Amsterdam

General courses

2005 Career perspectives, Wageningen Graduate Schools, Wageningen
2005 Master class Funding, Talent days, NWO, Den Haag
2004 Techniques for writing & presenting a paper, Business School Wageningen
2004 Design and carrying out of your PhD project, Radboud University Nijmegen
2003 Advanced conversation, University Language Centre, Nijmegen
2002 Education competence, Radboud University Nijmegen
2002 PhD student week, VLAG, Bilthoven

Optional activities

2003 Human Nutrition Wageningen University, PhD study tour to Australia

CURRICULUM VITAE

Carolien Annika Bouwman was born on 31 July 1979 in Kampen and grew up in Dronten, the Netherlands. In 1997, she passed secondary school (Atheneum) at the Ichthus College in Kampen In the same year, she started the study Human Nutrition & Health at Wageningen University. As part of that study, she conducted a research project in Biomedical Sciences at the Community Health Service (GGD) and the National Institute for Public Health (RIVM) about the subjective hunger feelings and the leptine, glucose, and insulin metabolism in adult men. The other research project in Clinical Epidemiology, she performed at the University Medical Centre Utrecht about the symptoms, dietary adaptations and quality of life in patients after surgery. She was three times student-assistant for two practical trainings in 2001 and 2002. In May 2002, she participated in the European study tour to Germany and Poland. She obtained the MSc degree in Human Nutrition & Health in June 2002.

In July 2002 she started as a Junior Researcher on the PhD research project entitled 'vitamin A equivalency of β-carotene in humans' at the Radboud University Nijmegen Medical Centre, department of Gastroenterology & Hepatology under supervision of Professor Clive West and Dr. Ton Naber. Since the passing away of Clive West (27 August 2004) Dr. Ton Naber and Professor Gertjan Schaafsma were the supervisors. She joined the educational programme of the Graduate School VLAG. She participated in teaching of Medicine and Biomedical Sciences students in Nijmegen and Human Nutrition students in Wageningen. In November 2003 she participated in the PhD study tour to Australia; they visited universities and research institutes in Sydney, Canberra and Melbourne. In November 2004, she was selected to give a poster presentation at the congress of the International Vitamin A Consultative Group in Lima, Peru. In July 2005, she was selected to give an oral presentation at the International Symposium on Carotenoids in Edinburgh, Scotland. In March 2014, she was an invited speaker and participant in the Technical Meeting 'Assessing vitamin A safety in large scale nutrition intervention programmes: setting the research agenda', at the International Atom Energy Agency (IAEA) in Vienna, Austria.

Since 2006 she is a registered Nutritionist A at the Dutch Academy for Nutritional Sciences and after the thesis defence she can apply for the Nutritionist B registration. From January to December 2007, she worked fulltime as Nutrition Scientist Europe at Mead Johnson Nutrition in Nijmegen. From June 2008 to June 2013, she worked as Clinical Study Manager/Researcher at Danone Research -Centre for Specialised Nutrition in Wageningen. From August 2013 to August 2014, she worked as Senior Researcher Nutrients at FrieslandCampina Innovation Centre in Wageningen.

She is married to Wim Van Loo and has two children; Laurens (born in 2010) and Maarten (born in 2012).

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